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DOMESTIC & INTERNATIONAL



The Controller of Patents
The Patent Office
Kolkata

October 04, 2019

Kind Attn: Mr. Jayant Anand
Ld. Controller of Patents & Designs

Dear Sir,

Re: Response to First Examination Report dated March 26, 2019
Patent Application No. 1336/KOL/2013 dated November 26, 2013
Entitled: "A METHOD FOR CONTROLLING RUST"
Applicant: UPL LIMITED.
Opponent: MRS. VISHAKHA REKHI.
Our Ref: PII00644

This has reference to the Pre-grant Opposition filed by **MRS. VISHAKHA REKHI.**

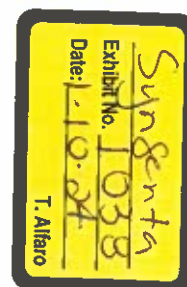
We refer to our letter dated May 21, 2019, pointing out that under Rule 55 (3), which dictates the procedure for pre grant opposition/representation, the Ld. Controller is required to give a notice to the applicant regarding the representation for opposition after consideration of the same by him. The applicant is time bound from receipt of such notification for filing the reply statement along with evidence within three months' time from the date of notice as required under Rule 55(4).

It is respectfully submitted that in view of the above, by our letter of May 21, 2019, we had requested that a notice regarding the representation be sent to us under Rule 55 to enable filing of the reply statement within three months from the date of notice.

We have as of now not received a notice in respect of the opposition filed by MRS. VISHAKHA REKHI, however, to expedite disposal of the application, we are enclosing herewith a Reply statement and evidence to the opposition filed by MRS. VISHAKHA REKHI.

Kolkata Marin Burn Business Park Suite # 501, BP Block, Sector V Salt Lake, Kolkata 700 091, India p: +91 33 4055 1111 f: +91 33 4055 1122 e: info@patentindia.com	New Delhi G48, LOF, Laxmi Nagar II Ground Floor New Delhi 110 024, India p: +91 11 4100 8738 / 4109 7455 f: +91 11 4100 7455 e: info@patentindia.com	Mumbai 202 Eicon Chambers Behind Saki Naka 1st Exchange Mumbai 400 072, India p: +91 22 2652 2900 / 2402 f: +91 22 2652 2901 e: info@patentindia.com	Hyderabad 108 Block B, Kalyan Towers Kalyan Nagar Hyderabad 500 004, India p: +91 40 3076 1294 f: +91 40 3052 3392 e: info@patentindia.com	Bengaluru Suite # 1001, 10th Floor A Wing, Mittal Tower, MG Road Bengaluru 560 001, India p: +91 80 4372 1870 f: +91 80 2558 6479 e: info@patentindia.com
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Registered Office: S Harihar Mukherjee Road, Kolkata 700 025, India | p: +91 33 2455 7100 | f: +91 33 2455 7101 | e: info@patentindia.com





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Patent & Trademark Attorneys

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Kolkata	New Delhi	Mumbai	Hyderabad	Bengaluru
Metro Bunk Business Park Block # 301, BP Block, Sector V Salt Lake, Kolkata 700 091, India p: +91 33 4055 1111 f: +91 33 4055 1122 m: cal@patentindia.com	G4B, LGF, Lajpat Nagar II Ground Floor New Delhi 110 024, India p: +91 11 4610 8738 / 4109 7055 f: +91 11 4109 7455 n: del@patentindia.com	202 Eileen Chambers Behind Saki Naka Tel Exchange Mumbai 400 072, India p: +91 22 2852 2901 / 2902 f: +91 22 2852 2908 m: bom@patentindia.com	108 Block B, Kurchal Towers Khanwarabadi Hyderabad 500 004, India p: +91 40 3078 1294 f: +91 40 3052 3392 m: hyd@patentindia.com	Suite # 1006, 10th Floor A Wing, Midol Tower, MG Road Bengaluru 560 001, India p: +91 80 4372 1870 f: +91 80 2558 6999 m: bnl@patentindia.com

Registered Office: 5 Harish Mukherjee Road, Kolkata 700 025, India | p: +91 33 2455 7464 / 85 / 86 | f: +91 33 2455 7467 / 89 | m: cal@patentindia.com

DUPLICATE FIRST PAGE



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The Ld. Controller is requested to kindly take the same on record.

Yours faithfully,

DR. SANCHITA GANGULI

IN/PA No. 625

Of S. Majumdar & CO.

Applicant's agent

CC: Mrs. Vishakha Rekhi
A-903 Julian Alps,
Ivory Towers CHS Ltd.,
Bhakti Park, Wadala East,
Mumbai 400037
Cell: 9892092090
Email: vishakha.rekhi@gmail.com

**BEFORE THE CONTROLLER OF PATENTS,
KOLKATA**

In the matter of The Patents Act, 1970 *as amended by* The Patents (Amendment) Act 2005,

And

In the matter of The Patents Rules, 2006 and The Patent (Amendment) Rules, 2019,

And

IN THE MATTER of Patents Application No. **1336/KOL/2013** dated **November 26, 2013** made in the name of **UPL LIMITED** having its principal place of business at UPL House, 610 B/2, Bandra Village, Off Western Express Highway, Bandra-East, Mumbai-400051, State of Maharashtra, India and also having place of business at Agrochemical Plant, Durgachak Haldia – 721 602, Midnapore Dist. West Bengal, India.

.....Applicant

And

IN THE MATTER of opposition of the grant of a patent thereto by Representation under Section 25(1) by **Mrs Vishakha Rekhi**, having address at A-903 Julian Alps, Ivory Towers CHS Ltd., Bhakti Park, Wadala East, Mumbai 400037, India.

.....Opponent

**REPLY STATEMENT IN SUPPORT OF THE APPLICANT AND IN RESPONSE TO
THE OPPOSITION FILED UNDER RULE 55(4) FOR THE INDIAN PATENT
APPLICATION NUMBER 1336/KOL/2013**

ABOUT THE APPLICANT:

1. We, **UPL LIMITED** (erstwhile United Phosphorus Limited), having its principal place of business at UPL House, 610 B/2, Bandra Village, Off Western Express Highway, Bandra-East, Mumbai-400051, State of Maharashtra, India and also having place of business at Agrochemical Plant, Durgachak Haldia – 721 602, Midnapore Dist. West Bengal, India. The Applicant named above, state that we desire to contest the opposition entered by the Opponent named above, and set out below the statement in support of said patent application.
2. The applicant is a Company incorporated under the Indian Companies Act, 1956, carrying on business, inter alia, of manufacture of various products relating to agrochemicals. The applicant company is and has been engaged in the manufacture of, inter alia, a wide range of products that include fumigants, fungicides, insecticides, rodenticides, and herbicides.
3. The opponent has entered an opposition under Section 25(1) to the present application and has filed representation by way of opposition. The applicant has read and understood the documents filed and relied upon by the opponent and set out below the grounds on which the opposition is contested.
4. It is stated that save and except what are specifically admitted herein or are borne out from the records, all contentions, allegations and averments in the said Opposition Statement shall be deemed to be denied and disputed as if the same have been specifically dealt with herein and set out as scriatim.
5. The applicant denies and disputes the various statements and allegations made by the opponent barring what are matters of record and what are specifically admitted by the applicant.
6. The applicant now provides paragraph wise reply to the Representation filed by the opponent.
7. With reference to paragraphs 3.1, the applicant states that almost all of the countries where corresponding applications have been filed have granted this patent with different levels of claim scope, and the applicant has filed divisional application for

claiming the restricted scope of claims in those countries. The fact that almost all of these substantively examining countries have granted this patent speaks volumes about the inventive merit of this application.

8. With reference to paragraphs 3.2, the applicant states that the fact that India is an agricultural country means that further research into agrochemical industry must be encouraged, and granting patents on applications such as the present one efficiently and quickly would go a long way in encouraging research in this field. This will not only ensure food safety for the growing population in the country, but will also ensure increased investment in the agrochemical research.

The subject application relates to an improved fungicidal combination and describes the synergistic effects of the fungicidal combination claimed herein. This combination is especially suitable for controlling undesired fungus, and thus helps in improving the productivity of farmers in the agriculture dependent regions of the country. Keeping the needs of the farmers in mind, the applicant named above invented this improved combination in order to make this synergistic fungicidal combination available to the farmer, which is intended to benefit the farmers in the country. The applicant company UPL Ltd spent a huge sum on the invention and research on this combination in order to be able to benefit the farmers.

Keeping this inventive idea in mind, and the expected benefit to the farmers and agricultural productivity in the country, the applicant herein named invented this improved combination and filed the instant patent application.

Meanwhile, the applicant has learnt of several unauthorized people selling spurious and ineffective products covered by the present claims to the gullible farmers. This has two-fold disadvantages to the applicant, to the farmer and to the country at large. Firstly, these unauthorized sellers are illegally usurping the product of the applicant's focussed research to find this synergistic fungicidal combination for the farmers. Secondly, and more seriously, these spurious products are ineffective to target the desired fungus, thereby decreasing the farmer's productivity and his yield. Thirdly, the continuous usage of these spurious and ineffective products increases the cross-resistance of these fungi to the fungicides in general due to the ineffective use of the combination and the unstable copycat products. This leads to the fungicides degrading during the storage of these spurious products before it reaches the farmers, thus defeating the entire purpose of keeping the farmer community first in the development of the country.

The applicant herein above is the patent applicant of the improved and synergistic fungicidal combination. Applicant is desirous of acting against these unauthorized sellers of spurious products, who are harming both the farmer and the country, and for this reason the Ld. Controller is requested to grant a patent swiftly on this pending application.

9. With reference to paragraphs 4, it is submitted tht the same relates to jurisdiction of the impugned application been Patent Office, **Kolkata** and the same been admitted.
10. With reference to paragraphs 5.1, the opponent has merely set out the ground on which the frivolous opposition is based.
11. With reference to paragraphs 5.2, the applicant states those are matters on record and do not call for any comment.
12. With reference to paragraphs 5.3, it is stated that the applicant has filed amendments to the claims in response to the first examination report issued by the Indian Patent Office, which may kindly be taken on record. A copy of the amended claims is annexed herewith as **Annexure A**.
13. With reference to paragraphs 6.1 (a), the applicant states that it merely set out the prior arts alleging that the present invention has been anticipated, prior to the filling of the impugned application.
14. With reference to paragraphs 6.1 (b), it is stated that **US2005/0032903A** discloses the method for controlling rust diseases in legumes such as soybeans by treatment of the aerial part of the plant legumes with a formulation containing strobilurin (claims 1 and 2). **US'903** also discloses that such strobilurin fungicides could be used in combination with **another i.e. one other** fungicide, which may either be a contact fungicide or a systemic fungicide such as a conazole fungicide or a SDHI fungicide alternatively. This means that **US'903** teaches use of only one of the second group of fungicides i.e. only envisages a dual combination but not a triple combination.
15. It is stated that the opponent has deliberately tried to misrepresent the disclosure of this document. **US'903** never teaches the use of a second as well as a third fungicide, which is required according to the present invention. Even in paragraphs [0040] and [0082] (example), particularly the combination of strobilurins and azoles have been disclosed, but use of a contact fungicide such as mancozeb has not been disclosed. Page 2, paragraphs [0023], [0031] and [0037], teaches the combination of strobilurins with other fungicides such as mancozeb and chlorothalonil, but this disclosure does not include a conazole fungicide within the same combination.

16. Thus, it is clear from the above discussion that US'903 does not envisage a triple combination of fungicides as claimed in the present invention.
17. With reference to paragraphs 6.1 (c), it is stated that **Brazilian Patent PI0601469** describes a composition comprising an oil dispersed agricultural sprays composition comprising: 1) at least one fungicidal formulation which comprises a dithiocarbamate, triazole, morpholine or strobilurin compound; and 2) an oil soluble or oil compatible poly fatty acid-imine polymer.
18. A complete reading of BR'1469 suggest that primarily the composition disclosed in this reference relates to single active composition of either of dithiocarbamate, or of triazole, or of morpholine or of strobilurin compound (refer example 1-5 and 11-20) or of a dual combination of dithiocarbamate with either a triazole, or a morpholine or a strobilurin compound (i.e. double combination).
19. BR'1469 fails to disclose the triple combination of a multisite contact fungicide e.g. mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further this document also fails to disclose the use of such triple combination for treating soybean rust.
20. The opponent has deliberately tried to misrepresent the teachings of this prior art document. This document teaches agricultural sprays dispersed in oil comprising at least one fungicide formulation containing a compound dithiocarbamate, or a triazole, or a morpholine or a strobilurin, but the opponent has misrepresented this teaching as a fungicide formulation containing a compound dithiocarbamate, and a triazole, and a morpholine and a strobilurin.
21. It is stated that claim 11 of BR'1469 teaches a combination of dithiocarbamate formulation and formulation of triazole or formulation of morpholine or formulation of strobilurin. This finding is also supported by Table 1 at page 7 of the complete specification of BR'1469. Table 1 teaches combination of dithiocarbamate + triazole, morpholine or strobilurin wherein weight percentage of dithiocarbamate is 1-30, preferably 5-25 and weight percentage of any one of triazole, morpholine or strobilurin is 0.1-10, preferably 0.5-5. BR'1469 does not teach a combination of dithiocarbamate formulation and one or more formulation of triazole, morpholine or strobilurin (i.e. combination of 3 fungicidally active agents).
22. Thus, it is submitted that a mistaken representation of the prior art teaching is not sufficient to sustain a case of lack of novelty or even a *prima facie* case of obviousness.

23. With reference to paragraphs 6.1 (d), it is stated that WO2006/035316 discloses an aqueous composition suitable for applying fungicides to plant propagation materials comprising at least three fungicidally active compounds, as active ingredients, water and a blend of the following components, by weight: a) 2 - 10% of at least one surface-active agent, comprising a) 1) at least one anionic surfactant; b) 0 - 10% of at least one polymer selected from water-dispersible and water-soluble film-forming polymers; c) 4 - 20% of at least one inorganic solid carrier; and, optionally d) 3 - 20% of at least one antifreeze agent.
24. WO'316 provides a long laundry list of fungicidally active ingredient which may be combined together. However the main combination of interest for WO'316 is fludioxonil, difenconazole and mefenoxam.
25. WO'316 fails to disclose the triple combination of a multisite fungicide e.g. mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further WO'316 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference WO'35316 does not anticipate present invention.
26. WO'316 discloses useful compositions dispersion of fungicides comprising at least one or at least three suitable fungicides for seed treatment. First of all, it is not obvious to use a combination meant for seed treatment in the actual field. Secondly, the three fungicides to be combined according to WO'35316 are to be selected from a random list, and may not necessarily include a multisite fungicide with a dual combination including a conazole fungicide and a strobilurin fungicide. The preferred combinations according to this document are fludioxonil, mfenxam and azoxystrobin or difenconazole. Thus, this document does not teach the presence of a multisite fungicide such as mancozeb essentially, and considers strobilurins or conazoles as being alternatives to each other i.e. they are not taught to be used together.
27. This document fails to disclose the triple combination of Mancozeb with the combination of first and second systemic fungicides selected from quinone outside or inside inhibitors, demethylation inhibitors, or succinate dehydrogenase inhibitors. Further this document also fails to disclose the use of such triple combination for treating soybean rust.
28. With reference to paragraphs 6.1 (e), it is stated that US2005/0226902 discloses a chemical soil disinfestation method in which a fungicidally, insecticidally or

nematicidally active amount of a mixture M comprising a component a) of a1) 20 to 96% by weight of sulfur, a2) 4 to 80% by weight of a complexing agent selected from the group consisting of: nonpolymeric complexing compounds: EDTA (ethylenediaminetetraacetic acid), NTA (nitrilotriacetic acid), and, if appropriate, one or more crop protection agents b) and/or further additives c) is applied.

29. The main crux of the US'902 relates to combination of Sulfur with complexing agent and optionally any other active ingredient.

30. US'902 does not even disclose a combination of Sulphur with a dual combination of systemic fungicides. Further US'902 fails to disclose the triple combination of any other multisite fungicide with the combination of first and second systemic fungicide selected from quinone outside or inside inhibitors, demethylation inhibitors or succinate dehydrogenase inhibitors. Further US'902 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference US'902 does not anticipate present invention.

31. With reference to paragraphs 6.1 (f), it is stated that US2010/197741 allegedly claims a composition comprising fungicide boscalid (SDHI fungicide systemic), Chlorothalonil (fungicides of multiple sites) and at least one other fungicide.

32. The Applicant states that US'741 discloses a fungicidal composition for controlling phytopathogenic harmful fungi, comprising 1) Boscalid, 2) Chlorothalonil and 3) at least one fungicidally active compound III selected from groups A) to F):

A) an azole selected from

B) a strobilurin selected from

C) a carboxamide selected from

D) a heterocyclic compound selected from

E) a carbamate selected from

F) a fungicide selected from

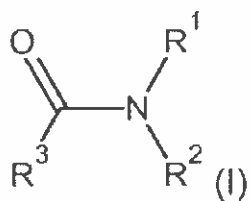
33. US'741 fails to disclose the triple combination of a multisite fungicide such as dithiocarbonate such as mancozeb with the combination with first systemi fungicide and second systemic fungicides selected from individual quinone outside or inside inhibitors, demethylation inhibitors or succinate dehydrogenase inhibitors. Further

US'741 also fails to disclose the use of such triple combination for treating soybean rust.

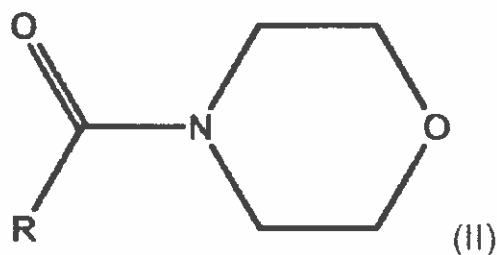
34. Applicant states that the amended claims presented with the response to First Examination report (**Annexure A**) do not include chlorothalonil as the contact fungicide. Therefore, there is no overlap of the amended claims of the present invention with the disclosure of US'741.
35. With reference to paragraphs 6.1 (g), it is stated that **WO2007/052256** teaches a pesticidal composition in the form of a water-dispersible granule comprising a) at least one pesticidal active ingredient, b) at least one surfactant, c) at least one dispersing agent, and optionally d) a filler, obtainable by a process comprising of mixing all the ingredients of the premix to obtain a homogenous premix; micronizing the premix; wetting the micronized premix; granulating the micronized wet premix; drying the granules and treating said granules with a surfactant agent.
36. This is a platform technology patent application. The main crux of the invention is to provide a water dispersible granule composition which may be applicable for number of active ingredients disclosed therein. WO'256 is not relevant to present invention as WO'256 fails to disclose the triple combination of a multisite fungicide with individual quinone outside or inside inhibitors, demethylation inhibitors or succinate dehydrogenase inhibitors. Further WO'256 also fails to disclose the use of such triple combination for treating soybean rust.
37. It is pertinent to note that the presently amended claims does not falls within the purview of WO'256. WO'256 discloses formulations containing a single active ingredient. The choice of the single active ingredient may be wide and may include the contact fungicide, the conazole fungicide, the strobilurin fungicide or the SDHI fungicide. Still, these fungicides are to be formulated in isolation according to WO'256, and are not to be used in any combination. Applicant fails to understand as to how this disclosure is even relevant to the subject matter of the present invention.
38. With reference to paragraphs 6.1 (h), it is stated that **US2012/003199** discloses a method for controlling harmful fungi, which method comprises subjecting plants to be protected against fungal attack to two or more sequential treatment blocks, where at least one treatment block comprises treating the plants with at least one synthetic fungicide and at least one treatment block comprises treating the plants with at least one biological control agent, with the proviso that the last treatment block comprises

treating the plants with at least one biological control agent selected from *Bacillus subtilis* and metabolites produced therefrom.

39. US'199 essentially relates to combination of a synthetic fungicide and a biological control agent such as *Bacillus subtilis*. Biological agents are critical and essential part of invention disclosed in US'199. The present invention does not disclose any biological agents to be used in the combination disclosed therein.
40. US'199 fails to disclose the triple combination of a multisite fungicide with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further US'199 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference D7 does not anticipate present invention.
41. US'199 discloses the list of synthetic fungicides that includes systemic fungicides, such as azoles, strobilurins, carboxamides, and fungicides from multiple sites. US'199 discloses that these fungicides are to be used in isolation, along with a biological control agent. A mere listing of these fungicides does not mean that these fungicides are taught to be used in combination. The opponent has deliberately attempted to misrepresent the teachings of US'199, whereas in reality, this document does not teach the use of more than one fungicide in combination.
42. The Applicant states that US'903, BR'469, WO'316, US'902, US'741, WO'256 and US'199 (Annexures II-VIII) does not anticipate the present invention and thus the present invention is novel over the aforesaid prior art. The Applicant states that these citations would not have been arisen had the opponent actually read the invention and the accompanying description of the invention without involving the element of hindsight.
43. With reference to paragraphs 6.2, the applicant states that the ground of prior claiming requires an exact overlap between the cited claims and the claims under examination. The claims of the cited Indian Application 3142/DELNP/2014 (Annexure XIX) relate to a composition comprising an agricultural a) active ingredient; b) an amide of the following formula (I):



c) an acyl morpholine compound of the following formula (II):



wherein R is H, CH₃ or C₂H₅.

44. The applicant states that the amended claims of the instant application neither include the amide of formula (I) or the acyl morpholine of the formula (II). Accordingly, there is no exact overlap between the claims of the present application and the claims of the cited art. Thus, there is no possibility that the presently claimed invention could even be *arguably* said to have been prior claimed in 3142/DELNP/2014.
45. The Applicant states that picking and choosing one feature from one claim of the cited art, and another feature from another claim of the cited art, is not a valid and proper manner of analysing the ground of prior claiming as has been done by the opponent. Moreover, merely listing the strobilurins, the triazoles, the carboxamides and the dithiocarbamates together side-by-side does not mean that the prior art teaches these fungicides to be used in combination. This only means that these fungicides were meant to be used separately (any one in isolation) together with the solvent system comprising the compounds of formula (I) or formula (II).
46. In contrast, and quite differently from the cited art, the present invention relates to a triple combination of fungicides, wherein at least two systemic fungicides and at least one multisite fungicide, must be simultaneously present within the same combination.
47. Accordingly, the ground of prior claiming does not stand at all and ought to be dismissed.
48. With reference to paragraphs 6.3 (a), it is denied that the present invention is obvious and lacks an inventive step, in view of the prior art from the patented and non-patented literature and products in market.

49. With reference to paragraphs 6.3 (b), it is stated that US2005/0032903A discloses the method for controlling rust diseases in legumes such as soybeans by treatment of the aerial part of the plant legumes with a formulation containing strobilurin (claims 1 and 2). US'903 also discloses that such strobilurin fungicides could be used in combination with **another i.e. one other** fungicide, which may either be a contact fungicide or a systemic fungicide such as a conazole fungicide or a SDHI fungicide alternatively. This means that US'903 teaches use of only one of the second group of fungicides i.e. only envisages a dual combination but not a triple combination.
50. It is stated that the opponent has deliberately tried to misrepresent the disclosure of this document. US'903 never teaches the use of a second as well as a third fungicide, which is required according to the present invention. Even in paragraphs [0040] and [0082] (example), particularly the combination of strobilurins and azoles have been disclosed, but use of a contact fungicide such as mancozeb has not been disclosed. Page 2, paragraphs [0023], [0031] and [0037], teaches the combination of strobilurins with other fungicides such as mancozeb and chlorothalonil, but this disclosure does not include a conazole fungicide within the same combination.
51. Thus, it is clear from the above discussion that US'903 does not envisage a triple combination of fungicides as claimed in the present invention.
52. With reference to paragraphs 6.3 (c), it is stated that **Brazilian Patent PI0601469** describes a composition comprising an oil dispersed agricultural sprays composition comprising: 1) at least one fungicidal formulation which comprises a dithiocarbamate, triazole, morpholine or strobilurin compound; and 2) an oil soluble or oil compatible poly fatty acid-imine polymer.
53. A complete reading of BR'1469 suggest that primarily the composition disclosed in this reference relates to single active composition of either of dithiocarbamate, or of triazole, or of morpholine or of strobilurin compound (refer example 1-5 and 11-20) or of a dual combination of dithiocarbamate with either a triazole, or a morpholine or a strobilurin compound (i.e. double combination).
54. BR'1469 fails to disclose the triple combination of a multisite contact fungicide e.g. mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further this document also fails to disclose the use of such triple combination for treating soybean rust.
55. The opponent has deliberately tried to misrepresent the teachings of this prior art document. This document teaches agricultural sprays dispersed in oil comprising at

least one fungicide formulation containing a compound dithiocarbamate, **or** a triazole, **or** a morpholine **or** a strobilurin, but the opponent has misrepresented this teaching as a fungicide formulation containing a compound dithiocarbamate, **and** a triazole, **and** a morpholine **and** a strobilurin.

56. It is stated that claim 11 of BR'1469 teaches a combination of dithiocarbamate formulation and formulation of triazole or formulation of morpholine or formulation of strobilurin. This finding is also supported by Table 1 at page 7 of the complete specification of BR'1469. Table 1 teaches combination of dithiocarbamate + triazole, morpholine or strobilurin wherein weight percentage of dithiocarbamate is 1-30, preferably 5-25 and weight percentage of any one of triazole, morpholine or strobilurin is 0.1-10, preferably 0.5-5. BR'1469 does not teach a combination of dithiocarbamate formulation and one or more formulation of triazole, morpholine or strobilurin (i.e. combination of 3 fungicidally active agents).
57. Thus, it is submitted that a mistaken representation of the prior art teaching is not sufficient to sustain a case of *prima facie* case of obviousness.
58. With reference to paragraphs 6.3 (d), it is stated that WO2006/035316 discloses an aqueous composition suitable for applying fungicides to plant propagation materials comprising at least three fungicidally active compounds, as active ingredients, water and a blend of the following components, by weight: a) 2 - 10% of at least one surface-active agent, comprising a) at least one anionic surfactant; b) 0 - 10% of at least one polymer selected from water-dispersible and water-soluble film-forming polymers; c) 4 - 20% of at least one inorganic solid carrier; and, optionally d) 3 - 20% of at least one antifreeze agent.
59. WO'316 provides a long laundry list of fungicidally active ingredient which may be combined together. However the main combination of interest for WO'316 is fludioxonil, difenconazole and mefenoxam.
60. WO'316 fails to disclose the triple combination of a multisite fungicide e.g. mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further WO'316 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference WO'35316 does not anticipate present invention.
61. WO'316 discloses useful compositions dispersion of fungicides comprising at least one or at least three suitable fungicides for seed treatment. First of all, it is not obvious to use a combination meant for seed treatment in the actual field. Secondly,

the three fungicides to be combined according to WO'35316 are to be selected from a random list, and may not necessarily include a multisite fungicide with a dual combination including a conazole fungicide and a strobilurin fungicide. The preferred combinations according to this document are fludioxonil, mefenxam **and** azoxystrobin **or** difenconazole. Thus, this document does not teach the presence of a multisite fungicide such as mancozeb essentially, and considers strobilurins or conazoles as being alternatives to each other i.e. they are not taught to be used together.

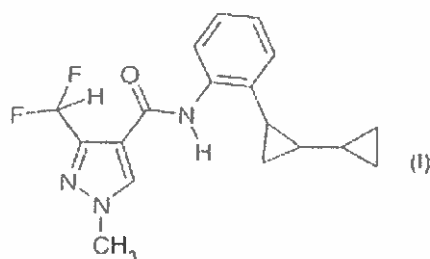
62. This document fails to disclose the triple combination of Mancozeb with combination of first and second systemic fungicides selected from quinone outside or inside inhibitors, demethylation inhibitors, or succinate dehydrogenase inhibitors. Further this document also fails to disclose the use of such triple combination for treating soybean rust.
63. With reference to paragraphs 6.3 (e), it is stated that US2005/0226902 discloses a chemical soil disinfestation method in which a fungicidally, insecticidally or nematocidally active amount of a mixture M comprising a component a) of a1) 20 to 96% by weight of sulfur, a2) 4 to 80% by weight of a complexing agent selected from the group consisting of: nonpolymeric complexing compounds: EDTA (ethylenediaminetetraacetic acid), NTA (nitrilotriacetic acid), and, if appropriate, one or more crop protection agents b) and/or further additives c) is applied.
64. The main crux of the US'902 relates to combination of Sulfur with complexing agent and optionally any other active ingredient.
65. US'902 does not even disclose a combination of Sulphur with a dual combination of systemic fungicides. Further US'902 fails to disclose the triple combination of any other multisite fungicide with combination of first and second systemic fungicide selected from quinone outside or inside inhibitors, demethylation inhibitors or succinate dehydrogenase inhibitors. Further US'902 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference US'902 does not anticipate present invention.
66. With reference to paragraphs 6.3 (f), it is stated that US2010/197741 allegedly claims a composition comprising fungicide boscalid (SDHI fungicide systemic), Chlorothalonil (fungicides of multiple sites) and at least one other fungicide.

67. The Applicant states that US'741 discloses a fungicidal composition for controlling phytopathogenic harmful fungi, comprising 1) Boscalid, 2) Chlorothalonil and 3) at least one fungicidally active compound III selected from groups A) to F):
- A) an azole
 - B) a strobilurin
 - C) a carboxamide
 - D) a heterocyclic compound
 - E) a carbamate selected
 - F) a fungicide selected
68. US'741 fails to disclose the triple combination of a multisite fungicide such as dithiocarbonate such as mancozeb with combination with first systemic fungicide and second systemic fungicides selected from individual quinone outside or inside inhibitors, demethylation inhibitors or succinate dehydrogenase inhibitors. Further US'741 also fails to disclose the use of such triple combination for treating soybean rust.
69. Applicant states that the amended claims presented with the response to First Examination report (**Annexure A**) do not include chlorothalonil as the contact fungicide. Therefore, there is no overlap of the amended claims of the present invention with the disclosure of US'741.
70. With reference to paragraphs 6.3 (g), it is stated that **WO2007/052256** teaches a pesticidal composition in the form of a water-dispersible granule comprising a) at least one pesticidal active ingredient, b) at least one surfactant, c) at least one dispersing agent, and optionally d) a filler, obtainable by a process comprising of mixing all the ingredients of the premix to obtain a homogenous premix; micronizing the premix; wetting the micronized premix; granulating the micronized wet premix; drying the granules and treating said granules with a surfactant agent.
71. This is a platform technology patent application. The main crux of the invention is to provide a water dispersible granule composition which may be applicable for number of active ingredients disclosed therein. WO'256 is not relevant to present invention as WO'256 fails to disclose the triple combination of a multisite fungicide with individual quinone outside or inside inhibitors, demethylation inhibitors or succinate

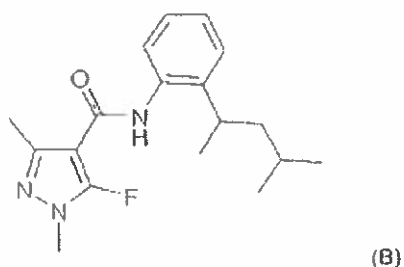
dehydrogenase inhibitors. Further WO'256 also fails to disclose the use of such triple combination for treating soybean rust.

72. It is pertinent to note that the presently amended claims does not falls within the purview of WO'256. WO'256 discloses formulations containing a single active ingredient. The choice of the single active ingredient may be wide and may include the contact fungicide, the conazole fungicide, the strobilurin fungicide or the SDHI fungicide. Still, these fungicides are to be formulated in isolation according to WO'256, and are not to be used in any combination. Applicant fails to understand as to how this disclosure is even relevant to the subject matter of the present invention.
73. With reference to paragraphs 6.3 (h), it is stated that US2012/003199 discloses a method for controlling harmful fungi, which method comprises subjecting plants to be protected against fungal attack to two or more sequential treatment blocks, where at least one treatment block comprises treating the plants with at least one synthetic fungicide and at least one treatment block comprises treating the plants with at least one biological control agent, with the proviso that the last treatment block comprises treating the plants with at least one biological control agent selected from *Bacillus subtilis* and metabolites produced therefrom.
74. US'199 essentially relates to combination of a synthetic fungicide and a biological control agent such as *Bacillus subtilis*. Biological agents are critical and essential part of invention disclosed in US'199. The present invention does not disclose any biological agents to be used in the combination disclosed therein.
75. US'199 fails to disclose the triple combination of a multisite fungicide with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further US'199 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference D7 does not anticipate present invention.
76. US'199 discloses the list of synthetic fungicides that includes systemic fungicides, such as azoles, strobilurins, carboxamides, and fungicides from multiple sites. US'199 discloses that these fungicides are to be used in isolation, along with a biological control agent. A mere listing of these fungicides does not mean that these fungicides are taught to be used in combination. The opponent has deliberately attempted to misrepresent the teachings of US'199, whereas in reality, this document does not teach the use of more than one fungicide in combination.

77. With reference to paragraphs 6.3 (i), it is stated that **WO2008/049575** teaches method for controlling Asian soybean rust comprising (α) applying a pesticidal composition (A) to a glyphosate tolerant soybean plant propagation material, and (β) applying a pesticidal composition (B) to the resulting soybean plant, part of plant and/or the locus thereof one or more times: i) before emergence, ii) after emergence, or iii) both (i) and (ii), provided that composition (A) comprises one or more of flutriafol, triticonazole, tebuconazole, ipconazole, epoxyconazole, orysastrobin, prothioconazole fluoxastrobin, azoxystrobin, furametpyr, cyproconazole, a compound of formula (I) and a compound of formula (B), and pesticide composition (B) comprises glyphosate; wherein compound (I) is represented by



or a tautomer of such a compound, and compound (B) is represented by formula



or a tautomer of such a compound.

78. WO'575 teaches use of glyphosate in combination with the fungicides such as flutriafol, triticonazole, tebuconazole, ipconazole, epoxyconazole, orysastrobin, prothioconazole fluoxastrobin, azoxystrobin, furametpyr, cyproconazole. There is no suggestion that mancozeb or dithiocarbamate fungicides or multi-site fungicides are an important addition to the composition. Moreover, the compounds flutriafol, triticonazole, tebuconazole, ipconazole, epoxyconazole, orysastrobin, prothioconazole fluoxastrobin, azoxystrobin, furametpyr, and cyproconazole are suggested to be alternatives to each other. They are not taught by WO'575 to be used in combination, which is the essence of the present invention. The present invention on the other hand, teaches the importance of multi-site fungicides as being an important element of the

present invention and does not mention the use of glyphosate. The inventors in WO'575 disclose a combination of fungicides with glyphosate for the control of Asian soybean rust in glyphosate resistant soybean. WO'575 fails to teach multi-site fungicides as an essential part of the invention, WO'575 fails to teach only foliar applications. The novel and inventive feature of the claimed combination reside in the use of multi-site fungicides such as dithiocarbamates in combination with demethylation inhibitor fungicides and Quinone outside or inside inhibitor fungicides for the treatment of soybean rust, specifically so as to synergize the effect of the combination of these actives when used to control Asian soybean rust.

79. WO'575 fails to disclose the triple combination of Mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further WO'575 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference WO'575 does not render the present invention obvious.

80. WO'575 teaches use of glyphosate in combination with the fungicides such as flutriafol, triticonazole, tebuconazole, ipconazole, epoxyconazole, orysastrobin, prothioconazole fluoxastrobin, azoxystrobin, furametpyr, cyproconazole. There is no suggestion that mancozeb or dithiocarbamate fungicides or multi-site fungicides are an important addition to the composition. Moreover, the compounds flutriafol, triticonazole, tebuconazole, ipconazole, epoxyconazole, orysastrobin, prothioconazole fluoxastrobin, azoxystrobin, furametpyr, and cyproconazole are suggested to be alternatives to each other. They are not taught by WO'575 to be used in combination, which is the essence of the present invention.

81. The present invention on the other hand, teaches the importance of multi-site fungicides as being an important element of the present invention and does not mention the use of glyphosate. The inventors in WO'575 disclose a combination of fungicides with glyphosate for the control of Asian soybean rust in glyphosate resistant soybean. WO'575 fails to teach multi-site fungicides as an essential part of the invention, WO'575 fails to teach only foliar applications. . The novel and inventive feature of the claimed combination reside in the use of multi-site fungicides such as dithiocarbamates in combination with demethylation inhibitor fungicides and Quinone outside or inside inhibitor fungicides for the treatment of soybean rust, specifically so as to synergize the effect of the combination of these actives when used to control Asian soybean rust.

82. WO'575 fails to disclose the triple combination of Mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further WO'575 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference WO'575 does not render the present invention obvious.
83. With reference to paragraphs 6.3 (j), it is stated that WO03/022049 discloses an agricultural composition comprising at least one agriculturally active compound; at least one alkyl polyglycoside; at least one anionic surfactant selected from a polyarylphenol polyalkoxyether sulfate and a polyarylphenol polyalkoxyether phosphate; and at least one basic compound; wherein the at least one anionic surfactant is neutralized to the inflection point in the titration curve with the at least one basic compound.
84. WO'049 is the platform technology patent. The main crux of the invention is to provide a composition comprising alkyl polyglycoside; alkyl polyglycoside and basic compound. WO'049 fails to disclose the triple combination of multisite fungicides such as mancozeb with individual quinone outside inhibitors, demethylation inhibitors and succinate dehydrogenase inhibitors. Further WO'049 also fails to disclose the use of such triple combination for treating soybean rust.
85. WO'049 merely lists the active ingredients that may be used in isolation in the disclosed formulation. It does not exemplify even a single formulation that comprises a combination of two or more active ingredients. WO'049 does not disclose that the compounds can be used alone or may be used in combinations of 2, 3, 4, 5, 6, 7 or more. The sentence at page 12, paragraph 3 is a mere speculative statement, but no exemplification has been provided. A mere speculative statement in the prior art in question is not enough to sustain a case of obviousness.
86. With reference to paragraphs 6.3 (k), it is stated that WO03/005816 discloses a method of producing lignin-based matrix microparticles for the controlled release of an agricultural active, the method comprising the steps of: forming an emulsion of an organic solution in an aqueous solution, wherein the organic solution contains a lignin derivative and an agricultural active in a volatile organic solvent and the aqueous solution contains an emulsifier; and removing the organic solvent, thereby producing microparticles having a matrix comprising the lignin derivative within which the agricultural active is distributed.

87. WO'816 is also a platform technology patent. The main invention lies in the process of forming a lignin-based matrix microparticles. All the examples relates to Imidacloprid microparticles. A laundry list of active ingredient is mentioned in WO'816 from which a person skilled in the art can pick and choose a active ingrdient and make a lignin-based matrix microparticles. WO'816 is nowhere relevant to present invention, and as such deserves to be discarded.
88. Further WO'816 also fails to disclose the use of such triple combination for treating soybean rust. In light of these difference WO'816 does not anticipate present invention.
89. WO'816 discloses the list of active compounds that includes agricultural fungicides of multiple sites, such as dithiocarbamates, phthalimides, sulphonamides, guanidine's and systemic fungicides, such as DMI: triazoles, Strobilurin compounds and their mixtures, but the opponent fails to mention that these fungicides are to be used in isolation. They are not taught to be used in combination with each other.
90. Applicant states that by repeatedly misrepresenting the contents of the cited prior art, the opponent mischaracterizes the present invention, as also mistakenly making out a case of obviousness where none exists. WO'816 never teaches using the fungicides in combination, which the present invention requires. A mere linear listing of the fungicides in question is not sufficient to sustain the ground of obviousness where the prior art does not teach using these fungicides in the exact combination which the presently amended claims require.
91. With reference to paragraphs 6.3 (I), it is stated that US2002/173529 discloses dual combinations i.e. combinations comprising only two active ingredients. The compound of formula (I) is US'529 is prothioconazole. US'529 thus discloses combinations comprising prothioconazole, and a specific triazone derivative of formula II, or tebuconazole, or propineb, or mancozeb, or azoxystrobin and so on.
92. There is no disclosure or hint in US'529 towards triple combinations. The present invention does not claim to have prepared combinations of prothioconazole and mancozeb for the first time. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'529 does not envisage any triple combination of fungicides.

93. With reference to paragraphs 6.3 (m), it is stated US2012/088665 again discloses irrelevant dual combinations. These combinations include a sulfur-containing triazole compound, and at least another fungicide selected from strobilurins, carboxamides, azoles, carbamates and dithiocarbamates, growth regulators, herbicides and insecticides. The presently claimed invention does not require the presence of a sulfur-containing triazole compound.
94. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'665 does not envisage any triple combination of fungicides.
95. The list of compound II includes systemic fungicides, but they are stated to be alternatives to each other and are never exemplified to have been used in combination. The opponent thinks that a mere listing of the fungicides in the prior art is sufficient to sustain the ground of obviousness, but this is not true. Applicant states that in order to sustain the ground of obviousness, more than a mere listing of the fungicides in question is required. It requires a teaching of how to combine these fungicides, which is absent in the present case. Thus, the ground of obviousness sought to be made by the opponent is unsustainable.
96. With reference to paragraphs 6.3 (n), it is stated US6,569,809 discloses a concentrated formulation comprising glyphosate and flumioxazin. US'809 further discloses that instead of glyphosate, certain other water soluble active ingredients may be present, and instead of flumioxazin also, certain other water insoluble herbicides may be present. It is not clear to the applicant as to how the disclosure of this reference is even remotely relevant to be subject matter of the present invention.
97. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'809 does not envisage any triple combination of fungicides.
98. The list of additional compounds disclosed in US'809 includes systemic fungicides, but they are stated to be alternatives to each other and are never exemplified to have been used in combination. The opponent thinks that a mere listing of the fungicides in the prior art is sufficient to sustain the ground of obviousness, but this is not true. Applicant states that in order to sustain the ground of obviousness, more than a mere listing of the fungicides in question is required. It requires a teaching of how to

combine these fungicides, which is absent in the present case. Thus, the ground of obviousness sought to be made by the opponent is unsustainable

99. With reference to paragraphs 6.3 (o), it is stated that US2012/0129696 relates to a method for increasing the level of free amino acids in the storage tissues of perennial plants by the application of a strobilurin compound, optionally along with another fungicide which may be carboxamides, azoles, carbamates and other inorganic compounds. Thus, it would have been absolutely clear to anybody who would have read US'696 that it relates to double combinations comprising a strobilurin and another fungicide.

100. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'696 does not envisage any triple combination of fungicides, nor its use for the control of soybean rust.

101. The opponent has again and again cited a mere listing of the fungicides in the prior art as being sufficient to sustain the ground of obviousness, but this is not true. Applicant states that in order to sustain the ground of obviousness, more than a mere listing of the fungicides in question is required. It requires a teaching of how to combine these fungicides, which is absent in the present case. Thus, the ground of obviousness sought to be made by the opponent is unsustainable.

102. With reference to paragraphs 6.3 (p), it is stated US2013/0309327 again discloses dual combinations. It discloses combinations of sulphur with a second active ingredient which is either strobilurin or a conazole, but according to the disclosure of US'327, it cannot be both strobilurin and conazole. Thus, it would have been absolutely clear to anybody who would have read US'327 that it relates to double combinations comprising sulphur and a second fungicide.

103. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'327 does not envisage any triple combination of fungicides, nor its use for the control of soybean rust.

104. With reference to paragraphs 6.3 (q), it is stated that US2008/269174 is irrelevant to the presently claimed subject matter. It discloses combinations comprising tebuconazole and at least one of another set of compounds which includes

azoxystrobin. The disclosed second set of fungicides does not include mancozeb. Thus, US'174 fails to disclose combinations comprising mancozeb, a strobilurin fungicide and a conazole fungicide. US'174 essentially discloses double combinations comprising tebuconazole and a second fungicide.

105. The present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. US'174 does not envisage any triple combination of fungicides, nor its use for the control of soybean rust.
106. Although US'174 discloses strobilurins, and other multisite contact fungicide in the list of possible fungicide B, it does not teach including more than one type of the fungicide B in combination with tebuconazole. The listing of strobilurin and multisite fungicides as fungicide B is in the alternative i.e. if one is used the other is not used. This is away from the concept of using them in a combination.
107. With reference to paragraphs 6.3 (r), it is stated that **New fungicides for grapevine powdery mildew management; Gary g. Grove and Mark E. Nelson; 2011** does not disclose fungicides for the control of soybean rust, but for grapevine powdery mildew. In its closest approach to the subject matter of the present invention, this article discloses the combination of tebuconazole and trifloxystrobin, but mancozeb or any other multisite fungicide at all is not disclosed to be used in this combination. In fact, this article does not teach any combination comprising mancozeb at all.
108. The Applicant further states that this article always refers to a double combination of fungicides i.e. either one multisite with one systemic fungicide, or two systemic fungicides. There is no disclosure of a triple combination of fungicides including at least one multisite fungicide with at least two systemic fungicides.
109. In contrast the present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides. This article does not envisage any triple combination of fungicides, nor its use for the control of soybean rust.
110. With reference to paragraphs 6.3 (s) and (t), it is submitted that to make such specific selection of one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides to reach at the composition of the present

invention an element of hindsight will be involved and it is therefore submitted that the present invention is not obvious for a person skilled in the art by the teaching of prior art as mentioned herein above alone or in combination with each other and therefore it is stated that the present invention involve “inventive step” as mentioned in section 2(1)(ja) of the Patents Act, 2005,

111. With reference to paragraphs 6.3 (u), it is stated in the view of the above submission the present invention is not obvious for a person skilled in the art in view of the combine teachings of the prior art documents Annexure II-XVIII to arrive at the composition claimed in amended claim 1 and method for treatment as claimed in amended claim 9.

112. The Applicant states that as evident, from above submission that the non-disclosure of the claimed combinations in the cited prior art implies that the present invention would have been non-obvious and involves an inventive step. It has been seen above that almost all of the above cited prior references disclose only a double combination of fungicides. None of the disclosed combinations envisage the presence of a multisite fungicide, specifically mancozeb, in this combination. None of the disclosed combinations recite a triple combination of fungicides including a multisite fungicide such as mancozeb. In contrast, the present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicide, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides.

113. Without prejudice to anything aforesaid, applicant states that the presently claimed invention involves an inventive step, and relies on the Expert Affidavit Dr. Gilson Oliveira in its entirety filed with the Response to the First Examination Report of the instant application as being part and parcel of the present reply statement filed with the Response to the First Examination Report of the instant application as being part and parcel of the present reply statement, and adopts the reasoning provided by Dr. Oliveira as a part of the applicant's reply statement, which are not repeated herein merely for the sake of brevity. Applicant annexed herewith a copy of affidavit of Dr. Gilson Oliveira as Annexure B.

114. Relying on the evidence set forth by Dr. Oliveira, applicant states that the presently amended claims involve an inventive step:

1. Because none of the cited prior art discloses a triple fungicidal combination comprising at least one multisite fungicide and at least two

systemic fungicides selected from a quinone outside inhibitor, a quinone inside inhibitor, a demethylation inhibitor and a succinate dehydrogenase inhibitor, or a method for treating soybean rust in a host leguminous plant by applying these combinations;

2. Because the prior art envisaged the dual combination of systemic fungicides without the multisite contact fungicides; and because the efficacy of these systemic fungicides had been reducing gradually with usage over the years;
3. Because the presence of a multisite fungicide as recited reversed this gradual decline in the fungicidal efficacy of the systemic fungicides;
4. Because the efficacy of cyproconazole + azoxystrobin mixture over six seasons reduced from 90% in 2003/04 to 2013/14 efficacy of only 41% control, and because of a multisite fungicide, this declining trend was reversed;
5. Because for cyproconazole + picoxystrobin mixture over six seasons, the efficacy reduced by 9.0% per year, reaching 37% control crop in 2013/14, and because of a multisite fungicide, this declining trend was reversed;
6. Because for epoxiconazole + pyraclostrobin mixture over six seasons, from the 2009/10 harvest the effectiveness of this mixture has been decreased by 12.6% per year, reaching the last harvest 23%, and because of the presence of a multisite fungicide, this trend was reversed;
7. Because from 2008/09 the average effectiveness of mixtures "cyproconazole + azoxystrobin", "cyproconazole + picoxystrobin" and "epoxiconazole + pyraclostrobin" over six crops has been reduced by 9.0% per year, reaching 37% in the last harvest, and because this trend was reversed due to the addition of a multisite contact fungicide;
8. Because the experimental results of Dr. Oliveira demonstrate that the addition of the multisite fungicide such as mancozeb fungicide not only enhances the percentage control, but also reverts the observed control to the original level of 80%;
9. Because the efficacy of these known dual combinations decayed by more than 50%, leading to the cancellation of these registrations by the Brazilian

regulatory authority MAPA, leading to the failure of these fungicides, and that this failure was cured only by the addition of a multisite contact fungicides;

10. Because an independent and unbiased study commissioned by EMBRAPA i.e. the Brazilian Agricultural Research Corporation, found post-filing of the present invention that the addition of a multisite fungicide e.g. mancozeb to the combination of demethylation inhibitor + quinone outside inhibitor reverses the decay of efficacy of the known combination of demethylation inhibitor + quinone outside inhibitor and reverts it to the original known efficacy or better efficacy;
11. Because it was observed that in the presence of mancozeb, there was 15 to 30% reduction in the percentage incidence of soybean rust;
12. Because these combinations comprising mancozeb in addition to a demethylation inhibitor and a quinone outside inhibitor were approved by EMBRAPA for the control of soybean rust, whereas 63 other existing products failed to successfully control Asian soybean rust in the absence of a multisite fungicide;
13. Because the resulting triple combination of a multisite contact fungicides and at least two systemic fungicides were also synergistic according to the Colby's formula;
14. Because it was found that mancozeb, a multisite fungicide, surprisingly enhances the efficacy in a triple combination (present invention) more than it does in a double combination (prior art). In the double combinations (prior art), mancozeb was found to actually antagonize the efficacy of either a strobilurin or a conazole fungicide, but its efficacy enhancing role was consistently greater than 20% or 30%, and being near about 50% - 60% when present in the triple combinations of the present invention;
15. Because the exact combination of fungicides as claimed is critical and the addition of an optional other insecticide tended to antagonize the combination;
16. Because fipronil, an insecticide, when sprayed together with Trifloxistrobin + Ciproconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluations of 7 days after third

- spray (7 DAC) evaluation, and thus the exactly claimed combination was critical for synergistic effect;
17. Because fipronil, when sprayed together with Azoxistrobin + Ciproconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluations of 7 DAC and thus the exactly claimed combination was critical for synergistic effect;
 18. Because fipronil, when sprayed together with Trifloxistrobin + Tebuconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluation of 7 DAC and thus the exactly claimed combination was critical for synergistic effect;
 19. Because antagonism was observed in most of the evaluations in mixtures of strobilurin fungicide + conazole fungicide + Mancozeb with Fipronil;
 20. Because the addition of fipronil an extraneous insecticide, was found to antagonize the fungicidal efficacy of a triple combination of fungicides consisting of mancozeb, a strobilurin fungicide and a conazole fungicide, and because the triple combination as claimed herein was critical in the presence of the three fungicides exactly as recited herein;
 21. Because the role of the multisite contact fungicides in dual combinations was substantially different and inferior vis-à-vis its role in triple combinations of the present invention;
 22. Because the addition of mancozeb, to either a strobilurin fungicide or a conazole fungicide, did not significantly enhance the efficacy of the strobilurin or the conazole fungicide in isolation, but in fact was almost always antagonistic, being mildly additive (about +1.1%) to being critically antagonistic (varying from about -1.5 to about -10.0), but the benefits of adding mancozeb to a double combination of systemic fungicides i.e. a combination comprising a conazole fungicide and a strobilurin fungicide, was unexpected and surprising;
 23. Because upon addition of mancozeb, to a strobilurin fungicide and a conazole fungicide combination, the resulting efficacy was surprisingly greater than what was expected from a knowledge of the improvement accruing to the efficacy of either the strobilurin fungicide or the conazole fungicide in isolation, and that it was consistently greater than 20% (i.e.

double the improvement in the isolated strobilurin or the conazole fungicide), mostly greater than 30% (i.e. triple the improvement in the isolated strobilurin or the conazole fungicide), and preferably greater than 50% or about 60% (i.e. 5 or 6 times the improvement in the isolated strobilurin or the conazole fungicide);

24. Because the association of mancozeb with commercial products based in mixtures of pyraclostrobin and epoxiconazole, picoxystrobin and ciproconazole and azoxystrobin and ciproconazole increased the yield synergistically;
25. Because the presence of a multisite contact fungicide such as mancozeb reduced the sensitivity reduction factor (SRF) of the systemic fungicides to less than 1.0;
26. Because it was mathematically found that for mancozeb, the calculated resistance factor (FR) was the lowest value compared to other products, which may suggest, due to its non-specific mode of action, that the active exerted a lower or no selection pressure;
27. Because it was found that the greater adoption of multisite fungicides (mancozeb) in Rio Grande do Sul State help to keep the sensitivity reduction factor of azoxystrobin, trifloxystrobin, ciproconazol and tebuconazol less than in Paraná State i.e. led to a better efficacy;
28. Because the increase in the sensitivity reduction factor in Parana state, where mancozeb use was not prevalent, was 90.62%, whereas in Rio Grande state where mancozeb use was prevalent, it was only 39.91%;
29. Because it was found that a lower multisite fungicide i.e. mancozeb adoption led to a greater resistance to the fungicides, in turn leading to lower fungicidal efficacy in Parana State, whereas, a greater mancozeb adoption led to a lower resistance to the fungicides, in turn leading to significantly higher fungicidal efficacy in Rio Grande do sul State;
30. Because for the first time, by way of the present invention, it was scientifically possible to correlate the sensitivity reduction factor to the adoption of mancozeb used in the treatment used to control the fungal infection;

31. Because the presence of a multisite contact fungicide surprisingly reduces the phytotoxicity due to the resulting triple combination;
32. Because from the phytotoxicity results, it was seen that 7 days after fourth spray (7 DAT D), a significant reduction of phytotoxicity using Mancozeb in association of a demethylation inhibitor fungicide namely Prothioconazole in combination with Trifloxystrobin was noted;
33. Because, it was found surprisingly found that mancozeb reduced the phytotoxicity of prothioconazole alone by 72.2% and 75.5%, but in a combination of systemic fungicides, mancozeb caused to reduce the phytotoxicity of the combination by 100%, 84.4% and 77.5% respectively, and thus because the contribution of mancozeb in reducing the phytotoxicity of a double combination of systemic fungicides is more than its contribution in reducing the phytotoxicity of single systemic fungicide in isolation;
34. Because, like mancozeb, chlorothalonil also increased the efficacy of a dual combination of systemic fungicides from between 1.5% to 20.0%, and more importantly, the trend of decay in the efficacy of the systemic fungicides combination was reversed;
35. Because, like mancozeb, copper oxychloride also increased the efficacy of a dual combination of systemic fungicides by about 25.0%, and more importantly, the trend of decay in the efficacy of the systemic fungicides combination was reversed;
36. Because of the secondary evidence set forth by Dr. Oliveira in the form of a reasonable expectation of a person skilled in the art;
37. Because prior to the publication of the present application, there had been a general scientific prejudice in the state of the art against the use of multisite fungicides in soybean rust control program, and because the publication of the present application decisively marked an end to this scientific prejudice, to the extent that the current thinking in the scientific community took a dramatic turn immediately after the publication of the present application;
38. Because Dr. Claudia Godoy's research publications prior to November 2015 being **Papers 1 – 15**, no mention could be found regarding the use of

a multisite fungicide for controlling soybean rust, but in **Papers 16 – 21** i.e. post publication of the present application, Dr. Godoy has independently confirmed what the present inventors found three years earlier i.e. in 2013 that the inclusion of a multisite contact fungicide in addition to the **mixture of triazoles and/or strobilurins and/or SDHIs** leads to surprisingly beneficial effect;

39. Because the post-published Paper 16 proves that in a combination of picoxystrobin + cyproconazole, the addition of multisite fungicides copper oxychloride (16%), chlorothalonil (15%), mancozeb (15%), copper sulfate (19%), propineb (19%), and fluazinam (20%) leads to a surprising increase in efficacy of the double combination of systemic fungicides;
40. Because Paper 16 also proves that this improvement was seen for the combination of azoxystrobin and SDHI fungicide benzovindiflupyr;
41. Because Papers 17 – 21 also demonstrate the increase in the efficacy of the systemic fungicide combination due to the addition of a multisite fungicide;
42. Because, as opinionated by Dr. Oliveira, the use of a multisite contact fungicide for treating soybean rust is a very unconventional idea, one that would not occur to a skilled agronomist without the benefit of hindsight, and that these multisite fungicides were being used historically for the control of soybean rust, but with the advent of strobilurin, conazole and SDHI class of fungicides, the multisite contact fungicides were superseded by the later discovered systemic fungicides, thus, reconsidering the use of multisite fungicides for soybean rust control would have been akin to moving backwards in technology, and thus unlikely to have been considered seriously by a person skilled in the art.
43. Because the pathogen for rust penetrates and colonizes the leaf tissue, forming necrotic lesions, and due to its penetrative nature, it has always been treated with systemic fungicides i.e. fungicides absorbed within the plant tissues while multisite contact fungicides have not been recommended, or have been discouraged, from being used in rust control;
44. Because the Preez PhD thesis clearly discourages the use of contact fungicides in rust control;

45. Because Papers 8 and 11 also concludes that the addition of a foliar fungicide treatment regimen did not have a favorable influence on the standard known treatment regime of **strobilurin + conazole** fungicides for the control of soybean rust, contrary to the present invention;
46. Because **Paper 11** concludes that - *Fungicide trials in India (Patil and Anahosur, 1998) identified several triazole compounds, hexaconazole, triadimefon, propiconazole and difenoconazole, which performed better than mancozeb, tridemorph and chlorothalonil*, which discussion clearly discourages a person skilled in the art from considering the use of contact fungicides in soybean rust control programs;
47. Because considering Dr. Claudia Godoy's publications on rust control before and after the publication of the present application, Dr. Oliveira concludes that the publication of this application did precipitate a turnaround in the collective thinking of the state of the art towards the role to be played by a multisite contact fungicide in the control of rust.

112 With respect to the ground of Section 25(1)(d) (paragraph 6.4 (a) to 6.4 (g)), it is stated that **None** of these cited prior commercial products envisages any triple combination of fungicides, nor its use for the control of soybean rust as explained below.

- Avtar (zineb 68% + hexaconazole 4% WP). MERGER (tricyclazole 18% + mancozeb 62% WP). Native (trifloxystrobin to 25% + tebuconazole to 50%). Unicorn DF (tebuconazole to 4.5% sulfur content to 70%).
- AVTAR is again a **dual combination** of fungicides. It discloses a combination of zineb and hexaconazole. Zineb is not mancozeb. Secondly, it does not recite the presence of a strobilurin fungicide.
- MERGER discloses a **double combination** comprising mancozeb and tricyclazole. It does not envisage the presence of a strobilurin fungicide in this combination.
- NATIVE discloses a **double combination** comprising trifloxystrobin and

tebuconazole. It does not envisage the presence of a multisite fungicide, specifically mancozeb, in this combination.

- UNICORN again discloses a **double combination** comprising tebuconazole and sulphur. It does not envisage the presence of a multisite fungicide, specifically mancozeb, or a strobilurin fungicide, in this combination.

113 In contrast the present invention relates to a triple combination comprising three fungicides: at least one multisite contact fungicides, and at least two systemic fungicides: selected from strobilurin fungicides, or conazole fungicides or succinate dehydrogenase inhibitor fungicides.

114 It is therefore stated that none of these cited prior commercial products envisages any triple combination of fungicides or its use for the control of soybean rust.

115 With respect to the ground of Section 25(1)(f) (paragraph 6.5 (a)), the applicant states that as demonstrate herein above the present invention is novel, inventive and technical advanced over the prior art and show economic significance and the present invention is not obvious for a person skilled in the art.

116 With respect to the ground of Section 25(1)(f) (paragraph 6.5 (b)), the applicant states that the opponent has alleged that the present invention does not involve any inventive step, is obvious and does not have any industrial application. It is stated that such allegations are totally baseless and without any support.

117 With respect to the ground of Section 25(1)(f) (paragraph 6.5 (c)), it is stated that the applicant has demonstrated herein above that the present invention is novel and inventive in view of the teachings of prior art (Annexure II, III, IV, V, VI, VII and VIII as well as Annexure IX to XXIII).

118 The Applicant states the inventive step of the present invention (significant feature/component/characteristic) is also supported by the Expert Affidavit of Dr. Gilson Oliveira whereas the opponent fail to file any expert evidence to show that the present invention does not involve any inventive step and is obvious for a person skilled in the art.

119 With respect to the ground of Section 25(1)(f) (paragraph 6.5 (d)), it is stated that the applicant has demonstrated herein above that the present invention is novel in view

of the teachings of prior art (Annexure II-VIII) and inventive in view of the teachings of Annexure II to XXIII.

- 120 With respect to the ground of Section 25(1)(f) (paragraph 6.5 (e)), it is respectfully submitted that According to **Section 3(d)** of the Patents Act, 1970 “*the mere discovery of a new form of a known substance which does not result in the enhancement of known efficacy of that substance or the mere discovery of any new property or new use for a known substance or of the mere use of a known process, machine or apparatus unless such known process results in a new product or employs at least one new reactant*” is not patentable under this Act.
- 121 It is submitted that the presently amended claims relates to a composition comprising atleast three fungicides (i.e. selected from multi-site contact fungicide, a first systemic fungicide; and an additional second systemic fungicide).
- 122 The Applicant relies upon the submission made hereinbefore to show that the presently amended claims are novel and inventive and the same is not reproduced below for the sake of brevity.
- 123 It is therefore submitted that presently amended claims are novel and inventive and possesses improved efficacy and therefore does not relates to new form of the known substance or new use of a known substance.
- 124 In view of the above submission the present invention does not attract clause (d) of Section 3(d) of the Patent Act.
- 125 The Applicant further relies upon the **Expert Affidavit of Dr. Gilson Oliveira** to demonstrate improved efficacy of the composition of the present invention over commercial known products.
- 126 With respect to **section 3 (e)** of the Patent Act, it is respectfully submitted that According to Section 3(e) of the Patents Act, 1970 “*a substance obtained by a mere admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance*” is not patentable under this Act.
- 127 It is submitted that the presently amended claims relates to a composition comprising atleast three fungicides (i.e. selected from multi-site contact fungicide, a first systemic fungicide; and an additional second systemic fungicide).
- 128 It is submitted that the addition of dithiocarbamate [multi-site contact fungicide] to mixture of strobilurin and triazole fungicides [demethylation inhibitor (DMI) + quinine outside inhibitor (QoI)] increase disease control and improved yield of plant. Complete specification at page 24 to 26 (Examples) shows that the addition of a

multi-site contact fungicide such as Mancozeb acted a synergist to the combination products registered for the treatment of Asian soybean rust.

- 129 The Applicant further relies upon **Expert Affidavit of Dr. Gilson Oliveira** to demonstrate the synergistic effect of the composition of the present invention. The opinion of Dr. Oliveira under the ground of inventiveness is relied upon herein to show that the presently claimed invention does not attract Section 3(e) of the Act.
- 130 It is therefore submitted that the composition claimed in the present invention is not a mere admixture and provides synergistic effect to control soybean rust.
- 131 With reference to **section 3 (h)** of the Patent Act, According to Section 3(h) of the Patents Act, 1970 "*a method of agriculture or horticulture*" is not patentable under this Act.
- 132 It is submitted that the presently amended claims relates to a composition comprising atleast three fungicides (i.e. selected from multi-site contact fungicide, a first systemic fungicide; and an additional second systemic fungicide) and a method for treating soybean rust in a host leguminous plant.
- 133 It is further submitted that the present invention as amended does not relate to a method of agriculture or horticulture per se, but relates to a method of for controlling fungus at a locus and therefore it is stated that the present invention does not attract clause (h) of Section 3.
- 134 With respect to the ground of Section 25(1)(g) (paragraph 6.6), it is stated that the impugned application sufficiently and fairly describe the invention in a manner so as to enable a person skilled in the art to perform or work the invention. The claims have been amended and are sufficiently based on the matter disclosed in the complete specification.
- 135 The applicant further states that in view of the data provided by Dr. Oliveira, the amended claims are now supported by sufficient experimental data with sufficient examples of fungicides from each selected class so as to make it more than plausible that the invention as described works as intended for the entire class of the selected fungicides.
- 136 In view of the above arguments it is stated that the complete specification and amended claims meet the sufficiency requirement of the complete specification in order to be performed present invention by a person skilled in the art.
- 137 With respect to the ground of Section 25(1)(g) (paragraph 6.7), it is respectfully submitted that the **petition under rule 137** is submitted herewith to condone the

delay in providing particulars of foreign filings corresponding to the instant application and fresh form 3 with updated status is submitted herewith the same may be kindly taken on record.

138 With respect to the ground of Section 25(1)(k) (paragraph 6.8) it is stated that the as explained hereinabove that the present invention is novel and inventive and therefore the present invention is not anticipated having regards to the knowledge, oral or otherwise, available within any local or indigenous community in India or elsewhere, in the world. It is therefore stated that the ground of 25(1) (k) ought to be dismissed.

139 In view of the above the applicant vehemently denies the allegations put forward by the opponent as misconstrued and baseless and without any merit and is totally directed to delay the grant of the present application. The opposition may accordingly be dismissed with exemplary cost against the opponent.

140 **The Applicant prays for:**

- (i) that the reply statement be taken on record along with the supporting evidence by Dr. Gilson Oliveria;
- (ii) that the opposition by way of representation be rejected in toto;
- (iii) that leave to provide further expert evidence, if required, be granted;
- (iv) that an early hearing be fixed;
- (v) that the opposed application be proceeded to grant;
- (vi) that exemplary cost against the opponent be awarded so as to demotivate others from indulging in such illegal and unethical activities; and
- (vii) Such other reliefs that the Ld. Controller considers fit and appropriate under the facts and circumstances of the case.

141 In the light of the above, the applicant denies all the allegations put forward in the representation by the Opponent and requests the Ld. Controller to dismiss the opposition.

142 We humbly pray that the application may be proceeded to grant.

Dated this the 04th day of October 2019



DR. SANCHITA GANGULI
IN/PA No. 625
Of S. Majumdar & Co.
(Applicant's Agent)

To
The Controller of Patents
The Patent Office Branch
Kolkata

ANNEXURE A

WE CLAIM:

1. A fungicidal composition comprising at least one multi-site contact fungicide; a first systemic fungicide and an additional second systemic fungicide, wherein
- 5 the multi-site contact fungicide is selected from:
- (i) copper fungicides selected from copper oxychloride, copper sulfate, copper hydroxide and tribasic copper sulfate (Bordeaux mixture);
 - 10 (ii) elemental sulfur;
 - (iii) dithiocarbamate fungicides selected from amobam, asomate, azithiram, carbamorph, cufraneb, cuprobam, disulfiram, ferbam, metam, nabam, tecoram, thiram, urbacide, ziram, dazomet, etem, milneb, mancopper, mancozeb, maneb, metiram, polycarbamate, propineb and zineb;
 - 15 (iv) phthalimide fungicides selected from folpet, captan and captafol;
 - (v) sulfamide fungicides selected from dichlofluanid and tolylfluanid;
 - (vi) guanidine fungicides selected from dodine, guazantine and iminoctadine;
 - 20 (vii) anilazine;
 - (viii) dithianon; or
 - (ix) combinations thereof.
- 25 the first and second systemic fungicides are selected from a quinone outside inhibitor, quinone inside inhibitor, demthylation inhibitor, and succinate dehydrogenase inhibitor; wherein:

- 5 (i) the quinone outside inhibitor is selected from fenamidone, famoxadone, and a strobilurin fungicide selected from the group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin, triclopyricarb, fenaminstrobin, pyraclostrobin and trifloxystrobin;
- 10 (ii) the demethylation inhibitor is selected from triflumizole, triforine, pyridinitrile, pyrifenox, fenarimol, nuarimol, triarimol and a conazole fungicide selected from the group consisting of climbazole, clotrimazole, imazalil, oxpoconazole, prochloraz, prochloraz-manganese, triflumizole, azaconazole, bitertanol, bromuconazole, cyproconazole, diclobutrazol, difenoconazole, diniconazole, diniconazole-M, epoxiconazole, etaconazole, fenbuconazole, fluotrimazole, fluquinconazole, flusilazole, flutriafol, furconazole, furconazole-cis, hexaconazole, imibenconazole, ipconazole, metconazole, myclobutanil, penconazole, propiconazole, prothioconazole, quinconazole, simeconazole, tebuconazole, tetraconazole, triadimefon, triadimenol, triticonazole, uniconazole, perfurazoate and uniconazole-P;
- 15 (iii) the quinone inside inhibitor is amisulbrom; and
- 20 (iv) a succinate dehydrogenase inhibitor selected from the group consisting of benodanil, flutolanil, mepronil, fluopyram, fenfuram, carboxin, oxycarboxin, thifluzamide, bixafen, fluxapyroxad, furametpyr, isopyrazam, penflufen, penthiopyrad, sedaxane and boscalid;
- 25 (v) combinations thereof.

provided that said first and second systemic fungicides are not same.

2. The fungicidal combination as claimed in claim 1, wherein the multi-site contact fungicide is mancozeb.
3. The fungicidal combination as claimed in claim 1 or claim 2, wherein the systemic fungicide is a combination of a quinone outside inhibitor and a demethylation inhibitor.
4. The fungicidal combination as claimed in claim 1 comprising:
a multi-site contact fungicide comprising mancozeb;
at least one strobilurin fungicide selected from the group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin, triclopyricarb, fenaminstrobin, pyraclostrobin and trifloxystrobin; and
at least one conazole fungicide selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole.
5. The fungicidal combination as claimed in claim 1 to 4, wherein the systemic fungicide is a combination of (i) a strobilurin fungicide selected from trifloxystrobin, picoxystrobin, azoxystrobin or pyraclostrobin; and (ii) a conazole fungicide selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole.
6. The fungicidal combination as claimed in claim 1 to 5, wherein:
(i) the strobilurin fungicide is trifloxystrobin and the conazole fungicide is prothioconazole; or
(ii) the strobilurin fungicide is picoxystrobin and the conazole fungicide is tebuconazole; or
(iii) the strobilurin fungicide is picoxystrobin and the conazole fungicide is cyproconazole; or

- (iv) the strobilurin fungicide is azoxystrobin and the conazole fungicide is cyproconazole; or
- (v) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is epoxiconazole; or
- 5 (vi) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is tebuconazole; or
- (vii) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is metconazole; or
- (viii) the strobilurin fungicide is trifloxystrobin and the conazole fungicide is cyproconazole, propiconazole or tebuconazole.
- 10

7. A fungicidal combination as claimed in claim 1 to 6 comprising mancozeband a strobilurin fungicide selected from the group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin, triclopyricarb, fenaminostrobin, pyraclostrobin and trifloxystrobin and at least one conazole fungicide selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole..

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8. The fungicidal combination as claimed in claim 1 to 7, wherein the strobilurin fungicide is azoxystrobin or kresoxim-methyl.

25 9. A method for treating soybean rust in a host leguminous plant, wherein the method comprises treating the plant at the locus of the infection with a fungicidal combination comprising at least one multi-site contact fungicide; a first systemic fungicide; and an additional second systemic fungicide, wherein

30 the multi-site contact fungicide is selected from:

- (i) copper fungicides selected from copper oxychloride, copper sulfate, copper hydroxide and tribasic copper sulfate (Bordeaux mixture);
 - (ii) elemental sulfur;
 - 5 (iii) dithiocarbamate fungicides selected from amobam, asomate, azithiram, carbamorph, cufraneb, cuprobam, disulfiram, ferbam, metam, nabam, tecoram, thiram, urbacide, ziram, dazomet, etem, milneb, mancopper, mancozeb, maneb, metiram, polycarbamate, propineb and zineb;
 - 10 (iv) phthalimide fungicides selected from folpet, captan and captafol;
 - (v) sulfamide fungicides selected from dichlofluanid and tolylfluanid;
 - (vi) guanidine fungicides selected from dodine, guazantine and iminoctaadine;
 - 15 (vii) anilazine;
 - (viii) dithianon; and
 - (ix) combinations thereof;
- 20 the first and second systemic fungicides are selected from a quinone outside inhibitor, quinone inside inhibitor, demthylation inhibitor, and succinate dehydrogenase inhibitor; wherein:
- (i) the quinone outside inhibitor is selected from fenamidone, famoxadone, and a strobilurin fungicide selected from the
 - 25 group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin, triclopyricarb,
 - 30 fenaminstrobin, pyraclostrobin and trifloxystrobin;
 - (ii) the demethylation inhibitor is selected from triflumizole, triforine, pyridinitrile, pyrifenoxy, fenarimol, nuarimol,

triarimol and a conazole fungicide selected from the group
 consisting of climbazole, clotrimazole, imazalil,
 oxpoconazole, prochloraz, prochloraz-manganese,
 triflumizole, azaconazole, bitertanol, bromuconazole,
 cyproconazole, diclobutrazol, difenoconazole, diniconazole,
 diniconazole-M, epoxiconazole, etaconazole, fenbuconazole,
 fluotrimazole, fluquinconazole, flusilazole, flutriafol,
 furconazole, furconazole-cis, hexaconazole, imibenconazole,
 ipconazole, metconazole, myclobutanil, penconazole,
 propiconazole, prothioconazole, quinconazole, simeconazole,
 tebuconazole, tetraconazole, triadimefon, triadimenol,
 triticonazole, uniconazole, perfurazoate and uniconazole-P;
 (iii) the quinone inside inhibitor is amisulbrom; and
 (iv) a succinate dehydrogenase inhibitor selected from the group
 consisting of benodanil, flutoianil, mepronil, fluopyram,
 fenfuram, carboxin, oxycarboxin, thifluzamide, bixafen,
 fluxapyroxad, furametpyr, isopyrazam, penflufen,
 penthiopyrad, sedaxane and boscalid; or
 (v) combinations thereof.

provided that said first and second systemic fungicides are not same.

10. The method as claimed in claim 9, wherein the multi-site contact fungicide is mancozeb.
11. The method as claimed in claims 9 or claim 10, wherein the systemic fungicide is a combination of a quinone outside inhibitor and a demethylation inhibitor.
12. The method as claimed in claim 9 wherein:
 a multi-site contact fungicide is mancozeb;

5 at least one strobilurin fungicide is selected from the group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin, triclopyricarb, fenaminstrobin, pyraclostrobin and trifloxystrobin; and

at least one conazole fungicide is selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole.

10 13. The method as claimed in any one of the claims 9 to 12, wherein the systemic fungicide is a combination of (i) a strobilurin fungicide selected from trifloxystrobin, picoxystrobin, azoxystrobin or pyraclostrobin; and (ii) a conazole fungicide selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole.

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14. The method as claimed in any one of the claims 9 to 13, wherein:

- (i) the strobilurin fungicide is trifloxystrobin and the conazole fungicide is prothioconazole; or
- 20 (ii) the strobilurin fungicide is picoxystrobin and the conazole fungicide is tebuconazole; or
- (iii) the strobilurin fungicide is picoxystrobin and the conazole fungicide is cyproconazole; or
- (iv) the strobilurin fungicide is azoxystrobin and the conazole fungicide is cyproconazole; or
- 25 (v) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is epoxiconazole; or
- (vi) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is tebuconazole; or
- 30 (vii) the strobilurin fungicide is pyraclostrobin and the conazole fungicide is metconazole; or

(viii) the strobilurin fungicide is trifloxystrobin and the conazole fungicide is cyproconazole, propiconazole or tebuconazole.

15. The method as claimed in any one of the claims 9 to 14 comprising:

5 mancozeb;
a strobilurin fungicide selected from the group consisting of azoxystrobin, mandestrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, pyraoxystrobin, dimoxystrobin, enestrobin, fluoxastrobin, kresoxim-methyl, metominostrobin, orysastrobin, picoxystrobin, pyrametostrobin,
10 triclopyricarb, fenaminstrobin, pyraclostrobin and trifloxystrobin; and
at least one conazole fungicide selected from prothioconazole, tebuconazole, cyproconazole, epoxiconazole, metconazole and tebuconazole.

15 16. The method as claimed in any one of the claims 9 to 15, wherein the strobilurin fungicide is azoxystrobin or kresoxim-methyl.

Dated this 26th day of November 2013

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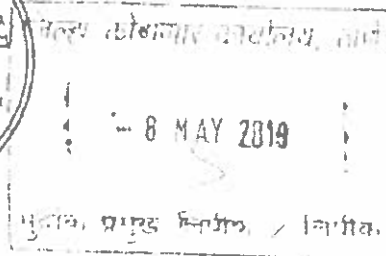
DR. SANCHITA GANGULI
IN/PA No. 625
Of S. Majumdar & Co.
(Applicant's Agent)



महाराष्ट्र MAHARASHTRA

2019

UY 341412



BEFORE THE CONTROLLER OF PATENTS,
THE PATENT OFFICE
KOLKATA

In the matter of The Patents Act, 1970
as amended by The Patents (Amendment)
Act 2005;



१. पुराना निवासी नोमनाली अनु. कम्पार/दिनांक	1 60 66
२. रसना कातर	
३. रसना नोमनाली कम्पार अडेल ना?	नोम/नाली
४. निरुपनीले लेखकपुत वरन-	UPL LIMITED UPL HOUSE 610 B/2, BANDRA VILLAGE, OFF. WESTERN EXPR. BANDRA (EAST), MU
५. पुस्तक विपत वेगनाये ना व सदी	
६. रसना अस्तपुत ताने ना, कता व ताने	
७. पुस्तक वस्तुनाये ना	
८. पुस्तक कुच कान	
९. पुस्तकगारक पुस्तक विपत ना व सदी व पुस्तक वस्तुनाये ना	विपत सा. पोतने पुस्तक विपत वेगनाये ना पुस्तक विपत वेगनाये ना पुस्तक विपत वेगनाये ना
१०. पुस्तकगारक पुस्तक विपत ना व सदी व पुस्तक वस्तुनाये ना	विपत सा. पोतने पुस्तक विपत वेगनाये ना पुस्तक विपत वेगनाये ना पुस्तक विपत वेगनाये ना

10 MAY 2019

70 MAY 2019

And

In the matter of The Patents
(Amendment) Rules, 2006 and as
amended in 2016;

And

In The Matter of Patent Application No.
1336/KOL/2013 dated November 26,
2013 in the name of UPL LIMITED,
Entitled "A METHOD FOR CONTROLLING
RUST", having its principal place of
business at Agrochemical Plant,
Durgachak Haldia - 721 602, Midnapore
Dist. West Bengal, India.

.....Applicant



AFFIDAVIT

I, Dr. Gilson Oliveira, solemnly affirm and declare as under:

1. I have worked on several projects in the field of agrochemicals. A copy of my resume is enclosed herewith as "Annexure A".
2. In view of my position, qualifications, experience and knowledge in the field of agrochemicals, I am competent to depose this affidavit.
3. I have been provided with the copy of Indian Patent Application No. 1336/KOL/2013 (referred herein after as '1336'), filed in the name of UPL LTD entitled "A METHOD FOR CONTROLLING RUST" of which I am one of the inventors.
4. The present invention relates to a fungicidal combination comprising at least one multisite fungicide and at least two systemic fungicides selected from a quinone outside inhibitor, a quinone inside inhibitor, a demethylation inhibitor and a succinate dehydrogenase inhibitor. The present invention also relates to a method for treating soybean rust in a host leguminous plant by applying these combinations. Accordingly, the present invention relates to a triple combination of fungicides essentially. One of the uses of this combination is against Asian soybean rust.



5. Prior to the present invention, the combination of systemic fungicides had been used, albeit without the multisite contact fungicides. The efficacy of these systemic fungicides had been reducing gradually with usage over the years. This gradual decline in the fungicidal efficacy of the systemic fungicides needed to be reversed.

Need for the invention:

6. Experiments were conducted at the Foundation MT, Rondonopolis, the University of Rio Verde and institutions participating in the Cooperative Testing of Fungicides (beginning in 2003/04). These experiments proved the reduction of control effectiveness by comparing the performance of conazole fungicides in the 2005/06 harvest with the harvest (2012/13). In the season 2005/06, the average rust control by conazole fungicides was 90.3%. After eight years, corresponding to the 2012/13 season, control of conazole fungicides was 52.0% with a reduction in efficacy of 42% (Table 1).

Table 1. Reduced soybean rust control by conazole fungicides.

S No.	Fungicide	Crops year		Percentage reduction
		2005/06	2012/13	
1	Cyproconazole	96.0	52.0	45.9

2	Epoxiconazole	80.0	40.0	40.0
3	Tebuconazole	95.0	64.0	32.8
4	Mean	90.3	52.0	42.0

7. Figure 1 below depicts the reduced soybean rust control efficiency using tebuconazole observed over eleven seasons:

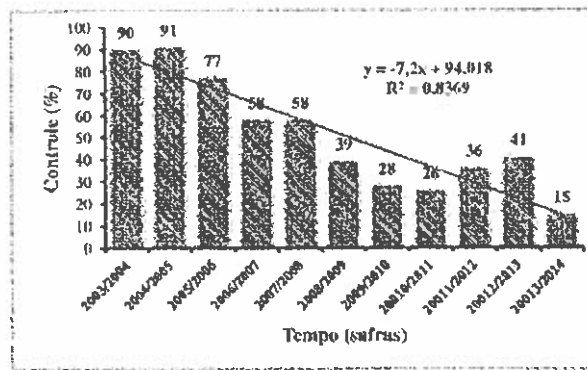
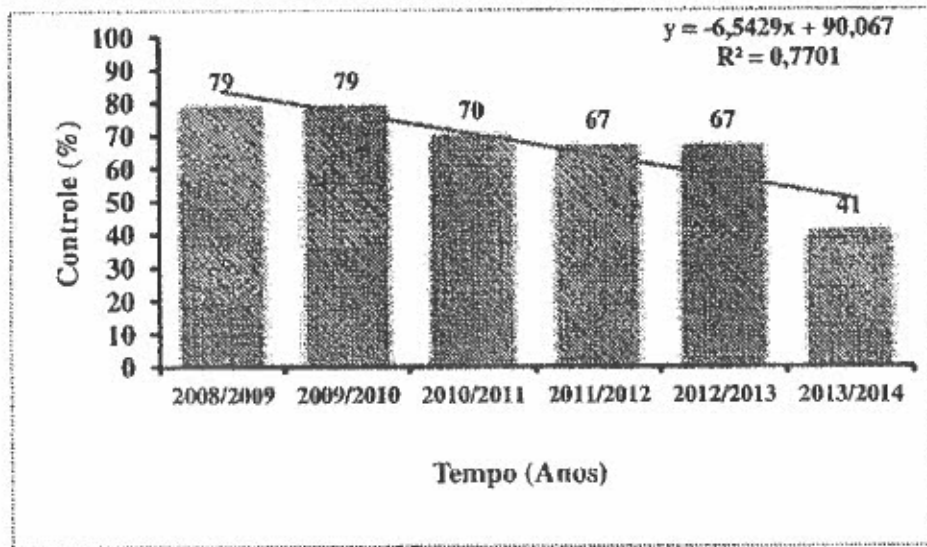


Figura 1

8. This example reinforces the gradual reduction of tebuconazole control over the use of crops covering the period beginning in 2004/05 to 2013/14. It was found that from the 2003/04 season, soybean rust control efficiency by tebuconazole, was reduced by 7.2% per year, using the best fit equation $y = 94.018 - 7.2x$.
9. Likewise, experiment was conducted to measure the reduced control of soybean rust by cyproconazole + azoxystrobin mixture over six seasons.

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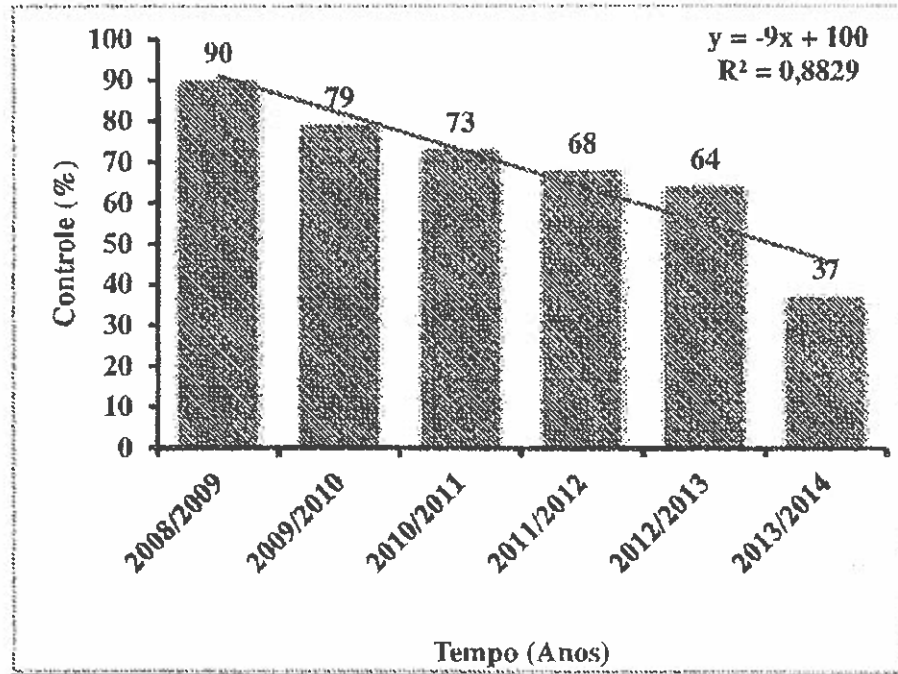


10. This mixture had been in use since 2003/04, when it had an efficiency of 90%.

11. Using the equation $y = 90.067 + -6.5429x$, it was calculated that from the 2007/08 harvest, the effectiveness of this mixture has been reduced by 6.54% per year, reaching the 2013/14 harvest only 41% control.

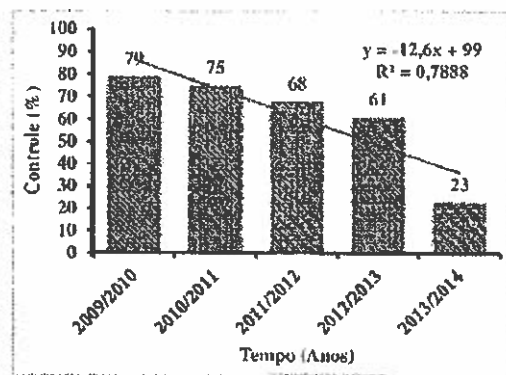
12. Figure 3 depicts the reduced control of soybean rust by cyproconazole + picoxystrobin mixture over six seasons.

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13. Using the equation $y = + 100.0 - 9.0x$, it was calculated that from 2008/09 the effectiveness of the mixture cyproconazole + picoxystrobin has been reduced by 9.0% per year, reaching 37% control crop in 2013/14.

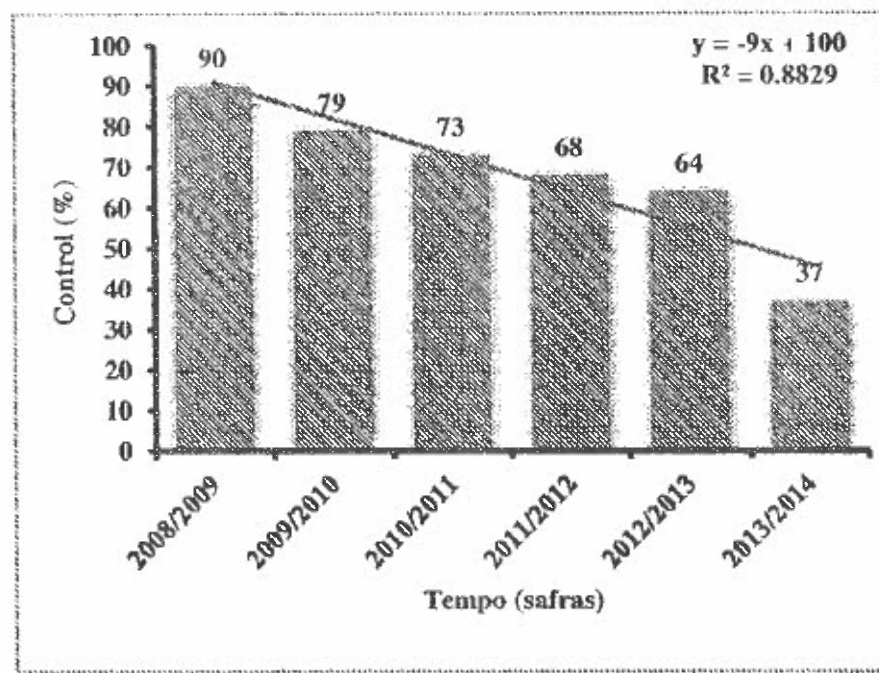
14. Figure 4 depicts the reduced control of soybean rust by epoxiconazole + pyraclostrobin mixture over six seasons.



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15. The mixture "epoxiconazole + pyraclostrobin" has been used since 2007/08. With the equation $y = + 99.0 - 12.6x$ [where y = control (%) x = the percentage of annual reduction of control], it was calculated that from the 2009/10 harvest the effectiveness of this mixture has been decreased by 12.6% per year, reaching the last harvest 23%.

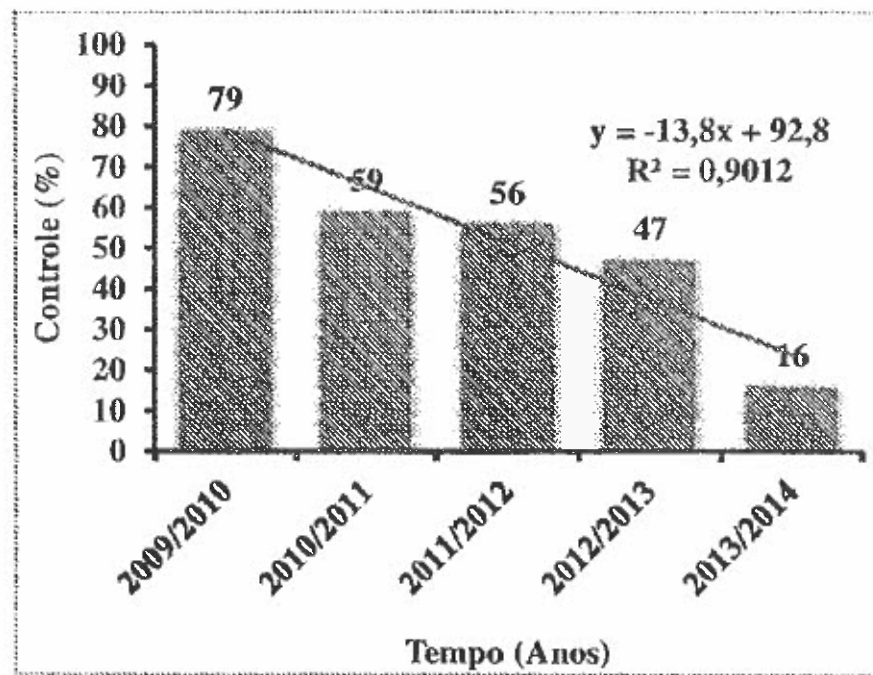
16. Figure 5 depicts the average reduction of soybean rust control by cyproconazole + azoxystrobin, epoxiconazole + pyraclostrobin and cyproconazole + picoxystrobin over six seasons.



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17. According to the equation $y = -9.0x + 100.0$, from 2008/09 the average effectiveness of mixtures "cyproconazole + azoxystrobin", "cyproconazole + picoxystrobin" and "epoxiconazole + pyraclostrobin" over six crops has been reduced by 9.0% per year, reaching 37% in the last harvest.

18. Figure 6 depicts the reduced control of soybean rust for azoxystrobin over six seasons.



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19. It was observed that from the 2008/09 crop, there was reduction in the control of Asian soybean rust using azoxystrobin and reached only 16% of control in the 2013/14 season.

20. Using the equation $y = + 92.8 - 13.8x$, it was determined that from 2009/10 the efficacy of this fungicide has been reduced by 13.8% per year and ending by reaching the 2013 harvest, at only 16% of control.

21. A study was conducted to determine the contribution of the addition of mancozeb to the known mixtures of "strobilurin + triazoles." The experiments were conducted in the field, in nine different locations where the contribution of the addition of mancozeb to the percentage control observed using mixtures of "strobilurin + triazoles" was observed.

22. Two doses were tested (1.5 and 2.0 kg / ha) with various numbers of mancozeb applications. The effect of mancozeb (1.5 kg / ha), incorporated into commercial mixtures was also tested.

23. The experiments were conducted with soybean cultivar Monsoy 9144 RR. Mancozeb formulation was used 750 WDG (water dispersible granules), commercially available, for application as mancozeb.



24. Table 2 lists the percentage control of soybean rust by mixing mixtures of "strobilurin + triazole" with and without added mancozeb, applied three times in two groups of phenological stages of soybean, evaluated by the area under the disease progress curve (AUDPC).

S No.	Mixture		Mancozeb		Difference in percentage control
			Without	With	
1	Cyproconazole Azoxystrobin	+	42.8	74.0	31.2
2	Cyproconazole Picoxystrobin	+	74.0	79.4	5.4
3	Tebuconazole Picoxystrobin	+	74.0	85.9	11.9
4	Mean		66.1	79.8	13.7

Severity in the control = 70%. CV = 4.60%.

25. Table 3 lists the percentage control of soybean rust by mixtures of "strobilurin + triazoles" with or without added mancozeb, applied three times at different growth stages and assessed the severity in leaflet.

S No.	Mixture	Mancozeb	
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		Without	With	Difference in percentage control
1	Cyproconazole + Azoxystrobin	68.6	89.8	21.2
2	Epoxiconazole + Pyraclostrobin	40.5	75.2	34.7
3	Tebuconazole + Picoxystrobin	64.4	79.0	14.6
	Mean	57.8	81.3	23.5

Severity in the control = 37.3%. CV = 6.45%.

26. From the experimental results, it was thus found that the addition of the mancozeb fungicide not only enhances the percentage control, but also reverts the observed control to the original level of 80%.

27. Thus, in view of the above results, it was surprisingly found that the addition of mancozeb increased control of rust with the conventional fungicide mixture of mixtures of "strobilurin + triazoles."

Dire need in the art:

28. The combination of a demethylation inhibitor and a quinone outside inhibitor has been known to be used for the control of Asian soybean rust, particularly in Brazil. However, it has been seen recently that the efficacy

of these combinations has decayed by more than 50%, leading to the cancellation of these registrations by the Brazilian regulatory authority MAPA. In fact, as many as 63 existing registrations for the control of Asian soybean rust were cancelled due to this decay in efficacy. <http://www.agricultura.gov.br/noticias/mapa-suspende-registro-de-63-fungicidas-para-controle-da-ferrugem-da-soja>.

29. This regulatory action was published on the Brazilian Official Gazette (Act 71), in December, 2016. The action was based on the evaluation of effectiveness reports and technical opinions by the Technical Commission of Agronomic Revaluation of Pesticides Formulated Products for the Control of *Phakopsora pachyrhizi* on Soy Culture.

30. Some of the products for which the existing registrations were cancelled were known combinations of demethylation inhibitors and quinone outside inhibitors. Some of these products for which the recommendations were suspended are:

Commercial Name	Composition	MAPA Register No.
ARCADIA	kresoxim-methyl + tebuconazole	8511



31. Accordingly, there has been a dire need in the state of the art for fungicides that can efficaciously control the Asian soybean rust in Brazil and other countries.

Solution presented by the invention:

32. Accordingly, a study was commissioned by EMBRAPA i.e. the Brazilian Agricultural Research Corporation, which is a state owned research agency affiliated with the Brazilian Ministry of Agriculture. The research paper published by GODOY, Claudia as a result of this study is annexed hereto and also offered as post published evidence in support of the inventive step of the present invention.

33. This research corroborates what the present invention found at its priority date (2012) i.e. the addition of a multisite fungicide e.g. mancozeb to the combination of demethylation inhibitor + quinone outside inhibitor reverses the decay of efficacy of the known combination of demethylation inhibitor + quinone outside inhibitor and reverts it to the original known efficacy or better efficacy.

34. The relevant data from the table 5 appearing in this table is reproduced hereinbelow:



Treatment	Dosage (g a.i. /Ha)	Severity (%)
Trifloxystrobin + Prothioconazole	60 + 30	24.4
Trifloxystrobin + Prothioconazole and mancozeb	60 + 30 + 2250	18.9
Trifloxystrobin + Prothioconazole and mancozeb	60 + 30 + 1500	21.2
Trifloxystrobin + Prothioconazole and mancozeb	60 + 30 + 2240	21.1
Trifloxystrobin + Prothioconazole and mancozeb	60 + 30 + 2240	20.1

35. Accordingly, it was observed that in the presence of mancozeb, there was 15 to 30% reduction in the percentage incidence of soybean rust. This confirms the inventive step inherent in the invention claimed in the present invention.

36. Importantly, these combinations comprising mancozeb in addition to a demethylation inhibitor and a quinone outside inhibitor were approved by EMBRAPA for the control of soybean rust. This combination was found to succeed where 63 other existing products failed to successfully control Asian soybean rust.



The combinations are synergistic:

37. Not only the presently claimed combinations revert the efficacy of the systemic fungicides to the pre-decay levels, the resulting triple combination of a multisite contact fungicides and at least two systemic fungicides are also synergistic. This synergistic correlation between these components of the combinations of the present invention was established by the application of the Colby's formula. A search in Google Search platform for the references that reverse-cite the Colby reference (S R Colby, "Calculating Synergistic and Antagonistic Responses of Herbicide Combinations", Weeds 15 (1967), pp 20 - 22, annexed hereto as "Annexure A") demonstrates that about 699 later-published papers cite this article as a reference, providing evidence that Colby method is a standard method for demonstrating synergy in the field of agrochemicals. Please see the Google Search printout that accompanies Annexure A.

38. In the Colby method, the expected (or predicted) response of a combination of fungicides is calculated by taking the product of the observed response for each individual component of the combination when applied alone divided by 100 and subtracting this value from the sum of the observed response for each component when applied alone. An unexpected enhancement in efficacy of the combination is then determined by

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comparing the observed response of the combination to the expected (or predicted) response as calculated from the observed response of each individual component alone. If the observed response of the combination is greater than the expected (or predicted) response, or stated conversely, if the difference between the observed and expected response is greater than zero, then the combination is said to be synergistic or unexpectedly effective.

39. Thus, when the combination of the present invention was analysed using this method, it demonstrated an observed - expected value of greater than zero which is indicative of an unexpected efficacy. The basis of demonstration of unexpected efficacy by comparison with the Colby formula is that fungicide (A) tested alone would kill a proportion of the target fungi and leave the remaining portion (a %) as survivors. Similarly, fungicide B tested alone will leave (b %) as survivors. When combined, A+B will, act independently on the target fungi (if unexpected activity is absent); component A leaving a% survivors, which survivors will be controlled by component B; which has an overall effect of $a\% \times b\% \times 100$. Subsequently, if the percent control is greater than that predicted by the Colby formula or stated conversely, if the difference between the observed control and the expected control is greater than zero; then unexpected enhancement in activity is acknowledged. The degree to which the difference is greater than



zero is not itself critical as long as it is greater than zero; however greater the difference, more significant is the enhancement or the unexpectedness in fungicidal control.

The following formula was used to calculate the expected activity of mixtures containing active ingredients, A and B:

$$\text{Expected (E)} = A + B - \frac{AB}{100}$$

Where

A = observed efficacy of active ingredient A at the same concentration as used in the mixture.

B = observed efficacy of active ingredient B at the same concentration as used in the mixture.

The following formula is used to calculate the expected activity of mixtures containing three active ingredients, A, B and C:

$$\text{Expected (E)} = A + B + C - \frac{(AB+AC+BC)}{100} + \frac{ABC}{10,000}$$

Where



A = observed efficacy of active ingredient A at the same concentration as used in the mixture.

B = observed efficacy of active ingredient B at the same concentration as used in the mixture.

C = observed efficacy of active ingredient C at the same concentration as used in the mixture.

40. Mancozeb is a multisite contact fungicide. It was surprisingly found that mancozeb surprisingly enhances the efficacy in a triple combination (present invention) more than it does in a double combination (prior art). In the double combinations (prior art), mancozeb was found to actually antagonize the efficacy of either a strobilurin or a conazole fungicide, but its efficacy enhancing role was consistently greater than 20% or 30%, and being near about 50% - 60% when present in the triple combinations of the present invention. Thus, the contribution of mancozeb in the presently claimed triple combinations was much greater than its contribution in the dual combinations of the prior art.

41. The photographic evidence, taken from the actual fields where such trials were conducted, also convincingly prove the advantage of the present invention.



42. Trials were conducted to evaluate the efficacy for control of *Phakopsora pachyrhizi* for ready mix products with strobilurin and triazole fungicides sprayed individually or in association with fipronil (which is an insecticide) and/or mancozeb for checking the effect of an additional insecticide fipronil in the mixture. The product formulations used in the trials were Priori Xtra, Sphere Max, Fox, Nativo, Barão, Unizeb Gold. The evaluations were performed for the target fungus *Phakopsora pachyrhizi* in soybean crop.

The exact combination as claimed is critical:

43. The first set of experiments were conducted at field station at Ituverava, São Paulo in Brazil. The soybean cultivar SYN 1366 C IPRO was sown on December 4. The plots of size 3 X 6 m were spaced 0.50 m apart. The soybean crop was seeded at 20 seeds/m, and fertilizer with the formulation 02.20.10 (02% of Nitrogen; 20% of Phosphorus; 10% of Potassium) 520 kg/ha was spread in the sowing. Each treatment was performed in four replications. Three sprays A, B and C were applied on January 11, January 31 (20 DAA) and February 18 (18 DAA). The air temperature during the three applications were noted to be 29.0, 27.4 and 24°C. On all the three days of application wind speed (2.1, 4.8 and 3.2 m/h), humidity (24, 67 and 86%), soil condition (wet, wet and wet), and stage of development of soybean (Stage of development of soybean plantas (BBCH standard); (62, 76, and 79) was noted. The spray nozzle was XR 80 015 using a spray



volume of 150 L/ha was used. Severity assessments were made as per the schedule mentioned in the results table and Tukey's test was performed to 5% in all of the evaluations. The following product matrix was used for the experiments:

PRODUCTS	Concentration	Formulation	RATES (Kg- L/ha)	Active ingredient (g of a.i./ha)
Check				
Barão	800	WG	0,1	Fipronil (80)
Aureo	720	EC	0,25%	Methyl ester (270)
Unizeb Gold	750	WG	1,5	Mancozeb (1125)
Sphere Max	375 & 160	SC	0,2	Trifloxystrobin (75) & Ciproconazole (32)
Priori Xtra	200 & 80	SC	0,3	Azoxistrobin (60) & Ciproconazole (24)

Nativo	100 & 200	SC	0,5	Trifloxistrobin (50) & tebuconazol (100)
Fox	150 & 175	SC	0,4	Trifloxistrobin (60) & Prothioconazole (70)

44. The results and observations were evaluated in the following manner. Firstly, the percentage severity of *Phakopsora pachyrhizi* was noted in each evaluation. From the percentage severity, the percentage actual observed control of *Phakopsora pachyrhizi* was calculated in each evaluation. The predicted control was calculated using the Colby's formula in each evaluation. The abbreviations DAA/B/C denote the number of days after sprays A/B/C on which the respective observation was taken.

S No	Products	Rate s (kg-	Timin g	Severit y (%) 7 DAC	Contro l (5) 7 DAC	Colby predicte d control	Inference
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		L/ha)					
1	Check	-	-	8.5 a	0.0	-	-
2	Fipronil	80.0 0	ABC	5.5 a	35.9	-	-
3	Mancozeb	1125	ABC	2.2 b	74.7	-	-
4	Mancozeb + Fipronil	1125 + 80	ABC	1.3 bcd	85.2	83.8	Synergistic
5	Trifloxystrobin + Cyproconazole + Mancozeb	75 + 32 + 1125	ABC	0.5 cde	93.7	-	-
6	Azoxystrobin + Cyproconazole + Mancozeb	60 + 24 + 1125	ABC	1.5 bc	82.9	-	-
7	Trifloxystrobin + Tebuconazole + Mancozeb	60 + 120 + 1125	ABC	0.8 bcde	91.1	-	-

8	Trifloxystrobin + Prothioconazol e + Mancozeb	60 + 70 + 1125	ABC	0.1 e	98.5	-	-
9	Trifloxystrobin + Cyproconazole + Mancozeb + Fipronil	75 + 32 + 1125 + 80	ABC	1.0 bcde	88.7	95.9	Antagonisti c
10	Azoxystrobin + Cyproconazole + Mancozeb + Fipronil	60 + 24 + 1125 + 80	ABC	1.0 bcde	88.0	89.0	Antagonisti c
11	Trifloxystrobin + Tebuconazole + Mancozeb + Fipronil	60 + 120 + 1125 + 80	ABC	0.6 cde	93.5	94.3	Antagonisti c
12	Trifloxystrobin +	60 + 70 +	ABC	0.2 de	97.3	99.0	Antagonisti c



	Prothioconazol e + Mancozeb + Fipronil	1125 + 80					
CV (%)				24.41t			

t = Transformed data for " $\arcsin((x/100)^{1/2})$ ";; means followed by the same letter or symbol did not differ by Tukey's test 5% of probability

45. Fipronil sprayed together with Trifloxistrobin + Ciproconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluations of 7 days after third spray (7 DAC) evaluation. Fipronil sprayed together with Azoxistrobin + Ciproconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluations of 7 DAC. Fipronil sprayed together with Trifloxistrobin + Tebuconazol + Mancozeb promoted antagonism in the control of *Phakopsora pachyrhizi* in the evaluation of 7 DAC. Thus, antagonism was observed in most of the evaluations in mixtures of strobilurin fungicide + conazole fungicide + Mancozeb with Fipronil.

46. Surprisingly, in the mixture of Mancozeb with Fipronil, synergism was observed in the evaluations at 7 DAC.



47. It was concluded from a comparison of the efficacy data in rows 2, 3 and 4 that the addition of the insecticide fipronil, an additional agrochemical, to the fungicide mancozeb was found to synergize the activity of the fungicide mancozeb. It would be reasonable for a person skilled in the art to expect that the addition of fipronil to other fungicide(s) would also synergize the fungicidal efficacy of those fungicide(s).

48. However, the data presented in rows 9 – 12 provide evidence to the contrary. The addition of fipronil was found to antagonize the fungicidal efficacy of a triple combination of fungicides consisting of mancozeb, a strobilurin fungicide and a conazole fungicide. This was contrary to the reasonable expectation of a person skilled in the art and surprising. It was even more surprising considering that the Colby's formula, in general, tends to underestimate antagonism by its very nature (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5541435/>).

49. Thus, it was concluded that the triple combination as claimed herein was critical in the presence of the three fungicides exactly as recited herein. Any additional insecticide or an additional agrochemical overrides the synergy accruing due to mancozeb, or due to the contact fungicide, and causes actual antagonism.



Role of multisite contact fungicides in dual combinations versus its role in triple combinations of the present invention:

50. A second trial was carried out to evaluate the efficacy of a strobilurin fungicide, a conazole fungicide and mancozeb in double combinations for control of *Phakopsora pachyrhizi*. This trial F18BRSOYB02SEF0601 was carried out using the products Priori, Unizeb Gold and Alto 100 on soybean crop for the main target *Phakopsora pachyrhizi* at Ituverava – SP. The target crop cultivar SYN 1366 C IPRO was sown on 4th December at a row spacing of 0.50 m with seed populated at 20 seeds/m. The trial was carried out in 4 replications in plot sizes of 3 X 6 m. Fertilizer with the formulation 02.20.10 (02% of Nitrogen; 20% of Phosphorus; 10% of Potassium) 520 kg/ha was spread in the sowing

Spray	A	B	C
Spray date	11 January	31 January	18 February
Interval during sprays (days)	-	20 DAA	18 DAA
Start time	12:45	09:18	10:29
Finish time	12:56	09:24	10:35
Air temperature(°C)	29,3	27,4	25,3
Humidity(%)	52	67	80

Wind speed (km/h)	8,5	6,4	6,4
Soil condition	Wet	Wet	Wet
Stage of development of soybean (BBCH)	R1 (62)	R5.1 (76)	R5.4 (79)
Nozzle	XR 80 015	XR 80 015	XR 80 015
Spray volume (L/ha)	150	150	150

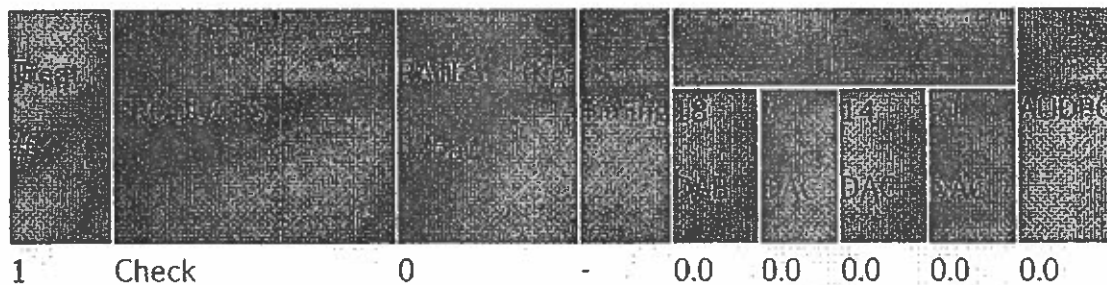
51. Thus, 3 sprays (ABC) were applied at interval of 21 days between first and second spray and 18 days between second and third spray. First spray was in R1 stage (Beginning of flowering). Severity assessments were made 21 days after spray A, 18 days after spray B, 7 and 14 days after the spray C. The Tukey's test to 5% was performed in all of the evaluations.

52. The results of severity of *Phakopsora pachyrhizi* in each evaluation was observed and tabulated:

Plot #	Treatments	RATES		Severity of Asian Soybean Rust (%)										AUDPC	
		(g)	(L/ha)	Prevalence	At 7 DAP	At 14 DAP	At 21 DAP	At 28 DAP	At 35 DAP	At 42 DAP	At 49 DAP	At 56 DAP			
1	Check	0	-	0.0	0.0	0.4	a	8.9	a	34.8	a	60.9	a	507.1	a

2	Mancozeb	1350.0	ABC	0.0	-	0.0	-0.0	c	1.5	b	9.3	c	17.	b	133.	c
		0								c			7	c	0	
3	Azoxistrobin	90,00	ABC	0.0	-	0.0	-0.2	a	3.8	b	21.3	b	23.	b	249.	b
								b					4	b	1	
4	Ciproconaz ol	60.00	ABC	0.0	-	0.0	-0.1	b	1.0	b	6.9	c	8.0	d	81.2	d
								c		c		d				
5	Azoxistrobin + Mancozeb	90 + 1350	ABC	0.0	-	0.0	-0.0	c	0.5	c	6.1	c	13.	c	89.8	c
												d	2	d		d
6	Ciproconaz ol + Mancozeb	60 + 1350	ABC	0.0	-	0.0	-0.0	c	0.7	c	3.7	d	7.9	d	56.9	d
C.V.(%)				0.0t	0.0	52.0	29.1	12.3	8.8t	8.2t						
					t	t	t	t								

53. The severity data was then used to calculate the percentage control of *Phakopsora pachyrhizi* in each evaluation.



2	Mancozeb	1350.00	ABC	94.9	82.9	73.3	71.0	73.8
3	Azoxistrobin	90.00	ABC	53.1	57.8	38.8	61.6	50.9
4	Ciproconazol	60.00	ABC	84.0	88.3	80.1	86.8	84.0
5	Azoxistrobin + Mancozeb	90 + 1350	ABC	96.0	93.9	82.4	78.4	82.3
6	Ciproconazol + Mancozeb	60 + 1350	ABC	99.4	92.2	89.2	87.0	88.8

54. The percentage control of each treatment was then compared with the Colby predicted control of *Phakopsora pachyrhizi* in each evaluation.

Tre at #	Product	Dose (g of a.i./ ha)	18 DAB		7 DAC		14 DAC		21 DAC		AUDPC	
			% Cont rol	Colby predic ted	% Cont rol	Colby predic ted	% Cont rol	Colby predic ted	% Cont rol	Colby predic ted	% Cont rol	Colby predic ted
1	Check		0.0		0.0		0.0		0.0		0.0	
2	Mancozeb	1350	94.9		82.9		73.3		71.0		73.8	
3	Azoxistrobin	90	53.1		57.8		38.8		61.6		50.9	

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4	Ciprocon azol	60	84.0		88.3		80.1		86.8		84.0	
5	Azoxistro bin	+90 +	96.0	97.6	95.9	92.8	82.4	83.7	78.4	88.9	82.3	87.1
	Mancozeb	1350										
	b											
Observed control												
- Expected control			- 1.6		- 1.3		- 10.5		- 4.8			
due to mancozeb												
6	Ciprocon azol	+60 +	97.4	99.2	92.2	98.0	89.2	94.7	87.0	96.2	88.8	95.8
	Mancozeb	1350										
	b											
Observed control												
- Expected control			- 0.2		- 5.8		- 4.5		- 8.8		- 7.0	
due to mancozeb												

55. It was thus concluded that the addition of mancozeb, to either a strobilurin fungicide or a conazole fungicide, did not significantly enhance the efficacy of the strobilurin or the conazole fungicide in isolation, but in fact was almost always antagonistic. The presence of mancozeb in the dual combinations was found to reduce the efficacy of the systemic fungicide. It

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varied from being mildly additive (about +1.1%) to being critically antagonistic (varying from about -1.5 to about -10.0). Even for the slightly additive scenario, the improvement in efficacy measured by the Colby's formula was about +1.1 when mancozeb was added to either the strobilurin fungicide (best Colby reading +1.1) or to the conazole fungicide (best Colby reading of -8.8 i.e. antagonistic) in isolation.

56. In contrast, the benefits of adding mancozeb to a double combination of systemic fungicides i.e. a combination comprising a conazole fungicide and a strobilurin fungicide, was unexpected and surprising. Thus, the efficacy of the resulting triple combination of fungicides was astoundingly unexpected and surprising.

57. This synergistic correlation between these components of the combinations of the present invention was established by the application of the Colby's formula.

58. Thus, when the combination of the present invention was analysed using this method, it demonstrated an observed - expected value of greater than zero which is indicative of an unexpected efficacy. The basis of demonstration of unexpected efficacy by comparison with the Colby formula is that fungicide (A) tested alone would kill a proportion of the target fungi and leave the remaining portion (a %) as survivors. Similarly, fungicide B tested alone will leave (b %) as survivors. When combined, A+B will, act



independently on the target fungi (if unexpected activity is absent); component A leaving a% survivors, which survivors will be controlled by component B; which has an overall effect of $a\% \times b\% \times 100$. Subsequently, if the percent control is greater than that predicted by the Colby formula or stated conversely, if the difference between the observed control and the expected control is greater than zero; then unexpected enhancement in activity is acknowledged.

59. In the same set of field conditions described above, trials were conducted to evaluate the importance of mancozeb in association with other fungicides in *Phakopsora pachyrhizi* control in soybean using mancozeb in association with the prior art products composed by mixtures of strobilurins and triazoles. The soybean variety was NA 7238 IPRO sown at a population density of 25 seeds/m in plot size of 3 x 6 m. The trials were carried out in 4 replications, and were treated with 5 sprays (ABCDE), V3, V5, R1, R5.1 and R5.4. The severity assessment was done using the Area Under Disease Progress Curve (AUDPC), from which the percentage control was calculated for each tested treatment and subsequently the crop was harvested. This percentage control was then applied to the Colby analysis reported herein.

60. The severity assessment was done using the Area Under Disease Progress Curve (AUDPC), from which the percentage control was calculated for each tested treatment and subsequently the crop was harvested. This percentage



control was then applied to the Colby analysis reported in the table appearing hereinbelow:

S No	Parameter	Mancozeb (1125 g/ha)	Strobilurin + Conazole	Strobilurin (S) + Conazole (C) + Mancozeb	Difference in Colby reading between actual and expected control originating due to addition of mancozeb (Conclusion)
	Assessment type (Dosage in g/ha)	Fungicidal Percentage control	Percentage control	Percentage control	
<i>Phakopsora pachyrhizi</i>					



1	S= Pyraclostrobin (66.5) C= Epoxiconazole (25)	74.7	8.1	Calc (Colby)	Actua l	+4.5; Synergistic
				76.7	81.2	
2	S= Trifloxystrobin (60) C= Prothioconazole (70)	0.0	66.9	Calc	Actua l	+23.7; Synergistic
				66.9	90.2	
3	S= Picoxystrobin (60) C=Cyproconazole (24)	0.0	63.6	Calc	Actua l	+20.9; Synergistic
				63.6	84.5	
4	S= Pyraclostrobin (66.5) C= Epoxiconazole (25)	0.0	57.9	Calc	Actua l	+35; Synergistic
				57.9	92.9	

5	S= Azoxystrobin (60)	0.0	60.3	Calc	Actua l	+25.5; Synergistic
	C= Cyproconazole (24)			60.3	86.8	
6	S=Pyraclostrobin (99.9)	0.0	70.4	Calc	Actua l	+21.3; Synergistic
	SDHI = Fluxapyroxad (50.1)			70.4	91.7	
Corynespora cassiicola						
7	S=Trifloxystrobin (60)	0.0	54.6	Calc	Actua l	+24.6; Synergistic
	C=Prothioconazol e (70)			54.6	79.2	
8	S=Picoxystrobin (60)	0.0	10.3	Calc	Actua l	+ 56.8; Synergistic

	C=Cyproconazole (24)			10.3	67.1	
9	S=Pyraclostrobin (66.5)	0.0	10.1	Calc	Actua l	+42.9; Synergistic
	C= Epoxiconazole (25)			10.1	53.0	
10	S=Azoxystrobin (60)	0.0	9.8	Calc	Actua l	+48.9; Synergistic
	C=Cyproconazole (24)			9.8	58.7	
11	S=Pyraclostrobin (99.9)	0.0	74.2	Calc	Actua l	+14.5; Synergistic
	C=Fluxapyroxad (50.1)			74.2	88.7	
<i>Phakopsora pachyrhizi</i>						
12	S = Azoxystrobin (60)	6.0	85.7	Calc	Actua l	+1.9; Synergistic

	SDHI = Benzovindiflupyr (30)			86.5	88.4	
13	S = Azoxystrobin (60)	6.0	41.2	Calc	Actua l	+22.2; Synergistic
	C = Cyproconazole (24)			44.7	66.9	
14	S=Picoxystrobin (60)	6.0	54.7	Calc	Actua l	+19.7; Synergistic
	C=Cyproconazole (24)			57.4	77.1	
15	S=Pyraclostrobin (116.55)	6.0	66.6	Calc	Actua l	+12.8; Synergistic
	SDHI = Fluxapyroxad (58.45)			68.6	81.4	

<i>Colletotrichum truncatum</i>						
16	S = Azoxystrobin (60)	12.8	87.2	Calc	Actua I	+4.0; Synergistic
				88.8	92.8	
	SDHI = Benzovindiflupyr (30)					
17	S = Azoxystrobin (60)	12.8	38.5	Calc	Actua I	+31.8; Synergistic
				46.4	78.2	
	C = Cyproconazole (24)					
14	S=Picoxystrobin (60)	12.8	51.3	Calc	Actua I	+31.5; Synergistic
				57.5	89.0	
	C=Cyproconazole (24)					
15	S=Pyraclostrobin (116.55)	12.8	87.2	Calc	Actua I	+ 6.1; Synergistic

	SDHI Fluxapyroxad (58.45)	=		88.8	94.9	
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61. It was thus concluded that the addition of mancozeb, to a strobilurin fungicide and a conazole fungicide combination was surprisingly greater than what was expected from a knowledge of the improvement accruing to the efficacy of either the strobilurin fungicide or the conazole fungicide in isolation. It was consistently greater than 20% (i.e. double the improvement in the isolated strobilurin or the conazole fungicide), mostly greater than 30% (i.e. triple the improvement in the isolated strobilurin or the conazole fungicide), and preferably greater than 50% or about 60% (i.e. 5 or 6 times the improvement in the isolated strobilurin or the conazole fungicide).

62. The effect of mancozeb supplementation was also evaluated for its effect on the crop yield. The crop yield was calculated for each tested treatment after the harvest. The yield data in each test result was converted to the percentage of increase in yield seen due to the treatment over the untreated crop to calculate the percentage yield gain parameter, rounded off to next round number. This percentage yield gain was then subjected to the Colby analysis reported in the table appearing hereinbelow:



S No.	Parameter	Mancozeb	Strobilurin + Conazole	Strobilurin (S) + Conazole (C) + Mancozeb	Conclusion
1	Untreated check	466.8			
2	S= Pyraclostrobin C= Epoxiconazole Yield kg soybean/ha	1376.4	780.6	2032.2	-
	Percentage yield gain (%) over the untreated crop	195	67	Calc 131.35	Actual 335 Synergistic

63. It was thus found that the association of mancozeb with commercial products based in mixtures of pyraclostrobin and epoxiconazole, picoxystrobin and ciproconazole and azoxystrobin and ciproconazole increased the control of *Phakopsora pachyrhizi*. More importantly, the combinations *per se* were found to be synergistic evidenced mathematically by the Colby's formula.

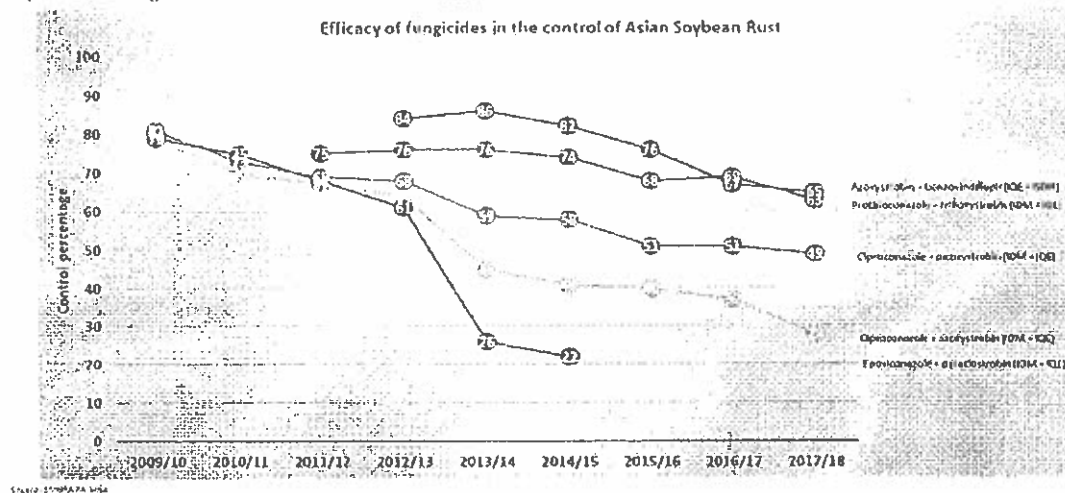
64. Equally surprisingly, it was further found that the association of mancozeb with pyraclostrobin and epoxiconazole, picoxystrobin and ciproconazole and azoxystrobin and ciproconazole increased the yield synergistically.

65. Surprisingly, in the absence of mancozeb, the fungicidal efficacy of these very fungicides was known to gradually decay with their usage over the years:

Technical Circulars EMBRAPA

Summary of Technical Circulars from the 2008/09 to 2017/18 harvest

Specific site fungicides decreased their control efficiency due to extensive use and strong selection pressure.



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66.The incorporation of mancozeb to these combinations reversed this trend.

67.Enclosed herewith the photographic evidence for the benefits accruing from the incorporation of mancozeb to the dual combination of strobilurin and conazole fungicides. The practical benefit is self-evident, and may be read as being part and parcel of this statement.

The presence of a multisite contact fungicide such as mancozeb reduced the sensitivity reduction factor (SRF) of the systemic fungicides:

68.The objective of these experimental trials was to estimate the LD₅₀ (Lethal Dose for 50% of population) and LD₉₅ (Lethal Dose for 95% of population) for azoxystrobin, trifloxystrobin, ciproconazole and tebuconazole for Asian Soybean Rust. A total of 69 samples of Asian Soybean Rust for different regions of Brazil were analyzed. Thus, it was made possible to calculate the sensitivity reduction factor (SRF) for each fungicide in each place in each season.

69.The results of the sensitivity reduction factor analysis were then correlated to the data on the adoption of the use of a multisite fungicide (mancozeb) in soybean. This data on the adoption of mancozeb was available from Sparks Market Research. This correlation between the sensitivity reduction factor and the degree of adoption of mancozeb was to establish the adoption of mancozeb in the trial program as a causation for the reduction in sensitivity reduction factor. The objective of these trials was thus to



establish whether the greater adoption of multisite fungicides e.g. mancozeb in one state i.e. Rio Grande do Sul State was indeed the causation to reduce the SRF of azoxystrobin, trifloxystrobin, ciproconazol and tebuconazol combinations less than in another state i.e. Paraná State. These results are described hereinafter.

70.The occurrence of resistance or less sensitivity of the rust agent (*P. pachyrhizi*) to fungicides makes it difficult to control the disease in the field, often requiring a greater number of applications or increase in the dosage of the fungicides or a shorter interval between them. Loss of sensitivity of the fungus to three different chemical groups of fungicides, namely demethylation inhibitors (DMIs) known as triazoles, outside quinone inhibitors (Qols) known as strobilurins, and most recently succinate dehydrogenase (SDHI) inhibitors, known as carboxamides have been studied and documented in Brazil (SCHMITZ et al., 2014; KLOSOWSKI et al., 2016, FRAC, 2017).

71.To date, fungicide applications represent one of the main tools in the management of soybean rust (*Phakopsora pachyrhizi*) due to the difficulty of resistant genetic material. When used preventively, fungicides can provide higher levels of disease control, while curative applications of triazoles and strobilurins may be compromised, reducing their residual power, as shown



by various field studies under our conditions (Godoy et al. (2007), Furlan (2011), and Julian (2006). In high potential inoculum conditions, when climatic periods are favorable, especially frequent rainfall and temperatures between 22 and 25 °C, the efficiency and residual fungicides usually decrease, resulting in greater difficulty of control (Yorinori, 2004). The great variability of the fungus from region to region may also influence the efficiency of chemical control.

72. Therefore, the present experiments were designed to monitor of the sensitivity of the pathogen populations through the "baseline" methodology adopted by the international FRAC to help in the direction of the anti-resistance strategies, providing a greater safety of use of the products within management.

73. Samples of soybean leaves with symptoms of Asian rust coming from Brazilian states of Rio Grande do Sul and Paraná were collected in the field to obtain uredospores. These were removed at the Phytopathology Laboratory of the Biological Institute for the fungicide sensitivity tests. A portion of the samples, about 20% of the samples in the form of uredospores were stored in a freezer for a period of 15 to 30 days for processing. This happens when there is coincidence of receiving many



samples in the same week, making it impossible to process the same day.

The others are processed soon after the spores are captured.

74. Following the methodology described by the FRAC (Fraction Resistance Committee for Fungicides) in Brazil (FRAC, 2008), unifoliated leaflets obtained from soybean plants of the cultivar BMX Potência cultivated in a greenhouse for 3 weeks were collected and taken to the laboratory to perform the chemical treatment. The leaflets are immersed for 3 seconds in fungicide syrups, which are pre-prepared in serial dilutions from a more concentrated stock solution (521 ppm ai) to obtain the different concentrations of active ingredients according to the scale logarithm of 0; 0.125; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0; 16; 32; 64; 128 and 256 ppm i.a. The use of the highest concentration (256 ppm i.a.) is required by the inclusion of the multisite in the tests, which generally has a lethal concentration higher than systemic fungicides. These concentrations were established to obtain 0 to 100% control of the disease, thus ensuring a good estimate of lethal concentrations (LC50 and CL95).

75. The fungicides tested were Ciproconazole (UPL 215 10% - 100gr i.a / L p.c.), Tebuconazole (Tebufort 20% - 200g i.a / L p.c.); Trifloxystrobin (Flint 50% - 500g i.a / L p.c.); Azoxystrobin (Amistar 50% - 500g i.a / L p.c.); Mancozeb (Unizeb Gold 75% - 750g ai / L pc) and Azoxystrobin +

Benzovindiflupir (Elatus WG 30% and 15% - 450g ai / L pc. After the chemical treatment by immersion, the leaflets were placed on two absorbent papers saturated with distilled water with the abaxial face facing upwards, inside Petri dishes of 15 cm in diameter. The inoculum of uredospores was then prepared, previously collected in glass bottles, beating the leaves and / or with the aid of a brush so that they would come off. A uredospore suspension was prepared (concentration of about 105 spores / mL of distilled water containing Tween 20) which was sprayed onto the leaflets with the aid of an airbrush for better uniformity of surface distribution. The plates with the inoculated leaflets were incubated in BOD for a period of 14 days under high humidity, 12-hour photoperiod and $24^{\circ} \pm 1^{\circ} \text{C}$ to ensure a good development of the symptoms of the disease. The symptoms were evaluated by the percentage of disease severity in each leaflet under a light plate (X-ray) and / or binocular magnifying glass, using a scale of notes according to Godoy et al. (2006). From these results the percentage of inhibition was estimated and the LC50 and CL95 estimated from the populations sampled. Four folioles were used per replicate, arranged in each plate, totaling 3 plates (repetition) per treatment, which were distributed completely randomized within the BOD. The estimates of lethal concentrations CL50 and CL95 for the fungicidal activity tests were performed using the logarithmic percentage regression - PROBIT (Table 1) of the relation between the values of inhibition percentage and the



concentrations tested. Calculations and graphs were compiled using the Excel program for Windows® version 2007. The data obtained from the samples showing rotting of the leaves during transportation or leaves without the presence of pustules or uredospores were not used.

76. The percent inhibition values at different concentrations of each fungicide (Table 2) and LC50 or LC95 varied for each isolate and fungicide, according to the region and time of sampling (Figures 3 to 53, Table 3) was observed and tabulated. The correlations (R^2) between the percent inhibition and the fungicide concentration were good, for all results. There are variations of responses between samples, within each fungicide, showing the variability of the population sampled. Differences were observed between the isolates for the same fungicide and among the fungicides, even though they were from the same group (Table 3). For the 50 samples processed, the mean values of LC50 and LC95 in ppm i.e., respectively were for UPL 215 (Ciprocozole): 1.61 and 27.48; Tebufort (Tebuconazole): 1.80 and 32.47; Flint (Trifloxystrobin): 0.63 and 10.21; Amistar (Azoxystrobin): 1.41 and 23.31; Unizeb Gold (Mancozeb): 12.52 and 41.28; and for the 15 samples tested for Elatus (Azoxystrobin and Benzovindiflupir): 1.38 and 25.16 (Table 4). The sensitivity of the isolates therefore varied according to each product, even though they were of the same chemical group. The values for Elatus were considered high in this crop, since until 2015/16 was considered the most



effective product for the control of rust, indicating a change in the behavior (sensitivity) of the populations of the fungus.

77. Table 1 below lists the mean concentrations (ppm ai) of the fungicides UPL 215, Tebufort, Amistar, Flint, Unizeb Gold (for 50 samples) and Elatus (for 15 samples) required to inhibit 50% (LC50) and 95% (LC95) of the symptoms of Asian rust:

Fungicidas	CL ₅₀ (ppm i.a.)	CL ₉₅ (ppm i.a.)
UPL 215 (50 amostras)	1,61	27,48
TEBUFORT (50 amostras)	1,80	32,47
AMISTAR (50 amostras)	1,53	26,13
FLINT (50 amostras)	0,63	10,21
UNIZEB GOLD (50 amostras)	2,52	41,28
ELATUS (15 amostras)	1,38	25,16
UPL 215 (15 amostras)	1,36	23,60
TEBUFORT (15 amostras)	1,56	30,99
AMISTAR (15 amostras)	1,37	25,72
FLINT (15 amostras)	0,63	10,44
UNIZEB GOLD (15 amostras)	2,07	39,03

78. Conclusions - There was a slight tendency to increase lethal concentrations of mancozeb when comparing the two crops (15/16 and 16/17). The values of lethal concentrations for Elatus were considered high in this crop, since until 2015/16 was considered the most effective rust control, indicating an evident change in their behavior due to the lower sensitivity of the fungus populations.

79. The fungicides tested were prothioconazole (UPL 2010 25% - 250g i.a / L p.c.), cyproconazole (UPL 215 10% - 100g i.a / L p.c.), tebuconazole (Tebufort 20% - 200g i.a / L p.c.); trifloxystrobin (Flint 50% - 500g i.a / L p.c.); azoxystrobin (Amistar 50% - 500g i.a / L p.c.); mancozeb (Unisex Gold 75% - 750g i.a / L p.c.) and azoxystrobin + benzovindiflupir (Elatus WG 30% and 15% - 450g i.a / L p.c.).

80. Table 2 below lists the mean concentrations (ppm ai) of the UPL 2010, UPL 215, Tebufort, Amistar, Flint, Unizeb Gold and Elatus fungicides required to inhibit 50% (LC50) and 95% (LC95) of Asian rust symptoms for 69 samples, 2017/18 harvest, compared to the 2016/17 harvest.

Fungicidas	CL ₅₀ (ppm i.a.)		CL ₉₅ (ppm i.a.)	
	2016/2017	2017/2018	2016/2017	2017/2018
UPL 2010	-	2,21	-	47,13
UPL 215	1,61	2,24	27,48	47,88

TEBUFORT	1,80	2,28	32,47	46,19
AMISTAR	1,53	1,83	26,13	40,35
FLINT	0,63	1,47	10,21	32,82
UNIZEB GOLD	2,52	3,27	41,28	59,29
ELATUS	1,38	1,44	25,16	31,49

81. It could be concluded that for mancozeb, the calculated resistance factor (FR) was the lowest value compared to other products, which may suggest, due to its non-specific mode of action, that the active exerted a lower or no selection pressure.

82. The activity data from these trials was then correlated with the data of adoptions of use of multisite in soybean given by Spark (Market research). This data was thus correlated with the SRF for each active ingredient. It was found that in the Paraná State the increase of SRF was greater for all the active ingredients in comparison with Rio Grande do Sul State. The Spark Market Research data revealed that the adoption of multisites fungicides for growers in Paraná State was less than the growers in Rio Grande do Sul State. The only plausible conclusion that could be drawn from such comparison was the lower adoption of multisites fungicides for growers in Paraná State than the growers in Rio Grande do Sul State meant that, and directly responsible for, the increase of SRF being greater in the Paraná State for all the active ingredients in comparison with Rio Grande

do Sul State. It was thus concluded that the greater adoption of multisite fungicides (mancozeb) in Rio Grande do Sul State in the last two seasons help to keep the SRF of azoxystrobin, trifloxystrobin, ciproconazol and tebuconazol less than in Paraná State i.e. led to a better efficacy. These results are in the table below.

Paraná State	2016/17 (8 samples)	33	6,85	5,44	183,88	152,38	
	2017/18 (24 samples)	39	9,79	16,79	272,58	247,83	

Increase in the SRF in Paraná State(%)			42,81	208,77	48,24	62,65	90,62
Rio Grande do Sul	2016/17 (6 samples)	55	5,44	4,39	192,00	186,00	
Rio Grande do Sul	2017/18 (9 samples)	68	6,34	9,93	200,67	209,00	
Increase in the SRF in Rio Grande do Sul State (%)			16,50	126,28	4,51	12,37	39,91
Values for LD50 for each active ingredient and each season was taken from a trial described above. Baseline values for Trifloxystrobin were taken from Blum 2009. Baseline values for Azoxystrobin, Tebuconazol and Ciproconazol were taken from Schmitz et al., 2013. This shift indicates the amount of sensitivity reduction for a fungicide.							

83.From these trials, the following inferences were scientifically drawn:

- (a) For azoxystrobin, the sensitivity reduction factor (SRF) increased by 42.82% in Parana State (due to lower mancozeb adoption), whereas in Rio Grande do Sul State, where the mancozeb adoption was significantly higher, the SRF increase was significantly lower at 16.50%. This lower increase in the SRF in Rio Grande do Sul State was attributed to the greater adoption in the state.

- (b) For trifloxystrobin, the sensitivity reduction factor (SRF) increased by 208.77% in Parana State (due to lower mancozeb adoption), whereas in Rio Grande do Sul State, where the mancozeb adoption was significantly higher, the SRF increase was significantly lower at 126.28%. This lower increase in the SRF in Rio Grande do Sul State was attributed to the greater adoption in the state.
- (c) For tebuconazole, the sensitivity reduction factor (SRF) increased by 48.24% in Parana State (due to lower mancozeb adoption), whereas in Rio Grande do Sul State, where the mancozeb adoption was significantly higher, the SRF increase was significantly lower at 4.51%. This lower increase in the SRF in Rio Grande do Sul State was attributed to the greater adoption in the state.
- (d) For ciproconazole, the sensitivity reduction factor (SRF) increased by 62.65% in Parana State (due to lower mancozeb adoption), whereas in Rio Grande do Sul State, where the mancozeb adoption was significantly higher, the SRF increase was significantly lower at 12.37%. This lower increase in the SRF in Rio Grande do Sul State was attributed to the greater adoption in the state.
- (e) For the total fungicide control values, the sensitivity reduction factor (SRF) increased by 90.62% in Parana State (due to lower mancozeb adoption), whereas in Rio Grande do Sul State, where the mancozeb adoption was significantly higher, the SRF increase was significantly



lower at 39.91%. This lower increase in the SRF in Rio Grande do Sul State was attributed to the greater adoption in the state.

84. It was thus concluded that a lower multisite fungicide i.e. mancozeb adoption led to a greater resistance to the fungicides, in turn leading to lower fungicidal efficacy in Parana State. In contrast, a greater mancozeb adoption led to a lower resistance to the fungicides, in turn leading to significantly higher fungicidal efficacy in Rio Grande do Sul State. Therefore, for the first time, it was scientifically possible to correlate the sensitivity reduction factor to the adoption of mancozeb used in the treatment used to control the fungal infection, which is a new scientific finding. This is a new scientific finding.

The presence of a multisite contact fungicide surprisingly reduces the phytotoxicity due to the resulting triple combination:

85. It has been well known and established that the fungicidal efficacy of the systemic fungicide combinations i.e. especially of combinations of prothioconazole or tebuconazole with strobilurin fungicides has been on a gradual downtrend since the onset of resistance to these fungicides. The reducing fungicidal efficacy of the systemic fungicides requires the application of a larger amount of these fungicides in order to achieve an appreciable fungicidal efficacy. This use of a greater amount of these fungicides leads to an unintended side-effect i.e. greater phytotoxicity on

the desired crop plants due to the larger dosage of these fungicides. Greater the quantity of the systemic fungicides used, greater was the observed phytotoxicity.

86.This problem needed a solution.

87.Now turning to the surprising effect of the present invention, experiments were conducted to evaluate the effect of mancozeb in reduction of phytotoxicity caused by a demethylation inhibitor fungicide namely prothioconazole and tebuconazole, and a quinone outside inhibitor, specifically trifloxystrobin. These experiments were conducted in Brazil in tropical savannah climatic zone on *Glycine max* crop variety NS 7209 IPRO. The crop was directly seeded at a planting density of 380000 S/ha. These trials were conducted in four replications in randomized complete block study design. Prothioconazole was used at 85 g/ha, and mancozeb at 1125 g/ha. The active ingredients were foliar sprayed on the crop at predetermined treatment intervals.

88.The 4 applications were carried out with a 14 day interval, starting at the beginning of flowering (R1 / R2) cycle. The disease severity assessments were carried out on at least 10 trifoliate leaves / plot before each application and at 7, 14 and 21 days after last application (D). One of the replication was also evaluated for plot defoliation. At the end of the



assessments, the area under the disease progress curve (AUPDC) was calculated for severity and its respective control by the Abbott formula.

89. The phytotoxicity measurements were evaluated before each application and 7 days after last application. The measurements used a 0 to 100% scale, wherein 0 means absence of symptoms and 100 total death of plants. In the end, the crop was harvested in each 5m² plots and the productivity (kg / ha) and thousand grain weight was determined. In each tested plot, a fourth standard application for all treatments was made at 21 days after the last application except for control.

90. The combination used was a formulation A comprising trifloxystrobin + tebuconazole. A 0.40 kg-L/ha of this formulation delivered 60 g/ha trifloxystrobin and 70 g/ha prothioconazole, while 0.60 kg-L/ha delivered 75 g/ha trifloxystrobin and 105 g/ha prothioconazole, and while 0.80 kg-L/ha delivered 120 g/ha trifloxystrobin and 140 g/ha prothioconazole. Mancozeb was added to the various treatments varying from 1.5 kg/ha till 3.5 kg/ha.

S No.	Product	Rates (kg-L/ha)	Phytotoxicity %		
			15 DAT A	15 DAT B	7 DAT C
1	Untreated	-	0.0	0.0	0.0
2	A	0.40	1.9	2.4	2.7

3	A + Mancozeb	0.40 + 1.5	0.0	1.1	1.6
4	A + Mancozeb	0.40 + 2.0	0.0	1.0	1.3
5	A + Mancozeb	0.40 + 2.5	0.0	0.0	1.2
6	A	0.6	3.5	4.6	6.1
7	A + Mancozeb	0.60 + 1.50	0.0	1.2	1.9
8	A + Mancozeb	0.60 + 2.0	0.0	1.1	1.9
9	A + mancozeb	0.60 + 2.5	0.0	1.5	2.5
10	A	0.8	4.8	6.6	8.4
11	A + Mancozeb	0.80 + 1.50	0.0	1.1	2.6
12	A + Mancozeb	0.80 + 2.00	0.0	1.5	2.5
13	A + Mancozeb	0.80 + 2.50	0.0	1.1	2.5

14	A + Mancozeb	0.80 + 3.00	0.0	0.9	2.2
15	A + Mancozeb	0.80 + 3.50	0.0	1.1	1.9

91. From the phytotoxicity results brought out above, 7 days after fourth spray (7 DAT D), a significant reduction of phytotoxicity using Mancozeb in association of a demethylation inhibitor fungicide namely Prothioconazole in combination with Trifloxystrobin was noted.

92. It was also found that greater the initial phytotoxicity seen in the combination of the systemic fungicides, greater was the benefit of adding mancozeb in the predetermined recited quantity to the phytotoxic combination of systemic fungicides. In other words, the use of mancozeb as a fungicidal safener was greater in the combinations which were more phytotoxic.

93. The addition of mancozeb suprisingly reduced phytotoxicity in a demethylation inhibitor fungicide, namely prothioconazole or tebuconazole, in combination with a strobilurbin.



S No.	Product	Rates (L- kg/ha)	Phytotoxicity %					AUDPC
			15 DAT A	15 DAT B	7 DAT C	14 DAT C	21 DAT C	
1	Untreated	-	3.7	21.9	43.4	65.3	84.5	1143.5
2	A	0.40	0.1	3.5	9.2	20.2	21.3	236.6
3	A + mancozeb	0.40 + 1.50	0.0	3.0	7.3	16.8	20.1	210.4
4	A + mancozeb	0.40 + 2.00	0.0	2.3	6.2	14.2	19.6	185.3
5	A + mancozeb	0.40 + 2.50	0.0	2.3	5.6	13.5	18.4	179.7
6	A	0.60	0.0	2.6	5.5	18.5	17.2	194.4
7	A + mancozeb	0.60 + 1.5	0.0	2.3	5.3	14.7	16.1	178.2
8	A + mancozeb	0.60 + 2.00	0.0	2.3	5.1	12.4	12.8	162.9
9	A + mancozeb	0.60 + 2.50	0.0	2.2	4.6	11.1	12.8	150.5
10	A	0.80	0.0	2.4	4.4	18.1	14.6	179.4
11	A + mancozeb	0.80 + 1.50	0.0	2.4	4.4	12.6	14.5	157.7

12	A + mancozeb	0.80 + 2.00	0.0	2.0	5.1	12.0	12.4	151.1
13	A + mancozeb	0.80 + 2.50	0.0	2.2	4.3	10.3	11.0	144.9
14	A + mancozeb	0.80 + 3.00	0.1	2.7	4.7	10.7	11.6	155.5
15	A + mancozeb	0.80 + 3.50	0.0	2.7	6.2	9.7	10.9	154.8

94. Another trial was conducted to evaluate the effect of mancozeb in reduction of phytotoxicity caused by prothioconazole @ 70 g/ha. The trial was conducted in Rio Verde, Goias (Brazil) in tropical savannah climatic zone. The target crop was *Glycine max* NS 7209 IPRO variety. The planting was done by seeding on 14th December. The seeds were planted at a rate of 16 seeds per metre, at a depth of 5 cm with the seeds being spaced at 50 cm from each other. The emergence rate was noted on December 21, and the harvest date of April 8. The planting density was maintained at 320000 seeds per hectare. The plots were convention-tilled and the study was conducted in 4 replications in randomized complete block study design.

95. Three sprays were carried out with a 14 day interval, starting at the beginning of flowering (R1 / R2). Disease severity assessments were carried

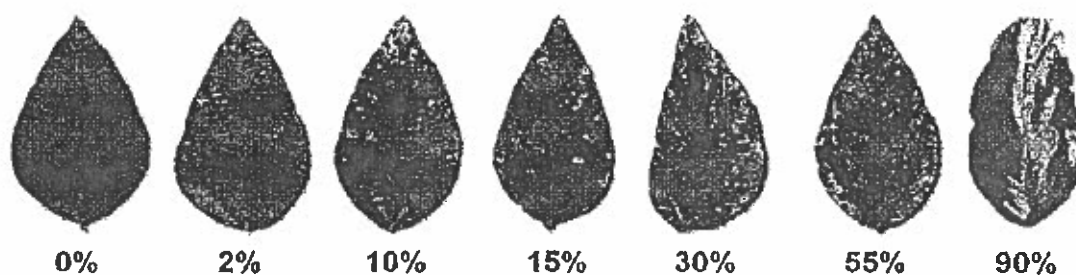


out on at least 10 trifoliate leaves / plot before each application and at 7, 14 and 21 days after last application (D). An evaluation of plot defoliation was carried out in R6 replication. At the end of the assessments, the area under the disease progress curve (AUPDC) was calculated for severity and its respective control by the Abbott formula. Each application was evaluated 7 days after last application for phytotoxicity using a scale of 0 to 10 scale, which 0 means absence of symptoms and 10 total death of plants. Lastly, the harvest of 5 m² was performed and determined the productivity (kg / ha) and thousand grain weight, correcting the humidity to 13%.

Phytotoxicity scale:

	No symptoms of phytotoxicity
	1 to 10% of foliar area affected
	11 to 20% of foliar area affected
	21 to 30% of foliar area affected
	31 to 40% of foliar area affected
	41 to 50% of foliar area affected
	51 to 60% of foliar area affected
	61 to 70% of foliar area affected
	71 to 80% of foliar area affected
	81 to 90% of foliar area affected
	91 to 100% of foliar area affected

Handwritten signature or mark



Agro Carregal Pesquisa e Proteção de Plantas, 2016

Fonte: Agro Carregal, 2016.

Results:

Table 3

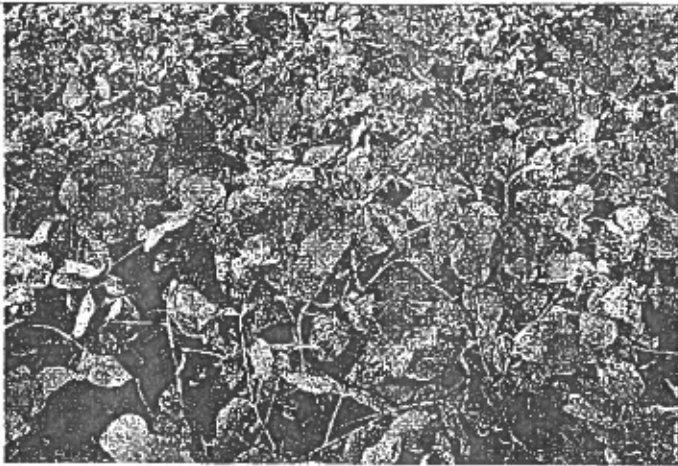
S No.	Active ingredient (g/ha)	Timing	Phytotoxicity (%)			Yield (bags/ha)
			15 DAT A	15 DAT B	7 DAT C	
1	Untreated	-	0.0	0.0	0.0	36.6 c
1	Trifloxystrobin 60 + Prothioconazole 70 + Mancozeb 750	ABC	0.0	0.0	2.0	46.6 b
3	Trifloxystrobin 60 +	ABC	0.0	0.0	2.0	54.2 a

	Prothioconazole 70 + Mancozeb 1125					
4	Trifloxystrobin 60 + Prothioconazole 70 + Mancozeb 1500	ABC	0.0	0.0	1.0	56.1 a
5	Trifloxystrobin 60 + Prothioconazole 70 + Mancozeb 1875	ABC	0.0	0.0	1.0	56.1 a
6	Trifloxystrobin 60 + Prothioconazole 70 + Mancozeb 2250	ABC	0.0	0.0	1.0	56.8 a
7	Trifloxystrobin 60 + Prothioconazole 70	ABC	0.0	3.0	5.0	45.7 b

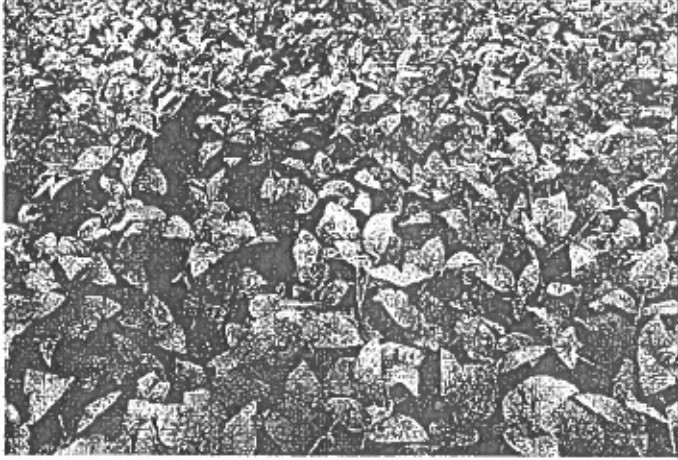
CV (%)	5.20
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15 DAT A – 15 days after spray A i.e. first spray. Means followed by the same letter or symbol did not differ by Scott-Knott test 5% of probability. 1 bag = 60 kg of soybean.

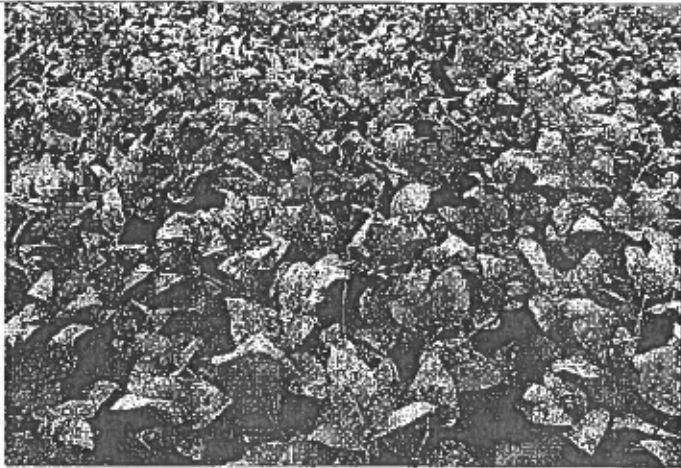
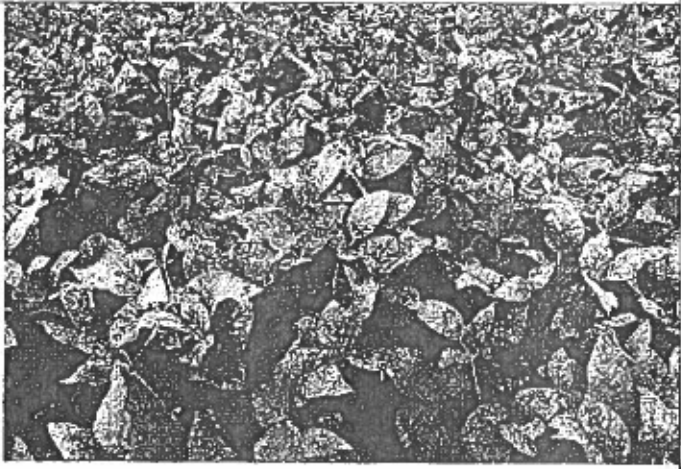
Photographic evidence:

S	Active ingredient (g/ha)	Timin g	
No			
1	Untreated		

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1	Trifloxystrobin 60 + Prothioconazol e 70 + Mancozeb 750	ABC	
3	Trifloxystrobin 60 + Prothioconazol e 70 + Mancozeb 1125	ABC	
4	Trifloxystrobin 60 + Prothioconazol e 70 + Mancozeb 1500	ABC	



5	Trifloxystrobin 60 + Prothioconazol e 70 + Mancozeb 1875	ABC	
6	Trifloxystrobin 60 + Prothioconazol e 70 + Mancozeb 2250	ABC	
7	Trifloxystrobin 60 + Prothioconazol e 70	ABC	

96. About results of phytotoxicity, 7 days after third spray (7 DAT C), it was possible to note a significant reduction of phytotoxicity using Mancozeb in association of Prothioconazole & Tryfloxistrobin.

Role of mancozeb in reducing the phytotoxicity of a triple combination versus in a dual combination:

97. The present invention uses a multisite fungicide with a combination of systemic fungicides. The combination of systemic fungicides comprises tebuconazole or prothioconazole and a strobilurin fungicide. The contact fungicide mancozeb is added to the systemic fungicide combination to decrease the phytotoxicity of the combination. This finding was an unexpected and hitherto unknown property of contact fungicides like mancozeb.

98. The combination of these systemic fungicides causes more phytotoxicity than the solo individual use of those fungicides. It has been surprisingly found that the contribution of mancozeb in reducing the phytotoxicity of a double combination of systemic fungicides is unexpectedly more than its contribution in reducing the phytotoxicity of single systemic fungicide in isolation.



99. Experiments were performed at Ituverava, Brazil in tropical savannah climatic zone. The target crop was NS 7209 IPRO variety of soybean. The crop was planted at a planting rate of 19 seeds per metre at a depth of 5 cm, to a total of 380000 seeds per hectare. The inter-row spacing was maintained at 50 cm. The experiments were performed in randomized complete block study design with 4 replications. The treatments were sprayed to the trial plots to ensure a foliar placement of the applied fungicides. Water was used as the carrier for diluting the applied fungicidal formulations to 150 L/ha.

100. 4 applications (ABCD) of the respective treatments were carried out with a 14 day interval, starting at the beginning of flowering (R1 / R2). The disease severity assessments were carried out on at least 10 trifoliate leaves / plot before each application and at 7, 14 and 21 days after last application (D). In R6, an evaluation of plot defoliation was performed. At the end of the assessments, the area under the disease progress curve (AUPDC) for severity and its respective control by the Abbott formula was calculated. Phytotoxicity was evaluated before each application and 7 days after last application, using a 0 to 100% scale, wherein 0 means absence of symptoms and 100 total death of plants. The harvest of 5 m² was performed and the productivity (kg / ha) and thousand grain weight was determined.



101. In all the experiments described below, Aureo has been used as an adjuvant. Aureo is the trademark for the soybean oil methyl ester adjuvant available from Bayer. It does not have any fungicidal efficacy of its own, but is commonly used in tank-mix trials to modify the surface tension of the fungicide/mixture to enable it to be applied to the test site (<http://dx.doi.org/10.1590/0100-5405/2020>). In any event, its effect would be uniform across all the experiments described herein, so that any benefits ascribable to Aureo would have evened out across all the treatments.

102. In the data described below, the percentage reduction in phytotoxicity due to mancozeb was calculated as follows. The phytotoxicity for the substance A with and without mancozeb was noted. For example, if the phytotoxicity of substance A was "a" without mancozeb, and "b" with mancozeb, the percentage reduction in phytotoxicity for the substance due to mancozeb was calculated as $[(a-b)/a*100]$.

103. It is well known that conazole fungicides are phytotoxic to the desired crop, at the required dosage at which they exhibit fungicidal properties. Moreover, the presence of a strobilurin fungicide seems to aggravate the problem of phytotoxicity (doi:10.4141/CJPS2012-181, annexed hereto E1).



This was also corroborated by the presently described experiments, wherein it was found that the phytotoxicity due to a combination of conazole and strobilurin fungicides was much higher than the phytotoxicity due to a conazole fungicide alone.

104. Regarding the phytotoxicity of conazole fungicides, E1 draws the following conclusions, thereby establishing the phytotoxic side-effects of conazole or conazole + strobilurin fungicides.

- *Dichlorprop/2,4-D mixed with tebuconazole caused up to 15% injury when applied early and up to 29% injury when applied late.*
- *Bromoxynil/MPCA mixed with tebuconazole injured wheat up to 15% when applied early but only 10% when applied late. Other herbicide and fungicide mixes caused a lower level of injury.*
- *The fungicide tebuconazole caused the highest level of injury when mixed with herbicides and injury was particularly high with dichlorprop/2,4-D.*
- *The fungicide tebuconazole applied in a tank-mix with a herbicide caused the highest level of visible injury compared with the other fungicide-herbicide combinations.*



- *Of the four fungicides, tebuconazole increased injury the most with each of the three herbicides evaluated.*
 - *The addition of azoxystrobin/propiconazole to bromoxynil/MPCA was injurious at 7 of 8 site years with injury level as high as 7.3% at Elora in 2009.*
 - *In both 2009 and 2010, all tank-mixes that include the fungicide tebuconazole, consistently caused visible injury greater than either of the single components of the mixture applied alone.*
 - *Tebuconazole caused injury when mixed with dichlorprop/2,4-D, bromoxynil/MPCA and pyrasulfotole/bromoxynil.*
 - *In previous studies, tebuconazole was reported to reduce the efficacy of certain herbicides when applied as a tank-mix; however, no injury or yield reductions were observed (Lancaster et al. 2005a, b).*
 - *In contrast, tebuconazole phytotoxicity has been reported in wheat and in other crops such as cocoa and rockmelon (Holderness 1990; Vawdrey 1994; Hunt et al. 2008). This fungicide is known to be inherently toxic to plants at rates required for adequate fungicidal action (Pedersen 2007; Hunt et al. 2008). It is conceivable that the phytotoxicity observed when herbicides were mixed with tebuconazole was due to a reciprocal action.*
- 

- *The fungicide or components of its commercial formulation may increase uptake of the herbicides, while components of the herbicide formulations could also enhance tebuconazole leaf penetration, accelerating its phytotoxic action. This double action would account for the specificity of injury we observed with each tebuconazole/herbicide tank-mix (Figs. 1 to 3).*
 - *Interestingly, of all the combinations that were tested in this research, bromoxynil/MPCA+tebuconazole was the only registered tank-mix found on both product labels (Bayer CropScience Inc. Suite 100, 3131 114th Avenue S.E., Calgary, AB), and was one of the most injurious tank-mixes in this study.*
 - *Strobilurin fungicides have been documented to cause crop injury when tank-mixed with certain herbicides (Stump et al. 2002; Robinson and Nurse 2009). The phytotoxic effect of strobilurin fungicides is enhanced when tank mixes include products that solubilize the plant cuticle (such as surfactants and some pesticides) and facilitate increased passage of strobilurin into the living plant tissue instead of remaining on the leaf surface, resulting in injury (Stump et al. 2002; Vincelli 2002; Agrios 2005).*
- 

- *In fact, the product label for azoxystrobin/propiconazole issues a warning that phytotoxic effects may be encountered when azoxystrobin+propiconazole is tank-mixed with products formulated as emulsifiable concentrates (Syngenta Crop Protection Canada, Inc., 140 Research Lane, Guelph, ON).*

105. The present invention provides a solution to this problem of phytotoxic side-effects of conazole or conazole + strobilurin fungicides.

106. Now turning to the present experiments, the following is a summary of the observations noted during this trial:

Treatment Protocols:

Treatment No.	Product	Concentration	Formulation type	Dosage	Active (g of a.i./ha)	Spraying timing
1	Untreated	-	-	-	-	-

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2	UPL 2030 FP	480	SC	0.177	Prothiocona zole (85)	ABC D
	Aureo	720	EC	0.25 %	Soybean methyl ester (270)	ABC D
3	UPL 2030 FP	480	SC	0.177	Prothiocona zole (85)	ABC D
	Unizeb Gold	750	WG	1.5	Mancozeb (1125)	ABC D
	Aureo	720	EC	0.25 %	Soybean methyl ester (270)	ABC D
4	Fox	150 + 175	SC	0.5	Trifloxystrobi n (75) + Prothiocona zole (87.5)	ABC D

07

	Aureo	720	EC	0.25 %	Soybean methyl ester (270)	ABC D
5	Fox	150 + 175	SC	0.5	Trifloxystrobin (75) + Prothioconazole (87.5)	ABC D
	Unizeb Gold	750	WG	1.5	Mancozeb (1125)	ABC D
	Aureo	720	EC	0.25 %	Soybean methyl ester (270)	ABC D

Phytotoxicity results:

S N o.	Active	Phytotoxicity (%)					
		14 DAT A	% reduction in phytotoxi	7 DA T C	% reduction in phytotoxi	7 DA T D	% reduction in phytotoxi

			city due to mancoze b		city due to mancoze b		city due to mancoze b
1	Untreated	-	0.0	0.0	-	0.0	-
2	Prothiocona zole	0.0	-	11. 8	-	26. 5	-
3	Prothiocona zole + mancozeb	0.0	-	3.3	72.3	6.5	75.5
4	Prothiocona zole + Trifloxystrobi n	8.5	-	22. 5	-	40. 0	-
5	Prothiocona zole + trifloxystrobi n + mancozeb	0.0	100.0	3.5	84.4	9.0	77.5

C		13.2	-	9.6	-	7.3	
V		4t		1t		8t	
(5)							

DAA/B/C - Days After Spray A/B/C/D; t = Transformed data for
 $\text{"arcsen}((x/100)^{1/2})$;

107. Thus, it was found surprisingly found that mancozeb reduced the phytotoxicity of prothioconazole alone by 72.2% and 75.5%, but in a combination of systemic fungicides, mancozeb caused to reduce the phytotoxicity of the combination by 100%, 84.4% and 77.5% respectively. Thus, the contribution of mancozeb in reducing the phytotoxicity of a double combination of systemic fungicides is more than its contribution in reducing the phytotoxicity of single systemic fungicide in isolation.

S No.	Active	Percent severity for <i>Phakopsora</i> <i>pachyrrhizi</i>	AUDPC
1	Untreated	100.0	609.1
2	Prothioconazole	5.8	24.4
3	Prothioconazole + Mancozeb	2.1	8.1

On

4	Prothioconazole + Trifloxystrobin	1.2	4.3
5	Prothioconazole + Trifloxystrobin + Mancozeb	0.5	2.1
	CV (%)	7.97t	12.08t

DAA/B/C - Days After Spray A/B/C/D; t = Transformed data for $\arcsin((x/100)^{1/2})$; t* = Transformed data for $(x+k)^{1/2}$ with k = 1

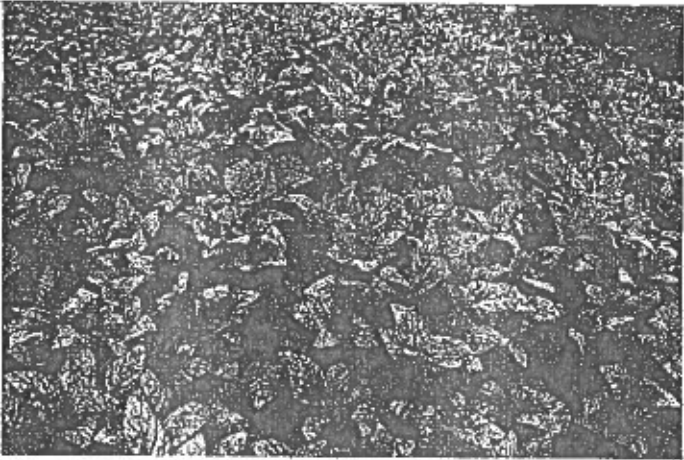
108. Thus, mancozeb also surprisingly reduced the percentage incidence of *Phakopsora pacchyrrzhi* and area under disease progress curve for the combination of systemic fungicides. This means that not only mancozeb reduced the phytotoxicity problem seen with conazole or conazole + strobilurin fungicides, it also enhanced the disease control i.e. fungicidal effect exerted by these fungicides.

109. The addition of mancozeb not only reduced the phytotoxicity of the systemic fungicides in combination quantitatively, but there was a qualitative visual reduction in the phytotoxicity in the plots treated additionally with mancozeb.

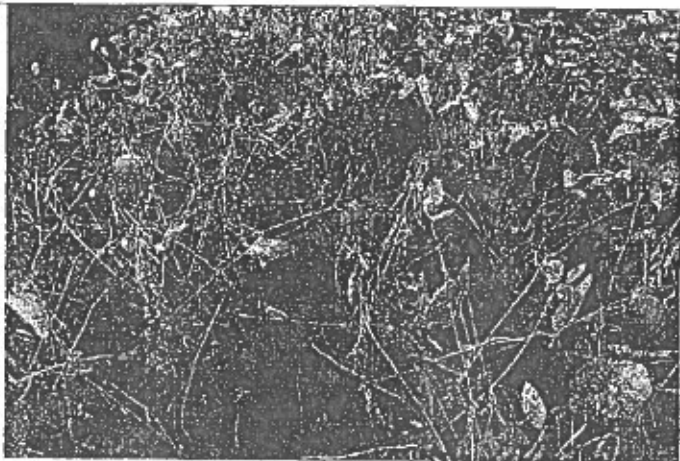
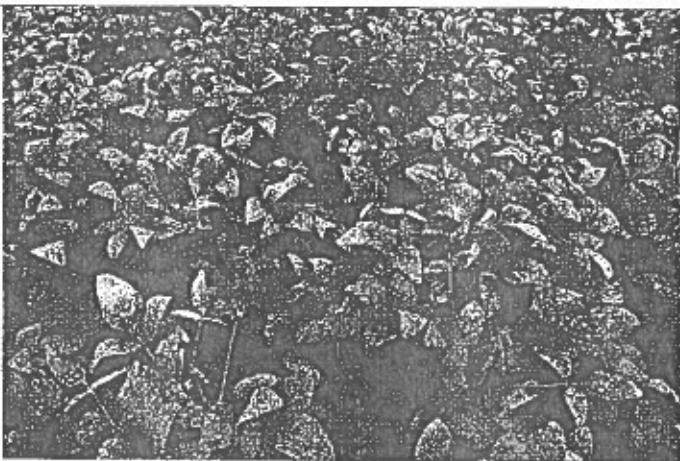
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S No	Treatment	Field observation
14 days after spray C		
1	Untreated	
2	Prothioconazole	

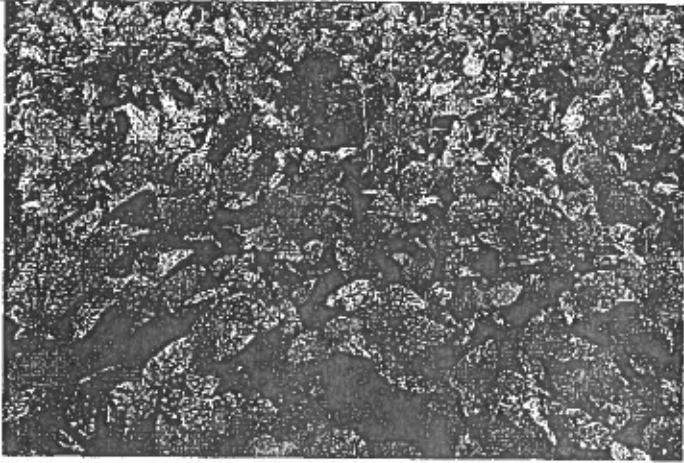
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3	Prothioconazol e + Mancozeb	
4	Prothioconazol e + Trifloxystrobin	
5	Prothioconazol e + Trifloxystrobin + Mancozeb	
14 days after spray D		

01

1	Untreated	
2	Prothioconazole	
3	Prothioconazole + Mancozeb	

On

4	Prothioconazol e + Trifloxystrobin	
5	Prothioconazol e + Trifloxystrobin + Mancozeb	

110. Thus, it was concluded that the combination of Prothioconazole with Trifloxystrobin caused much greater level of phytotoxicity in comparison with Prothioconazole sprayed in isolation. In general, the combination of an azole fungicide with a strobilurin fungicide was more phytotoxic. More importantly, the percentage of reduction of phytotoxicity by using mancozeb was greater in the mixture of Prothioconazole & Trifloxystrobin in comparison with the use of mancozeb with Prothioconazole in isolation in the most part of evaluations. This was more beneficial because of the



higher rate of phytotoxicity in the treatment using Prothioconazole & Trifloxystrobin.

111. Thus, it was surprisingly found that the greater the phytotoxic effect seen due to the treatment with a combination of systemic fungicides, greater was the beneficial effect seen due to the introduction of mancozeb in reducing this aggravated phytotoxicity.

Use of multisite contact fungicides in general

112. Similar trials as described above were carried out using other multisite contact fungicides. These experiments were carried out to corroborate the same advantages were achieved using these other multisite fungicides as achieved using mancozeb.

113. The trial protocol for these experiments was the same as that using mancozeb, hence not described again. In each of the following experiments, the percentage severity of Asian soybean rust and the area under disease progress curve (AUDPC) were transformed to calculate the percentage control values and reported. These results of percentage control achieved are reported below:

Use of chlorothalonil as the multisite contact fungicide;

A

114. The dosage of trifloxystrobin was 70 g/ha (500 WG formulation), tebuconazole 100 g/ha (200 EC formulation), while chlorothalonil was used at 1080 g/ha (720 SC formulation). In these experiments, DAA/B/C meant days after treatment A, B and C respectively.

S No.	Mixture	Chlorothalonil		Difference in percentage control
		Without	With	
1	Trifloxystrobin + Tebuconazole @ 21 DAA	97.1	98.6	+ 1.5
2	Trifloxystrobin + Tebuconazole @ 18 DAB	68.1	88.1	+ 20.0
3	Trifloxystrobin + Tebuconazole @ 7 DAC	72.9	78.8	+ 5.9

115. Thus, in each of these experiments, a positive correlation between the introduction of chlorothalonil and the percentage control of Asian soybean rust was seen.

Use of copper oxychloride as the multisite contact fungicide:

116. Copper oxychloride 588 SC was applied at 0.5 kg-L/ha. Pyraclostrobin 250 EC was applied at 0.32 L/ha, whereas metconazole 90 EC was applied

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at 0.55 L/ha. In these experiments, DAA/B/C meant days after treatment A, B and C respectively.

S No.	Mixture		Copper Oxychloride		Difference in percentage control
			Without	With	
1	Pyraclostrobin + Metconazole @ 18 DAB		61.6	85.7	+ 24.1%
2	Pyraclostrobin + Metconazole @ 7 DAC		53.4	80.8	+ 27.4%
3	Pyraclostrobin + Metconazole (AUDPC; % Control)		57.5	83.2	+ 25.7%

117. Thus, in each of these experiments, a positive correlation between the introduction of copper oxychloride and the percentage control of Asian soybean rust was seen.

Secondary evidence in the form of a reasonable expectation of a person skilled in the art:

118. Dr. Claudia Vieira Godoy is a prolific researcher, currently positioned as Senior Researcher at Brazilian Agricultural Research Corporation, EMBRAPA. She has published about 95 papers till date, a majority of which (50) relate

to soybean rust and its fungicidal control. A list of all her research publications is enclosed herewith. It is beyond doubt that Dr. Godoy is an expert in the art of soybean production and related fungicidal control. She is a person skilled in the art.

119. The present invention claims a priority date of 26.11.2013, whereas the first country to publish it was the Indian Patent Office on 29.05.2015. Dr. Godoy's publications before 2013, and after 2015, also stand a testimony to the inventiveness of the present application.

120. The first paper (Paper 1) referred to herein outlines the changes in performance of soybean rust fungicides over the years and the new management strategies adopted in Brazil. This paper explains the network of fungicides from 2003 – 2009, and provides perspectives for 2009/10. This would mean that the contents of this paper outlines the current thinking in the art of soybean rust control before 2009/10, i.e. before the publication date of the present publication.

121. Page 13 of Paper 1 lists the fungicides in use for soybean rust control from 2002/03 till 2009/10. This list includes 6 triazoles, 2 strobilurins, 5 triazoles + strobilurins and 1 triazole + benzimidazole. Page 16 onwards lists DMIs + Qols as one of the preferred soybean rust management strategies during 2003/04 till 2009/10. Page 24 of Paper 1 lists the sensitivity monitoring tests, which reveals that the increasing EC₅₀ values



denote that the efficacy of the fungicides known till then had been decreasing from 2005/06 till 2008/09. Page 28 of Paper 1 establishes that during 2007 – 2009, almost 80% of the soybean rust control was dominated by the DMI + Qol mixture even though its efficacy was reducing year by year. The industry was in dire need of a solution to this problem of reducing efficacy of known fungicides towards soybean rust.

122. There is no mention at all regarding the use of a multisite contact fungicide at all in these rust control strategies, i.e. before the publication of the present invention.

123. Paper 2 entitled Protective, curative and eradivative effects of fungicides to control soybean rust caused by *Phakopsora pachyrhizi* in greenhouse was published in November 2003. This Paper 2 investigates the protective, curative and eradicating effects of systemic fungicides (azoxystrobin 50 g ai / ha + nimbus 0.5%, carbendazin 250 g ai / ha, tebuconazole 100 g ai / ha, difenoconazole 50 g ai / ha and epoxiconazole 25 g ai / ha + pyraclostrobin 66.5 g ai / ha) evaluated in soybean (*Glycine max*) plants.

124. Table 1 of Paper 2 demonstrated that at 14 days after sowing, almost 90% control was seen with the combination of epoxiconazole + pyraclostrobin i.e. the concept of using a strobilurin + conazole fungicide in combination for the control of soybean rust was known and prevalent in 2003.



125. Till the knowledge existing in the state of the art till 2003, there is no mention at all regarding the use of a multisite contact fungicide at all in these rust control strategies, i.e. before the publication of the present invention.
126. Paper 3 was published around February 2004. Paper 3 also investigates the protective, curative and eradicating effects of systemic fungicides (azoxystrobin 50 g ai / ha + nimbus 0.5%, carbendazin 250 g ai / ha, tebuconazole 100 g ai / ha, difenoconazole 50 g ai / ha and epoxiconazole 25 g ai / ha + pyraclostrobin 66.5 g ai / ha) evaluated in soybean (*Glycine max*) plants.
127. Table 1 of Paper 3 also demonstrated that at 14 days after sowing, almost 90% control was seen with the combination of epoxiconazole + pyraclostrobin i.e. the concept of using a strobilurin + conazole fungicide in combination for the control of soybean rust was known and prevalent in 2004.
128. Till the knowledge existing in the state of the art till 2004, there is no mention at all regarding the use of a multisite contact fungicide at all in these rust control strategies, i.e. before the publication of the present invention.
129. Paper 4 was published in June 2005. On page 676 of this Paper 4, it is disclosed that – *Moreover, application of foliar fungicides is not a routine* 

practice on second-crop soybeans. Thus, this paper actually teaches a person skilled in the art away from using multisite contact fungicides for soybean rust control. Further, the very next paragraph of this paper teaches that – *Currently, strobilurin and triazole fungicides are being used.*

130. Therefore, till the knowledge existing in the state of the art till 2005, there is no mention at all regarding the use of a multisite contact fungicide at all, much less mancozeb, in these rust control strategies, i.e. before the publication of the present invention.

131. Paper 5 – Del Ponte, Emerson & Godoy, Claudia & Du Preez, E & van Rij, N & Caldwell, P & Yang, X. (2005). Predicting regional Asian soybean rust outbreaks in Brazil and South Africa. *Phytopathology*. 95. S23-S23 – was also published in June 2005 and has the same conclusions as the Preez thesis discussed above.

132. Preez is a part of a MSc thesis submitted by Eve Diane du Preez for her work done on control of soybean rust. Preez reports the efficacy of 11 fungicides for the control of *Phakopsora pachyrhizi*: azoxystrobin, triadimenol, flusilazole, triforine, mancozeb, epoxiconazole/pyraclostrobin, tebuconazole, flutriafol, flusilazole/carbendazim.

133. Preez quotes the studies attempted by Yang (1977) for different cultivars. The yield loss, calculated from the difference for mancozeb sprayed and unsprayed soybean yields, was 23, 24, 32 and 50% for the cultivars. Thus,

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this citation in Preez attempts to demonstrate the criticality of using mancozeb alone for controlling rust in wheat. Preez does not investigate the use of mancozeb as a synergist for soybean rust, nor the effects it has in synergizing the activity of the conazole + strobilurin fungicides in controlling soybean rust. Applicant never claims to have invented mancozeb for the first time or its usage in the control of Asian soybean rust for the first time. Applicant's invention resides in a combination comprising mancozeb and a double combination of systemic fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides. Preez or the Yang (1977) study quoted by Preez, does not contemplate mixing mancozeb with any systemic fungicides, less so, in a triple combination as in the present invention.

134. Preez again quotes a 1979 study by Ogle *et al.*, who conducted a field trial where soybeans were protected for different lengths of time. Yield reduction in the unsprayed treatment was 67.8% compared with the treatment that was sprayed fortnightly with mancozeb until harvest. Loss was associated with reduction in the number of pods, number of filled seeds, seed mass, seed yield per plant, reduced seed oil content (but not protein) and non-seed dry weight of plants. Again, this citation in Preez attempts to demonstrate the criticality of using mancozeb alone for controlling rust in wheat. Preez does not investigate the use of mancozeb as a synergist for soybean rust, nor the effects it has in synergizing the



activity of the conazole + strobilurin fungicides in controlling soybean rust. Applicant never claims to have invented mancozeb for the first time or its usage in the control of Asian soybean rust for the first time. Applicant's invention resides in a combination comprising mancozeb and a double combination of systemic fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides. Preez or the Ogle (1979) study quoted by Preez, does not contemplate mixing mancozeb with any systemic fungicides, less so, in a triple combination as in the present invention.

135. The data presented by Preez in the MSc thesis demonstrates that the lowest yield was obtained from the unsprayed control (92% disease; 16.60q.ha-l) which was not significantly different from mancozeb (79% disease; 18.11q.ha-l) tridemorph (73% disease; 19.47q.ha-l) and chlorothalonil (72% disease; 18.90q.ha-l) treatments. Thus, this study by Preez actually equates the effect of mancozeb usage with the untreated control, meaning thereby that mancozeb was not found to be useful for the control of soybean rust when used alone. The conclusions of this study would have actually dissuaded a person skilled in the art from combining mancozeb with a double combination of systemic fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides, and actually teaches away from the subject-matter of the present invention.
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136. Preez further discloses that research on the use of fungicides to control SBR dates back to the 1960s (Kitani *et al.*, 1960a, 1960b, 1960c; Chu and Chuang, 1961; Huang and Kiu, 1961; Wang, 1961) when protectant fungicides such as Bordeaux mixture, copper, dichlone, dinocap, limesulphur, mancozeb, maneb, wettable sulphur and zineb were tested. Applicant states that this teaching of Preez again favors the inventiveness positioning of the present inventors as despite being known since 1960s, no person skilled in the art contemplated using mancozeb, when used as a synergist, in combination with a double combination of systemic fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides.

137. Moreover, these studies cited by Preez are now 50-60 years old. It is an established principle of patent law that where a document may not be a realistic starting point for evaluating a later invention as prior art because it relates to outdated technology, and/or is associated with such well known disadvantages that the skilled person would not even consider trying to improve on it. Applicant states that these studies cited by Preez had been disregarded for more than 50 years by those skilled in the art, had never been used during that period as a basis for further development, was moreover completely silent about the extent of the indicated activity and, finally, did not even mention, let alone discuss, the relevant state of the art, did not represent the closest state of the art. Applicant states that it



would have been unrealistic to rely on these studies cited by Preez, without hindsight of the present invention, to try to combine mancozeb with other systemic fungicides of fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides to improve their properties. The technique of using mancozeb, along with other systemic fungicides selected from demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitor fungicides had not received any attention in the past 60 years, and it is not obvious as to why a person skilled in the art would consider such a strategy to improve the efficacy of known fungicides by using mancozeb as a synergist, after decades of this knowledge lying dormant.

138. Despite the aforesaid, even if Preez and the studies cited therein are considered as relevant prior starting points, applicant states that these studies are completely silent regarding the use of mancozeb in these combinations, or the quantity of mancozeb to be used which leads to a synergistic effect, or the precise triple combinations including mancozeb which are now being claimed.

139. Paper 6 was published in August 2007. This paper illustrates the use of the fungicide azoxistrobin + ciproconazole (60+24 g i.a./ha) + mineral oil (0.5%). There is no mention at all regarding the use of a multisite contact fungicide at all, much less mancozeb, in these rust control strategies, i.e.



before the publication of the present invention, especially for the control of soybean rust.

140. Paper 7 was published in October 2007, and is a review of the chemical control of soybean rust using the knowledge available in the state of the art till 2007. The objective of this paper was to test two active fungicides and five application times for the control of soybean rust. The experiments were carried out in Palmeira-PR, from November 2003 to May 2004, on cultivars CD 205 and BRS 133. The active ingredients tested were tetraconazole (50 g ai ha⁻¹) and pyraclostrobin (66.5 g ai ha⁻¹) + epoxiconazole (25g ia ha⁻¹).

141. This paper 7 concluded that treatments containing only triazole or a combination of strobilurin + triazole demonstrated a protective and curative effect with similar results in disease control. The unique application of the tetraconazole and pyraclostrobin + epoxiconazole fungicides showed higher efficiency when applied with low fungal severity.

142. Still, as of October 2007, there was no mention at all regarding the use of a multisite contact fungicide at all, much less mancozeb, in these rust control strategies, i.e. before the publication of the present invention, especially for the control of soybean rust.

143. Paper 8 was published in October 2008. This paper experimented with a different approach of treating seeds followed by chemical treatment for



optimum control of soybean rust. The seed seed was treated with carbendazim + thiram (30 + 70 g ai per 100 kg seed), or with fluquinconazol, at dose 50 g per 100 kg of seed. The seed treatment regimen was followed by following mixtures: pyraclostrobin + epoxiconazole and trifloxistrobin + tebuconazole.

144. This paper concludes that the treatment of the seed of soybean with fluquinconazol or with foliar fungicides of carbendazim + thiram did not delay the appearance of the first symptoms nor the evolution of the disease and did not presents a benefit in the management of rust. It was further concluded that the addition of a foliar fungicide treatment regimen did not have a favorable influence on the standard known treatment regime of strobilurin + conazole fungicides for the control of soybean rust.

145. The conclusion of this paper has two important ramifications for the subject-matter of the present invention: (i) It would have dissuaded any person skilled in the art from adding a multisite contact fungicide, much less mancozeb, in the known standard treatment regime of strobilurin + conazole fungicides for the control of soybean rust; and (ii) Secondly, as of October 2008, the general knowledge in the state of the art did not still consider the use of a multisite contact fungicide, much less mancozeb, in these rust control strategies, i.e. before the publication of the present



invention, especially for the control of soybean rust. D8 actually teaches away from the subject-matter of the present invention.

146. Paper 8_2 was published in October 2008 was presented similar conclusions as Paper 8 discussed above.

147. Paper 9 was published in February 2009. This paper teaches a premix of 60 g azoxystrobin ha-1 + 24 g cyproconazole ha-1 applied to different growth stages to control soybean rust. This paper again establishes that as of February 2009, the general knowledge in the state of the art did not still consider the use of a multisite contact fungicide, much less mancozeb, in these rust control strategies, i.e. before the publication of the present invention, especially for the control of soybean rust.

148. Paper 10, Scherm, H & Christiano, Rock & Esker, Paul & Del Ponte, Emerson & Godoy, Claudia. (2009). Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil. Crop Protection. 28. 774-782. 10.1016/j.cropro.2009.05.006., was published in September 2009.

149. This paper presented a meta-analytical synthesis of the results of 71 uniform fungicide trials containing 930 entries (specific fungicidal treatments) conducted in Brazil from 2003/2004 to 2006/2007. These experiments were conducted to: (1) quantify the overall efficacy of fungicidal treatments in reducing disease and yield loss; (2) determine to what extent fungicide efficacy depends on overall disease pressure, the number of spray 

applications, and the amount of disease present at the time of the first application; and (3) test for differences in efficacy among fungicide classes and specific active ingredients.

150. This paper concluded that triazole fungicides applied alone performed better than strobilurins alone, but there was a wide range in efficacy among individual triazoles, with prothioconazole and tebuconazole performing best and fluquinconazole and difenoconazole being least effective. Combinations of strobilurins with triazoles (especially those containing cyproconazole) improved disease and yield loss control compared with either class alone.

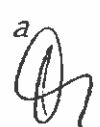
151. Therefore, Paper 10, tested 930 fungicide treatments for the control of soybean rust, and yet concluded that combinations of of strobilurins with triazoles was the best treatment available in the state of the art till September 2009. Later evidence further showed that even the efficacy of this best possible treatment by combinations of of strobilurins with triazoles was seen to be decaying with gradual decay over the years. Still, despite the 930 fungicides tested till September 2009, the general knowledge in the state of the art did not still consider the use of a multisite contact fungicide, much less mancozeb, in these rust control strategies, i.e. before the publication of the present invention, especially for the control of soybean rust.



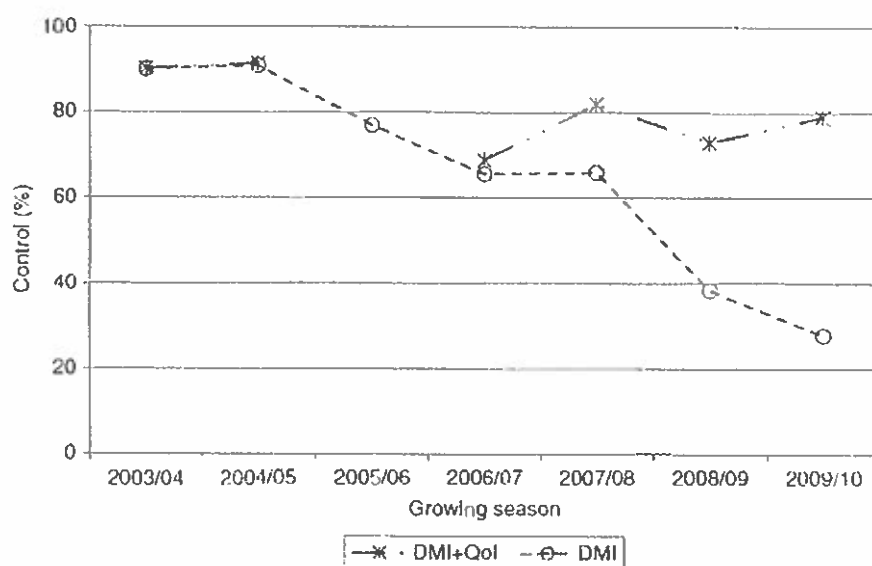
152. Paper 11 was published in December 2011. This paper is based on the premise that - *Fungicide trials in India (Patil and Anahosur, 1998) identified several triazole compounds, hexaconazole, triadimefon, propiconazole and difenoconazole, which performed better than mancozeb, tridemorph and chlorothalonil.* This discussion clearly discourages a person skilled in the art from considering the use of contact fungicides such as mancozeb in soybean rust control programs.

153. This paper further concludes that - *The mixtures of triazoles and strobilurin fungicides tend to be most consistent, providing less severe ASR and higher yields (Miles et al., 2003, 2007; Scherm et al., 2009), and that - The number of labelled fungicides to control ASR increased from 5, in 2002, to approximately 70, in 2010. Most of them belong to the DMI group, with several generic compounds. Qols were recommended alone in the first growing seasons only and, due to lower efficiency compared to triazoles alone (Scherm et al., 2009), they have been recommended only in mixtures with triazoles.*

154. This paper also, for the first time, quantitatively established a gradual decay in the efficacy of strobilurins, or conazoles or their mixtures towards the control of soybean rust with their use gradually over the years, by stating that - *Combinations of strobilurins with triazoles improved disease and yield loss control compared with either class alone. Even without a*



clear answer as to the reasons for the reduced performance of triazoles observed 7 years after the introduction of *P. pachyrhizi* in Brazil, it reached a level at which rust control with straight azoles became unsatisfactory. Since 2007, the use of straight azoles has been decreasing and the use of triazole–strobilurin mixtures was intensified as the major strategy to reduce the risk of resistance. The general knowledge in the state of the art, as of December 2011, did not still consider the use of a multisite contact fungicide, much less mancozeb, in these rust control strategies, i.e. before the publication of the present invention, especially for the control of soybean rust.



155. This again establishes that about 2 years before the priority date of the present invention, the general consensus in the state of the art was to consider the mixture of triazoles and strobilurins as the best available

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treatment for soybean rust. The present invention takes the state of the art forward from exactly this point where the knowledge existing in the state of the art culminated as of December 2011.

156. Paper 12 was published on September 4, 2014 i.e. after the priority date of the present application but before its earliest publication in India. Therefore, the contribution of the present invention i.e. the use of a multisite fungicide in combination with mixture of triazoles and/or strobilurins and/or SDHIs was not available to the authors of this paper yet.

157. This paper evaluated fungicides belong to the following groups: a) demethylation inhibitors; b) quinone oxidase inhibitors (Qol) and c) succinate dehydrogenase inhibitors (SDHI). The specific fungicides tested were: Tebuconazole 100 g ia ha⁻¹ (T2); cyproconazole 30 g ai ha⁻¹ (T3); azoxystrobin 50 g ia ha⁻¹ (T4); azoxystrobin + cyproconazole 60 + 24 g ai ha⁻¹ (T5); pyraclostrobin + epoxiconazole 66.5 + 25 g ai ha⁻¹ (T6); picoxystrobin + cyproconazole 60 + 24 g ai ha⁻¹ (T7); trifloxystrobin + prothioconazole 60 + 70 g ai ha⁻¹ (T8); picoxystrobin + tebuconazole 60 + 100 g ai ha⁻¹ (T9); azoxystrobin + flutriafol 62.5 + 62.5 g ai ha⁻¹ (T10); pyraclostrobin + metconazole 65 + 40 g ai ha⁻¹ (T11); azoxystrobin + tetraconazole 50 + 40 g ia ha⁻¹ (T12); pyraclostrobin + fluxapiroxade 99.9 + 50.1 g ia ha⁻¹ (T13); azoxystrobin + tebuconazole 72 + 96 g ai ha⁻¹ (T14).

-1 (T14); azoxystrobin + tebuconazole 62.5 + 120 g ai ha⁻¹ (T15); azoxystrobin + flutriafol 62.5 + 62.5 g ai ha⁻¹ (T16); metominostrobin + tebuconazole 63.2 + 94.8 g ai ha⁻¹ (T17); pyraclostrobin + epoxiconazole + fluxapiroxade 64.8 + 40 + 40 g ia ha⁻¹ (T18); bixafen + prothioconazole + trifloxystrobin 62.5 + 87.5 + 75 g ai ha⁻¹ (T19); azoxystrobin + benzovindiflupyr 60 + 30 g ai ha⁻¹ (T20).

158. This paper again does not envisage the use of a multisite fungicide, particularly mancozeb, additionally with the conventionally known mixtures for the control of soybean rust. This again establishes that even before the publication of the present application, the general consensus in the state of the art was to consider the mixture of triazoles and strobilurins as the best available treatment for soybean rust. In fact, there was scientific prejudice against using a multisite fungicide, particularly mancozeb, for the control of soybean rust. The present inventors have ventured against this scientific prejudice and achieved success.

159. Paper 13 was published in June 2015 i.e. barely 4 months prior to the publication of the present application in India.

160. Page 114, left hand column, second paragraph of this paper notes that
- the fungus started to show a decrease in sensitivity to Demethylation Inhibitors (DMIs) fungicides in Brazil from 2007 (Godoy, 2011). Since then,

only premixes of DMIs and Quinone outside Inhibitors (Qols) fungicides have been recommended to control the disease. Although Qols fungicides have never been recommended alone, due to low efficacy against soybean rust, in 2013, a decrease in efficacy was also observed in this group of fungicides, compromising the control of the premixes at different levels. Products consisting of new broad-spectrum Succinate Dehydrogenase Inhibitors (SDHIs) fungicides mixed with Qols were launched onto the market in 2013.

161. This paper again does not envisage the use of a multisite fungicide, particularly mancozeb, additionally with the conventionally known mixtures for the control of soybean rust. This again establishes that even before the publication of the present application, the general consensus in the state of the art was to consider the mixture of triazoles and strobilurins as the best available treatment for soybean rust. In fact, there was scientific prejudice against using a multisite fungicide, particularly mancozeb, for the control of soybean rust. The present inventors have ventured against this scientific prejudice and achieved success.

162. Paper 14 was published in July 2015, i.e. 4 months before the earliest publication of the present application in India. This paper tested the following mixtures: azoxystrobin, bixafen + prothioconazole + trifloxystrobin and trifloxystrobin + prothioconazole, tebuconazole, azoxystrobin +

benzovindiflupir, bixafen + prothioconazole + trifloxystrobin trifloxystrobin + prothioconazole and pyraclostrobin + epoxiconazole + fluxapiroxad. This paper again does not envisage the use of a multisite fungicide, particularly mancozeb, additionally with the conventionally known mixtures for the control of soybean rust. Paper 15 also draws similar conclusions from similar experiments and was published in the same month.

163. Paper 15 was published in May 2016. It was the first paper published after the earliest publication of the present application in India. This paper again discusses the fungicidal double and triple mixtures of demethylation inhibitors, quinone inhibitors and succinate dehydrogenase inhibitors for the control of soybean rust as the most current and effective strategy. In this regard, the disclosure of this paper is similar to the disclosure of papers 14 and 15 above.

164. Page 415, first paragraph of this paper, acknowledges the inventive contribution of the present invention for the first time by stating that -
Since effective fungicides with a new mode of action are not readily available, old multi-site fungicides, e.g. mancozeb, copper sulfate, chlorothalonil, which have low resistance risk, have been recently re-tested for P. pachyrhizi control in mixtures or tank mixtures to increase control efficiency and reduce the risk of resistance (Godoy et al., 2015b).



165. This paper still does not envisage the use of a multisite fungicide, particularly mancozeb, in mixture with the conventionally known mixtures for the control of soybean rust.

166. Paper 16 was published August 2016. It discusses results from trials done in 2015/16 crop i.e. the first trial which could be conducted full scale after the publication of the present application in India and in the PCT procedure. This paper discusses the role of a multisite fungicide in the soybean control program for the first time. Table 3 of this paper discloses for the first time, after the publication of the present application, regarding the role of copper oxychloride, chlorothalonil, mancozeb, copper sulfate, propineb, and fluazinam in addition to the long known mixture of triazoles and/or strobilurins and/or SDHIs for achieving a superior control of Asian soybean rust.



Tabela 3. Ingredientes ativos (i.a.), produto comercial (p.c.) e dose dos fungicidas avaliados em tratamentos associados para o controle de doenças na cultura da soja, safra 2015/16.

Ingrediente ativo (i.a.)	Dose g l.a. ha ⁻¹	Produto comercial (p.c.)	Dose kg p.c. ha ⁻¹
1 testemunha	-	Testemunha	-
2 picoxistrobina + ciproconazol ¹	60+24	Aproach [®] Prima	0,3
3 picoxistrobina + ciproconazol ¹ e oxicloreto de cobre	60+24 e 294	Aproach [®] Prima e Difere [®]	0,3 e 0,5
4 picoxistrobina + ciproconazol ¹ e clorotalonil	60+24 e 1080	Aproach [®] Prima e Previnil [®]	0,3 e 1,5
5 picoxistrobina + ciproconazol ¹ e oxicloreto de cobre	60+24 e 280	Aproach [®] Prima e PNR ²	0,3 e 0,4
6 picoxistrobina + ciproconazol ¹ e mancozebe	60+24 e 1500	Aproach [®] Prima e Unizeb Gold [®]	0,3 e 2,0
7 picoxistrobina + ciproconazol ¹ e sulfato de cobre	60+24 e 113,85	Aproach [®] Prima e PNR ²	0,3 e 0,75
8 picoxistrobina + ciproconazol ¹ e propinebe	60+24 e 1400	Aproach [®] Prima e PNR ²	0,3 e 2,0
9 picoxistrobina + ciproconazol ¹ e fluazinam	60+24 e 500	Aproach [®] Prima e Frownicide [®]	0,3 e 1,0
10 picoxistrobina + ciproconazol ¹ e clorotalonil	60+24 e 1000	Aproach [®] Prima e Bravonil 600	0,3 e 2,0
11 picoxistrobina + ciproconazol ¹ e clorotalonil	60+24 e 1000	Aproach [®] Prima e PNR ²	0,3 e 2,0
12 picoxistrobina + ciproconazol ¹ e mancozebe	60+24 e 2000	Aproach [®] Prima e PNR ²	0,3 e 2,5
13 azoxistrobina + benzovindiflupir ¹	60+30	Elatus [®]	0,2
14 azoxistrobina + benzovindiflupir ¹ e oxicloreto de cobre	60+30 e 294	Elatus [®] e Difere [®]	0,2 e 0,5
15 azoxistrobina + benzovindiflupir ¹ e clorotalonil	60+30 e 1080	Elatus [®] e Previnil [®]	0,2 e 1,5
16 azoxistrobina + benzovindiflupir ¹ e oxicloreto de cobre	60+30 e 280	Elatus [®] e PNR ²	0,2 e 0,4
17 azoxistrobina + benzovindiflupir ¹ e mancozebe	60+30 e 1500	Elatus [®] e Unizeb Gold [®]	0,2 e 2,0
18 azoxistrobina + benzovindiflupir ¹ e sulfato de cobre	60+30 e 113,85	Elatus [®] e PNR ²	0,2 e 0,75
19 azoxistrobina + benzovindiflupir ¹ e propinebe	60+30 e 1400	Elatus [®] e PNR ²	0,2 e 2,0
20 azoxistrobina + benzovindiflupir ¹ e fluazinam	60+30 e 500	Elatus [®] e Frownicide	0,2 e 1,0
21 azoxistrobina + benzovindiflupir ¹ e clorotalonil	60+30 e 1000	Elatus [®] e Bravonil 600	0,2 e 2,0
22 azoxistrobina + benzovindiflupir ¹ e clorotalonil	60+30 e 1000	Elatus [®] e PNR ²	0,2 e 2,0
23 azoxistrobina + benzovindiflupir ¹ e mancozebe	60+30 e 2000	Elatus [®] e PNR ²	0,2 e 2,5

¹ Adicionado Nabisus 0,6 L ha⁻¹; ² PNR = produto não registrado para a cultura da soja, RET III

167. This paper notes a marked reduction in severity of soybean rust and yield of soybean in treatments which were inclusive of a multisite contact fungicide in addition to the mixture of triazoles and/or strobilurins and/or SDHIs.

Tabela 5. Severidade da ferrugem, porcentagem de controle (C) em relação à testemunha sem fungicida, produtividade e porcentagem de redução da produtividade (RP) em relação ao tratamento com a maior produtividade, para os diferentes tratamentos. Média de 20 ensaios para severidade e 21 para produtividade. Safra 2015/16.

Tratamento Ingrediente ativo (i.a.)	Dose g L ⁻¹ ha ⁻¹	Severidade (%)	C (%)	Produtividade kg ha ⁻¹	RP (%)
1 testemunha	-	78,5 A	0	2175 I	36
2 picoxistrobina + ciproconazol ¹	60+24	43,8 B	44	2654 H	21
3 picoxistrobina + ciproconazol ¹ e oxícloreto de cobre	60+24 e 294	32,9 DE	58	2823 DEF	16
4 picoxistrobina + ciproconazol ¹ e clorotalonil	60+24 e 1080	31,6 E	60	2876 DE	15
5 picoxistrobina + ciproconazol ¹ e oxícloreto de cobre ²	60+24 e 280	32,9 DE	58	2780 EFG	18
6 picoxistrobina + ciproconazol ¹ e mancozebe	60+24 e 1500	31,6 E	60	2876 DE	15
7 picoxistrobina + ciproconazol ¹ e sulfato de cobre ²	60+24 e 113,85	37,0 C	53	2737 FGH	19
8 picoxistrobina + ciproconazol ¹ e propinebe ²	60+24 e 1400	36,1 C	54	2750 FGH	19
9 picoxistrobina + ciproconazol ¹ e fluazinam	60+24 e 500	34,1 D	57	2711 GH	20
10 picoxistrobina + ciproconazol ¹ e clorotalonil	60+24 e 1000	33,8 D	57	2793 EFG	17
11 picoxistrobina + ciproconazol ¹ e clorotalonil ²	60+24 e 1000	34,0 D	57	2841 DEF	16
12 picoxistrobina + ciproconazol ¹ e mancozebe ²	60+24 e 2000	29,2 F	63	2932 D	13
13 azoxistrobina + benzovindiflupir ¹	60+30	18,3 G	77	3186 BC	6
14 azoxistrobina + benzovindiflupir ¹ e oxícloreto de cobre	60+30 e 294	15,2 JK	81	3195 BC	5
15 azoxistrobina + benzovindiflupir ¹ e clorotalonil	60+30 e 1080	15,6 IJK	80	3238 BC	4
16 azoxistrobina + benzovindiflupir ¹ e oxícloreto de cobre ²	60+30 e 280	16,4 IJK	79	3147 C	7
17 azoxistrobina + benzovindiflupir ¹ e mancozebe	60+30 e 1500	15,2 JK	81	3377 A	0
18 azoxistrobina + benzovindiflupir ¹ e sulfato de cobre ²	60+30 e 113,85	16,8 GHJ	79	3176 BC	6
19 azoxistrobina + benzovindiflupir ¹ e propinebe ²	60+30 e 1400	17,5 GH	78	3192 BC	5
20 azoxistrobina + benzovindiflupir ¹ e fluazinam	60+30 e 500	17,5 GH	78	3205 BC	5
21 azoxistrobina + benzovindiflupir ¹ e clorotalonil	60+30 e 1000	16,8 GHJ	79	3219 BC	5
22 azoxistrobina + benzovindiflupir ¹ e clorotalonil ²	60+30 e 1000	17,0 GHI	78	3224 BC	5
23 azoxistrobina + benzovindiflupir ¹ e mancozebe ²	60+30 e 2000	15,0 K	81	3270 AB	3
C.V. %		10,5		6,6	

Médias seguidas de mesma letra, na coluna, não diferem entre si pelo teste de Tukey ($p=0,05$). ¹Adicionado Nitrôgeno 0,5 L ha⁻¹; ²PNR – produto não registrado para a cultura da soja, RET III.

168. Thus, it is clear from the trials done in 2015/16 crop i.e. the first trial which could be conducted full scale after the publication of the present application in India and in the PCT procedure that the addition of a multisite fungicide to the known mixture of triazoles and/or strobilurins and/or SDHIs leads to surprising enhancement in efficacy of these combinations, particularly for the control of soybean rust.

169. It is thus established that prior to the publication of the present application, there had been a general scientific prejudice in the state of the art against the use of multisite fungicides in soybean rust control program. The publication of the present application decisively marked an

Or

end to this scientific prejudice, to the extent that the current thinking in the scientific community took a dramatic turn immediately after the publication of the present application. Applicant states that the overcoming of this long held scientific prejudice is the strongest possible evidence of the inventiveness of the subject matter of the present invention. Applicant states that in view of this evidence, no other testimony regarding the inventiveness of the present claims is required and the claims deserve to be proceeded to grant straightaway.

170. A prejudice in any particular field relates to an opinion or preconceived idea widely or universally held by experts in that field. The present invention successfully overcomes this long held technical prejudice, which is indicative of non-obviousness of the present invention.

171. Paper 17 published in August 2016 again describes similar experiments as paper 16. This paper again studies the role of addition of a multisite fungicide to mixture of triazoles and/or strobilurins and/or SDHIs and draws the same conclusions.

172. Paper 18 published in August 2016, again reports similar trials with similar conclusions and is offered herewith as post-published evidence.



173. Paper 19 was published in July 2017. This paper again discusses the role of copper oxychloride, chlorothalonil, mancozeb, copper sulfate, propineb, and fluazinam in addition to the long known mixture of triazoles and/or strobilurins and/or SDHs for achieving a superior control of Asian soybean rust.

174. Paper 20 was published in October 2017. This paper studies the gradual decay in the efficacy of the long known mixture of triazoles and/or strobilurins and/or SDHs in the absence of a multisite fungicide, particularly mancozeb. Page 35 of this paper represents the downwards sloping efficacy graphs for the long known mixture of triazoles and/or strobilurins in the absence of a multisite fungicide. However, in the presence of a multisite fungicide such as mancozeb, the downward slope in the efficacy of the long known mixture of triazoles and/or strobilurins curve straightens. This paper is yet again an indication of the non-obviousness of the subject matter of the present invention.

175. Paper 21 was published in July 2017. This paper again discusses the role of a multisite fungicide in synergizing the efficacy of the mixture of triazoles and/or strobilurins and/or SDHs in control of soybean rust.



176. Tables 3 and 5 of this paper discloses the role of copper oxychloride, chlorothalonil, mancozeb, copper sulfate, propineb, and fluazinam in addition to the long known mixture of triazoles and/or strobilurins and/or SDHIs for achieving a superior control of Asian soybean rust.

Tabela 3. Ingredientes ativos (i.a.), produtos comerciais (p.c.) e doses dos fungicidas avaliados em tratamentos associados para o controle de doenças na cultura da soja, safra 2016/17.

1	testemunha			
2	trifloxistrobina + proclorazoxolol ¹	60 + 30	Fox ¹	0,4
3	trifloxistrobina + proclorazoxolol ¹ e clorotalonil ²	60 + 30 e 1080	Fox ¹ e PNA ³	0,4 e 1,5
4	trifloxistrobina + proclorazoxolol ¹ e oxiclóreto de cobre ²	60 + 30 e 560	Fox ¹ e PNR ³	0,4 e 0,8
5	trifloxistrobina + proclorazoxolol ¹ e mancozebe ²	60 + 30 e 2250	Fox ¹ e Unizub Gold ⁴	0,4 e 3,0
6	trifloxistrobina + proclorazoxolol ¹ e mancozebe ²	60 + 30 e 1500	Fox ¹ e PNR ³	0,4 e 2,0
7	trifloxistrobina + proclorazoxolol ¹ e mancozebe ²	60 + 30 e 2240	Fox ¹ e PNA ³	0,4 e 2,8
8	trifloxistrobina + proclorazoxolol ¹ e óxido cuproso ²	60 + 30 e 375	Fox ¹ e PNA ³	0,4 e 0,5
9	trifloxistrobina + proclorazoxolol ¹ e <i>Bacillus amyloliquefaciens</i> ²	60 + 30 e 3x10 ⁶ ufc mL ⁻¹	Fox ¹ e PNR ³	0,4 e 1,0
10	trifloxistrobina + proclorazoxolol ¹ e fluazinam ²	60 + 30 e 500	Fox ¹ e PNA ³	0,4 e 1,0
11	trifloxistrobina + proclorazoxolol ¹ e clorotalonil ²	60 + 30 e 1250	Fox ¹ e PNR ³	0,4 e 2,5
12	trifloxistrobina + proclorazoxolol ¹ e mancozebe ²	60 + 30 e 2240	Fox ¹ e PNA ³	0,4 e 2,8
13	trifloxistrobina + proclorazoxolol ¹ e hidróxido de cobre ²	60 + 30 e 807	Fox ¹ e PNR ³	0,4 e 1,5
14	trifloxistrobina + proclorazoxolol ¹ e oxiclóreto de cobre ²	60 + 30 e 294	Fox ¹ e PNA ³	0,4 e 0,5
15	trifloxistrobina + proclorazoxolol ¹ e oxiclóreto de cobre + mancozebe ²	60 + 30 e 201,6 + 300	Fox ¹ e PNR ³	0,4 e 1,0
16	azoxistrobina + benconvinclifluor ²	60 + 30	Elatus ¹	0,2

¹Adicionado Aureo 0,25% v/v. ²Adicionado Nimbus 0,5 L ha⁻¹. ³PNR: produto não registrado na cultura da soja. ⁴PNA: produto não registrado para o alvo *Phakopsora pachyrhizi*.

Tabela 6. Severidade da ferrugem, porcentagem de controle (C) em relação à testemunha sem fungicida, produtividade e porcentagem de redução de produtividade (RPI) em relação ao tratamento com a maior produtividade, para os diferentes tratamentos. Média de 28 ensaios. Safra 2016/17.

1	testemunha		72,7	A	0	2262	D	31
2	trifloxistrobina + proclorazoxol ¹	60 + 30	24,4	B	68	3087	BC	5
3	trifloxistrobina + proclorazoxol ¹ e clorotolona ²	60 + 30 + 1080	18,3	EF	75	3206	AB	2
4	trifloxistrobina + proclorazoxol ¹ e óxido de cobre ³	60 + 30 + 560	18,0	F	75	3196	AB	2
5	trifloxistrobina + proclorazoxol ¹ e mancozebe ⁴	60 + 30 + 2250	18,9	DEF	74	2233	A	1
6	trifloxistrobina + proclorazoxol ¹ e mancozebe ²	60 + 30 + 1500	21,2	BCDE	71	3142	ABC	4
7	trifloxistrobina + proclorazoxol ¹ e mancozebe ²	60 + 30 + 2240	21,1	CDEF	71	3196	AB	2
8	trifloxistrobina + proclorazoxol ¹ e óxido cuproso ⁴	60 + 30 + 375	19,4	DE ⁵	73	3202	AB	2
9	trifloxistrobina + proclorazoxol ¹ e <i>Raciburs amyloquefaciens</i> ¹	60 + 30 + 3 x 10 ⁴ ufc mL ⁻¹	24,3	BC	67	3055	C	6
10	trifloxistrobina + proclorazoxol ¹ e fluazinam ⁴	60 + 30 + 500	19,3	DEF	73	3161	ABC	3
11	trifloxistrobina + proclorazoxol ¹ e clorotolona ²	60 + 30 + 1250	18,8	DEF	74	2177	ABC	3
12	trifloxistrobina + proclorazoxol ¹ e mancozebe ⁴	60 + 30 + 2240	20,1	DEF	72	3263	A	0
13	trifloxistrobina + proclorazoxol ¹ e hidróxido de cobre ³	60 + 30 + 807	20,4	DEF	72	3171	ABC	3
14	trifloxistrobina + proclorazoxol ¹ e óxido de cobre ⁴	60 + 30 + 794	19,2	DEF	74	3192	AB	2
15	trifloxistrobina + proclorazoxol ¹ e óxido de cobre + mancozebe ⁴	60 + 30 + 201,6 + 300	19,7	DEF	73	3243	A	1
16	trifloxistrobina + benzenavadinilfula ⁴	60 + 30	21,9	BCD	70	3209	AB	2

Médias seguidas de mesma letra, na coluna, não diferem entre si pelo teste de Tukey ($p = 0,05$). ¹Adicionado Aureo 0,25% v/v.

²Adicionado Nubius 0,6 L ha⁻¹. ³Produto não registrado na cultura da soja. ⁴Produto não registrado para o alvo *Phakopsora pachyrhizi*.

177. This paper notes a marked reduction in severity of soybean rust and yield of soybean in treatments which were inclusive of a multisite contact fungicide in addition to the mixture of triazoles and/or strobilurins and/or SDHIs.

178. Incidentally, and quite interestingly, her research publications can be divided into two parts i.e. pre-November 2015 and post-November 2015. In all the papers discussed above being prior to November 2015 being Papers 1 – 15, no mention could be found regarding the use of a multisite fungicide for controlling soybean rust. In Papers 16 – 21 i.e. post publication of the present application, Dr. Godoy has independently confirmed what the present inventors found three years earlier i.e. in 2013 that the inclusion of a

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multisite contact fungicide in addition to the mixture of triazoles and/or strobilurins and/or SDHIs leads to surprisingly beneficial effect. Dr. Godoy is an independent researcher. Yet she has reconfirmed the findings of the present inventors by her own independent research. The post-published papers 16 – 21 establish that the addition of a multisite fungicide to the known mixture of triazoles and/or strobilurins and/or SDHIs leads to surprising enhancement in efficacy of these combinations, particularly for the control of soybean rust.

179. This clear demarcation between the papers 1 – 15 and papers 16 – 21 establish that prior to the publication of the present application, there had been a general scientific prejudice in the state of the art against the use of multisite fungicides in soybean rust control program. The publication of the present application decisively marked an end to this scientific prejudice, to the extent that the current thinking in the scientific community took a dramatic turn immediately after the publication of the present application, such that what was earlier considered as not being useful, is now being seen as the solution to all the problems of soybean crop. Applicant states that the overcoming of this long held scientific prejudice is the strongest possible evidence of the inventiveness of the subject matter of the present invention. Applicant states that in view of this evidence, no other testimony



regarding the inventiveness of the present claims is required and the claims deserve to be proceeded to grant straightaway.

180. A prejudice in any particular field relates to an opinion or preconceived idea widely or universally held by experts in that field. The present invention successfully overcomes this long held technical prejudice, which is indicative of non-obviousness of the present invention.

Post-published evidence

181. It is also evident from a review of the papers discussed above that after the first publication of the present invention, several independent research papers reconfirmed the findings of the present invention i.e. the addition of a multisite contact fungicide to the combination of systemic fungicides leads to a surprising enhancement of the efficacy of the resulting triple combinations.

Source : https://www.researchgate.net/publication/308451298 (Paper 16)				
S No.	Treatment	Percentage control	Difference in control due to introducing a	Percentage reduction in productivity

47

			multisite fungicide	
1	Untreated	0	-	36
2	Picoxystrobin + Cyproconazole	44	-	21
3	Picoxystrobin + Cyproconazole + Copper oxychloride	58	+14.0	16
4	Picoxystrobin + Cyproconazole + Chlorothalonil	60	+ 16.0	15
5	Picoxystrobin + Cyproconazole + Mancozeb	60	+16.0	15
6	Picoxystrobin + Cyproconazole + Copper sulfate	53	+9.0	19

7	Picoxystrobin	+	54	+10.0	19
	Cyproconazole	+			
	Propineb				
8	Picoxystrobin	+	57	+ 13.0	20
	Cyproconazole	+			
	Fluazinam				
9	Picoxystrobin	+	63	+ 19.0	13
	Cyproconazole	+			
	Mancozeb				
10	Azoxystrobin	+	77	-	6
	Benzovindiflupyr				
11	Azoxystrobin	+	81	+4.0	5
	Benzovindiflupyr	+			
	Copper oxychloride				
12	Azoxystrobin	+	80	+ 3.0	4
	Benzovindiflupyr	+			
	Chlorothalonil				

13	Azoxystrobin	+	81	+4.0	0
	Benzovindiflupyr	+			
	Mancozeb				
14	Azoxystrobin	+	79	+2.0	6
	Benzovindiflupyr	+			
	Copper sulfate				
15	Azoxystrobin	+	78	+1.0	5
	Benzovindiflupyr	+			
	Propineb				
16	Azoxystrobin	+	79	+2.0	5
	Benzovindiflupyr	+			
	Fluazinam				
Source : (Paper 17): https://www.researchgate.net/publication/306159053					
17	Picoxystrobin	+	56		15
	Tebuconazole				
18	Picoxystrobin	+	64	+6.0	10
	Tebuconazole	+			
	Mancozeb				
Source: Paper 19 - https://www.researchgate.net/publication/320702727					

19	Picoxystrobin + Tebuconazole	59	-	17
20	Picoxystrobin + Tebuconazole + Mancozeb	63	+4.0	12
Source - Paper 21 : https://www.researchgate.net/publication/320702665				
21	Untreated	0	-	31
22	Trifloxystrobin + Prothioconazole	66	-	5
23	Trifloxystrobin + Prothioconazole + Chlorothalonil	75	+9.0	2
24	Trifloxystrobin + Prothioconazole + Copper oxychloride	75	+9.0	2
25	Trifloxystrobin + Prothioconazole + Mancozeb	74	+8.0	1

26	Trifloxystrobin	+	73	+7.0	2
	Prothioconazole	+			
	Copper sulfate				
27	Trifloxystrobin	+	73	+7.0	3
	Prothioconazole	+			
	Fluazinam				
28	Trifloxystrobin	+	74	+8.0	3
	Prothioconazole	+			
	Chlorothalonil				
29	Trifloxystrobin	+	72	+6.0	3
	Prothioconazole	+			
	Copper hydroxide				
30	Trifloxystrobin	+	74	+ 8.0	2
	Prothioconazole	+			
	Copper oxychloride				
31	Trifloxystrobin	+	73	+7.0	2
	Prothioconazole	+			
	[Copper oxychloride +	+			
	Mancozeb]				

182. Thus, it has been surprisingly found that multisite contact fungicide, particularly mancozeb, surprisingly enhance the efficacy in a triple combination (present invention) more than it does in a double combination (prior art). In the double combinations (prior art), mancozeb was found to mostly antagonize the efficacy of either a strobilurin or a conazole fungicides but its efficacy enhancing role was consistently greater than 20% or 30%, and being near about 50% - 60% when present in the triple combinations of the present invention. Thus, the contribution of mancozeb in the presently claimed triple combinations was much greater than its contribution in the dual combinations of the prior art.

183. The photographic evidence attached herewith also convincingly prove the advantage of the present invention.

184. The class of multisite contact fungicides appears to work in a similar manner. They all provide a protective coating on the leaf. Therefore, it is believed that other multisite contact fungicides would work if mancozeb is found to work in a given environment.

185. Additionally, the use of a multisite contact fungicide for treating soybean rust is a very unconventional idea, one that would not occur to a skilled agronomist without the benefit of hindsight. These multisite fungicides were being used historically for the control of soybean rust, but with the advent of strobilurin, conazole and SDHI class of fungicides, the multisite contact



fungicides were superseded by the later discovered systemic fungicides. Therefore, reconsidering the use of multisite fungicides for soybean rust control would have been akin to moving backwards in technology, and thus unlikely to have been considered seriously by a person skilled in the art. The present inventors have ventured against this conventional thinking.

186. The pathogen for rust penetrates and colonizes the leaf tissue, forming necrotic lesions. Due to its penetrative nature, it has always been treated with systemic fungicides i.e. fungicides absorbed within the plant tissues. Multisite contact fungicides have not been recommended, or have been discouraged, from being used in rust control.

187. The Preez PhD thesis clearly discourages the use of contact fungicides in rust control. Paper 8 also concludes that the addition of a foliar fungicide treatment regimen did not have a favorable influence on the standard known treatment regime of strobilurin + conazole fungicides for the control of soybean rust. Paper 11 concludes that - *Fungicide trials in India (Patil and Anahosur, 1998) identified several triazole compounds, hexaconazole, triadimefon, propiconazole and difenoconazole, which performed better than mancozeb, tridemorph and chlorothalonil.* This discussion clearly discourages a person skilled in the art from considering the use of contact fungicides in soybean rust control programs.



188. As is evident from Dr. Claudia Godoy's publications on rust control before and after the publication of the present application, I say that the publication of this application did precipitate a turnaround in the collective thinking of the state of the art towards the role to be played by a multisite contact fungicide in the control of rust.

189. There is clear evidence that such use would be against the conventional thinking, and it did cause a turnaround in the collective thinking in the state of the art.

190. Interestingly, on delving deeper into Dr. Godoy's research, it was found that the content in her research papers could be clearly demarcated into two portions, the one before the publication of the present application and the other post- publication. In the pre-publication papers, she talks about soybean control by Qols or DMIs + SDHIs, without any reference to contact fungicides, particularly mancozeb. In her post-publication papers, she has dwelt only upon the effect of contact fungicides on these combination mixtures for the control of soybean rust. The dividing line between the change in her approach was exactly the publication date of the present invention.

191. It is appropriate to say that before the present invention was published, there was a technical prejudice against the use of contact fungicides as no scientist even mentioned it in any of her research, and suddenly after

the present application was published, the recommended fungicidal control regimen took a dramatic turn to include contact fungicides in the soybean rust control programs.

192. The statements made above are true to my knowledge and belief and that I have not suppressed any material fact.

Dated this 7th day of June 2019

Gilson Aparecido Hermenegildo de Oliveira
CPF 056147508-35

(Deponent)



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Gilson Hermenegildo de Oliveira

Estrada Ageo Mariano de Lima Km 4
 12935-000 – Vargem – SP – Brazil
 +55 11 9906-6519 mob. +55 11 4598-0392 home
gilson.holiveira@icloud.com

Brazilian
 01/22/1963

INNOVATION / BUSINESS DEVELOPMENT / MARKETS / PRODUCTS

PROFESSIONAL PROFILE

Executive with 33-year experience in Innovation, sales, product development, markets and agribusiness strategy sectors. Agronomist engineer observing the customer needs, always developing solutions in a "win/win" basis. High mobility and knowledge of the main regions throughout Brazil, intensively working with soy, corn, sugar cane, cotton, wheat, coffee, citrus, beans, and vegetables.

PERSONAL FEATURES

- Clearness and objectivity (organizing and structuring) focused on action and results;
- Expertise to lead teams composed by different people from different organizational levels;
- Excellent interpersonal relationship and ability to work with all departments;
- Flexibility and experience in driving groups in conflicting or changing situations;
- Strategic view in circumstances that require complex solution;
- Motivator, showing alternatives with knowledge, evidences and risk evaluation;
- Someone who looks for change, assuming discussed and calculated risks in an environment.

MAIN PROFESSIONAL ACHIEVEMENTS

- **Development of the platform "SPX" (Sucking Pests)**
 As the MMX again opened a new positioning in the market such that the UPL can lead the thread. So expert (acephato) was renovated and in just 2 years repositioning is the market leader with a potential sales of USD 150 Mio-in addition to an increase in profitability in 30%. New product launches to suckers on the same platform LCM.
- **Creation of protective fungicides segment**
 This segment opens a market in large crops in Brazil for a USD 1.5 Bio. Unizeb Gold is the main product of UPL with sales around USD 200 Mio.
- **Product positioning (Nativo – winter cereal application) – 6.5 point growth of market share, 33% increase in sales in 2007.** The "Nativo" product formula was improved and partnerships were established with official authorities in order to recommend adequate varieties for each kind of technology. Results were shown in field and internal teams were trained. *Bayer CropScience, 2006 to 2008.*
- **Fungicide segment focus changing to row crops (soy) – Sales passed from over US\$ 86.8 M in 2006 to US\$ 126.7 M in 2007, (46%, with market share changing from 15% to 21%).** Focus has been changed from curative to preventive applications. Internal and external teams were trained to work in the field and to create advertisements in the communication media. *Bayer CropScience, 2006 to 2008.*
- **Product revitalization (Baysiston / Creation of "Muito Mais..." Program – "Much More...") – Sales increased from US\$ 30 M to US\$ 54 M.** "Marketing Awards 2006" as Best Marketing global strategy (the first time that Brazil wins this award). Focus on the value of the product and not on the price. The dose of the product was changed in order to invigorate the plant instead of controlling pests. A new launch of product was performed with credible people, recycling sales team. *Bayer CropScience, 2005 to 2006.*
- **Portfolio reorganization - Creation of "PINBa" Program: Integrated Prevention Bayer – Contribution margin duplication from US\$ 1.8 M to US\$ 3.6 M, market share advance from 18% to 24%, establishing Bayer as market leader in Oomycetes (fungicide) market.** Portfolio rationalization excluding non-strategic products and including new products through known molecules, creation of PINBa (showing the benefits of treat crops preventively), patent defense through fungicide mixing, training all sales team to a new strategy. *Bayer CropScience, 2005 to 2006.*
- **Product (Acaricide) Group Strategy Realignment (Acaricides) / Envidor Launch – Sales increasing from US\$ 7 M to US\$ 24 M in the third year of Sales (343% growth), contribution margin increased from 55% to 78%.** Brazil strategy to Acaricides was recognized by the head office as the best and was completely introduced in other countries. Once in 5th place in market, it became leader in two years. Creation of a "Premium Price"; twice the competitor price, emphasizing quality on mites handling. It was created an internal sales campaign, field demonstration and controlled applications, to avoid any kind of strategy damage or misunderstanding. *Bayer CropScience, 2003 to 2004.*
- **Insecticide portfolio restructuring / New product building, Molecules LCM – With a contribution margin growth from 51% to 60%, CropStar reaches US\$ 40 M in sales by the second year, Connect**

exceeds US\$ 20 M and Imidacloprid exceeds US\$ 100 M. "Marketing Awards 2007" to the project. Portfolio rationalization, determination of target products with low margins, creation of "anti-genérico" (anti-generic) strategy to the main molecule (Imidacloprid), expansion of sale conception (selling "benefits" instead of product). **Bayer CropScience, 2003 to 2005.**

- **Sales Regional restructuring / Campinas** - From US\$ -2,7 M to US\$ +3,9 M in EBIT and Sales growth from US\$12 M to US\$17 M in the second year. 25% internal cost decreasing, saving US\$ 1 million already in first year. Results reached with local market potential deep analyses, resizing the sales potential area for each seller, with feasible targets, sales team training, changes focused on motivation and managing supply monthly at the dealer. **Bayer Plant Protection, 1999 to 2002.**
- **Product Launch / Brand Positioning in market** - Sales of US\$40 M in four years. The Challenge was to launch Imidacloprid, a product Five times more expensive than its competitors. A strategy was developed with a clear molecule view (Life Cycle Management)/ a new mode was created in treatment of plants against viruses vector and suckers ("drench" system), a campaign was developed among other tobacco companies (extremely hard market), with agronomists trained to ground the ideas about using the new product. **Bayer Plant Protection, 1994 to 1998.**
- **Strategic planning / Insecticide Segmentation Reorganization (Marketing)** - Increase of contribution margin from 45% to 65% in two years. Market deep analysis, products division by action /target group, definition of strategy to be followed in front of sales team internally motivated. **Bayer Plant Protection, 1992.**
- **Product Application Development / Product Launch (Marketing)** - Bulldock (the best product in its category until today), in the third sales year, was set as the market leader with sales of US\$4 M and a contribution margin of 85%. In a very competitive market and with low prices, researches were developed considering plant health and not just the effects in pests, what made the product a market leader until today. **Bayer Plant Protection, 1989.**
- **Researches for new product application development (Folicur)** - Sales increased from US\$ 18 M to US\$ 25 M in one year. Expansion of fungicide application to other cultures as vegetables and beans, used only in winter cereals up to that moment. **Bayer Plant Protection, 1988.**

PROFESSIONAL BACKGROUND

• **UPL**

Portfolio & Innovation Manager

Set the Apr 2015 2017

Responsible for Portfolio areas where expanded forms the platform of insecticides herbicides and seed treatments (SPX, WMX, STX). Consolidation of UPL as creator of protective fungicides in segment large crops. Introduction of the new concept of "suking pest" in the market of insecticides with new expert positioning. Inovations through Creation of marketing tools such as UPLider, CooperUP (promotion), UPLand (territorial control), UPField (DEMO control), UP2P (projects and products). Implementation of the strategies in the field through the "market development" (16 people). Specialization in building teams (internal & external)

• **UPL**

Fungicide Product Manager

Sep 2011 to Aug 2014

Responsible for the development of the strategy of Mancozeb and its mixtures. Creation of the MMX platforms based on "management of resistance". The MMX strategy was the basis of the growth of the UPL in Brazil and your greatest support was the creation of the EAGLE TEAM-group of scientists who support the project.

• **DVA**

Marketing Manager

Nov 2008/Aug 2011

Responsible for the strategic planning of the DVA with 6-year-old vision. Working the market access in different regions of Brazil (South-Distr/Coop, Center-Dist/Coop/DV and Cerrado Dist/Pools/DV). Customer-focused vision-understanding the customers' needs. Leadership in the process of innovation and creation of new registration/products/projects until the release and first-year sales. Strong leadership and strategic training of teams for implementation of strategies with a focus on profitability.

Bayer S/A – from 1985 to 2008

▪ **Fungicide Product Manager**

Aug 2004 / Apr 2008

Responsible for global strategy of main fungicides applied in main cultures: soy, wheat, corn cotton, and vegetables among others. Creation of new concepts, strategies, market differentiation and segmentation. Strategic planning of an amount over US\$200 M, creation of innovative products to protect the markets. Author of using programs and structured offers. Responsible to prepare the budget focusing on profitability improvement, working with COGs until EBIT. Development of team/partnership works with others company departments. Segment total amount: US\$ 1.200 M.

▪ **Insecticides and TS Product Manager**

Jun 2002 / Jul 2004

Responsible for insecticide portfolio reorganization when Aventis was acquired, because of the great specialization in the market. Value development of insecticide products of portfolio. Development and market

defense through innovations, among other applied techniques. Margin Expansion (IGM) and "cash flow". Product "Phase Out", replacing with better-gain strategies. "Life Cycle Management" of all portfolios (LCM of CNIs, won the International Market Award).

▪ **Local Sales Manager**

Jul 1999 / Jun 2002

Responsible for local Campinas Office, leading a 12-people team. Main markets: Vegetables & flowers, coffee, citrus and cereals. His challenge was the team and the distribution system restructuring. Improvement of process result, training about the new system focusing on company profitability and channels, team motivation. Strong partnerships with channels that are still market leaders in Local Agribusiness were developed. Responsible for a sales amount of US\$ 17 M.

- **PF Product Manager – São Paulo, SP.** **1993**
- **Agro III Sales Technical Representative – Guairá, SP.** **1992**
- **Research Agronomist III – Bragança Pta., SP.** **1991**
- **Development Agronomist III – Campinas, SP.** **1988 / 1991**
- **Regional Agronomist I – Palotina, PR.** **1985 / 1988**

EDUCATION

Agronomic Engineering - F.A.Z "Manuel Carlos Gonçalves" - 1984

registration No. 4210, TEBUCO NORTOX MAP registration No. 11108, TEBUCONAZOL 200 EC AGRIA MAP MAP No. 8216 TEBUCONAZOLE CCAB 200 EC MAP registration No 9412, TEBUFORT MAP registration No. 1710, TEBUHELM MAP registration No. 7406, TEBUZOL 200 EC MAP registration No. 9509, TENAZ 250 SC MAP registration No. 2811, TREASURE MAP registration No 4912, TRIADE MAP registration No 2600, TRIFOLI MAP registration No 4908, TRINITY 250 SC MAP registration No 15508, VERDADERO 600 WG MAP registration No 5003, VIRTUE registration MAP No 1197, YODA registration MAP No. 14814, ZOOM registration MAP No. 14907.ZOOM registration MAP No. 14907.

More information to the press:

Lebna Landgraf (MTb 2903-PR)

Embrapa Soybean

soy.imprensa@embrapa.br (<mailto:soy.imprensa@embrapa.br>)

Telephone: 4333716061

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Research 95

About

95
Research items
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Research Experience

Jun 2002

Brazilian Agricultural Research Corporation (EMBRAPA)

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Position

Senior Researcher



Current institution

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Current position

Researcher

Rafael Moreira Soares's Lab

Lab head



Rafael Moreira Soares

Lab members (2)



Top co-authors

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Marcelo Giovanetti Caneri
State University of Londrina



Mauricio Conrado Meyer
Brazilian Agricultural Research Corporation (EMBRAPA)



Hercules Diniz Campos



Cláudia B Pimenta
Institute of Technical Assistance and Rural Extension of Paraná



Edson P. Boraes



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Co-authors

[View All](#)

Marcelo Giovanetti Canteri
State University of Londrina



Mauricio Conrado Meyer
Brazilian Agricultural Research Corporation (EMBRAPA)



Hercules Diniz Campos



Cláudia B Pimenta
Institute of Technical Assistance and Rural Extension of Paraná



Edson P. Borges

Followers

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Phumelela Peace Mwelasi
University of KwaZulu-Natal



Edson Luis Furtado
São Paulo State University



Odair José Kuhn
State University Of The West Of Paraná



Nédio Rodrigo Tormen
University of Brasília



Edison Ulisses Ramos Junior
Brazilian Agricultural Research Corporation (EMBRAPA)

Following

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Ivani Lopes
Brazilian Agricultural Research Corporation (EMBRAPA)



Fernando Adegas
Brazilian Agricultural Research Corporation (EMBRAPA)



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Brazilian Agricultural Research Corporation (EMBRAPA)



Arne Jensen
Ruhr-Universität Bochum



Eduardo SG Mizubuti
Federal University of Viçosa (UFV)

Projects

Projects (2)

Monitoring the effectiveness of fungicides in soybean rust

Project

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1 Update

Integrated management of plant diseases

Project

View

1 Update

Research

Research Items (95)

Meta-analytic modeling of the decline in performance of fungicides for managing soybean rust after a decade of use in Brazil

Article Full-text available Oct 2017

Felipe Dalla Lana · Pierce Anderson Paul · Claudia VIEIRA Godoy · [...] · Emerson M Del Ponte

An apparent decline in fungicide performance for the control of soybean rust in Brazil has been reported but the rate at which it has occurred has not been formally quantified. Control efficacy and yield response to three fungicides applied as single active ingredients (ai) -azoxystrobin (AZOX), cyproconazole (CYPR), and tebuconazole (TEBU) -and four applied as mixtures-AZOX + CYPR, picoxystrobin + CYPR, pyraclostrobin +...

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Efficiency of fungicides for control of white mold (*Sclerotinia sclerotiorum*) in soybean, in the 2016/17 harvest: summary results of cooperative trials

Technical Report Full-text available Sep 2017

Mauricio Conrado Meyer · Hercules Diniz Campos · Claudia VIEIRA Godoy · [...] · Wilson Venancio

This technical report brings the results from the network trials for chemical control of white mold (*Sclerotinia sclerotiorum*) on soybean in Brazil, crop season 2016-2017 (in Portuguese). Also available at: <http://ainfo.cnptia.embrapa.br/digital/bitstream/item/164554/1/CT-133-OL.pdf>

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Efficiency of fungicides for the control of the target spot, *Corynespora cassiicola*, in the 2016/17 crop: summary results of cooperative trials

Technical Report Full-text available Jul 2017

Claudia VIEIRA Godoy · Carlos M Utimada · Mauricio Conrado Meyer · [...] · Valtemir J. Carlin

This paper reports the results from the network trials for fungicide efficiency to control target spot (*Corynespora cassiicola*) on soybean in Brazil, crop season 2016-2017 (in Portuguese). Also available at: <https://ainfo.cnptia.embrapa.br/digital/bitstream/item/162647/1/CT-130-OL.pdf>

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Efficiency of multisite fungicides and biological product in the control of soybean rust, *Phakopsora pachyrhizi*, in the 2016/17 crop: summary results of cooperative trials

Technical Report Full-text available Jul 2017

Claudia VIEIRA Godoy · Carlos M Utimada · Mauricio Conrado Meyer · [...] · Wilson Venancio

(*Phakopsora pachyrhizi*) on soybean in Brazil, crop season 2016-2017 (in Portuguese). This paper reports the results from the network trials for protectant fungicide efficiency evaluation to control Asian soybean rust (*Phakopsora pachyrhizi*) on soybean in Brazil, crop season 2016-2017. Also available at: <https://ainfo.cnptia.embrapa.br/digital/bitstream/item/162646/1/CT-131-OL.pdf>

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Efficiency of fungicides for the control of soybean rust, *Phakopsora pachyrhizi*, in the 2016/17 crop: summary results of cooperative trials

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(Phakopsora pachyrhizi) on soybean in Brazil, crop season 2016-2017 (in portuguese). This article is a stub. You can help Wikipedia by expanding it. Also available at: <https://ainfo.cnptia.embrapa.br/digital/bitstream/item/162648/1/CT-129-ferrugem-OL.pdf>

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The recombinant pea defensin Drr230a is active against impacting soybean and cotton pathogenic fungi from the genera Fusarium, Colletotrichum and Phakopsora

Article [Full-text available](#) Dec 2016

Ariane Ferreira Lacerda · Rafael Perseghini Del Sarto · Marília Santos Silva · [...] · Maria Fátima Grossi-de-Sá

Plant defensins are antifungal peptides produced by the innate immune system plants developed to circumvent fungal infection. The defensin Drr230a, originally isolated from pea, has previously been shown to be active against various entomopathogenic and phytopathogenic fungi. In the present study, the activity of a yeast-expressed recombinant Drr230a protein (rDrr230a) was tested against impacting soybean and cotton...

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Cooperative assays for biological control of Sclerotinia sclerotiorum in soybean crop: summary results of the 2015/2016 crop

Technical Report [Full-text available](#) Oct 2016

Mauricio Conrado Meyer · Hercules Diniz Campos · Claudia VIEIRA Godoy · [...] · Juliatti FERNANDO Cezar

White mold occurs in the soybean crop in several regions of Brazil, but especially in those with average night temperatures below 20 °C and with high rainfall frequency in the period between the end of the reproductive phase and the beginning of soybean formation. It is estimated that about 23% of the area planted with soybeans in Brazil is infested by Sclerotinia sclerotiorum, the causal agent of the disease. A hallmark of the...

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Efficiency of fungicides for the control of the target spot, Corynespora cassiicola, in the 2015/16 crop: summary results of cooperative trials

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Efficiency of fungicides for white mold control (Sclerotinia sclerotiorum) in soybeans, in the 2015/2016 harvest: summary results of cooperative trials

Technical Report [Full-text available](#) Sep 2016

Mauricio Conrado Meyer · Hercules Diniz Campos · Claudia VIEIRA Godoy · [...] · Wilson S Venancio

White mold is a disease caused by the fungus Sclerotinia sclerotiorum and, according to the results of the cooperative tests of chemical control of the last six crops, causes average reductions of 21% in soybean yield, and can reach up to 70% in some crops isolated (Meyer et al., 2014, MEYER et al., 2015a, MEYER et al., 2015b). According to surveys conducted by CONAB in the 2015/16 crop year (CONAB, 2016), and considerin...

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Efficiency of fungicides for the control of soybean rust, Phakopsora pachyrhizi, in the 2015/16 crop: summary results of cooperative trials
Soybean rust, caused by the fungus Phakopsora pachyrhizi, is one of the most severe diseases affecting soya, with damage varying from 10% to 90% in the different geographic regions where it was reported (YORINORI et al., 2005; HARTMAN et al., 2015). The initial symptoms of the disea...

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Efficiency of multisite fungicides in the control of soybean rust, Phakopsora pachyrhizi, in the 2015/16 crop: summary results of the cooperative trials Londrina, PR August, 2016

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Mobile Application for Asian Soybean Rust Tracking in Brazil

Conference Paper

Full-text available

Jul 2016 · VII Workshop on Computing Applied to the Management of the Environment and Natural Resources

José Henrique Debastiani Andreis · Felipe Borella · Willington Pavan · [...] · Jose Mauricio Cunha Fernandes

Brazil is the second-largest soybean producer. With the arrival of Asian soybean rust in the Western Hemisphere in 2001, a considerable amount of resources has been allocated to understand and control this important yield-limiting disease. Due to its rapid spread, in 2004, the federal government led an effort to create the Asian soybean rust consortium, with the main goal of coordinating research activities involving public an...

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Asian soybean rust in Brazil: past, present, and future

Article

Full-text available

May 2016

Claudia VIEIRA Godoy · Claudine Dinali · Santos Seixas · [...] · Access Orlando Amaral

Asian soybean rust, caused by the fungus *Phakopsora pachyrhizi*, is the most severe disease of the crop and can cause yield losses of up to 90%. The disease was first reported in Brazil in 2001. Epidemics of the disease are common in the country, where the fungus can survive year-round. Regulatory measures to reduce the inoculum between seasons and avoid late season soybean have been adopted to manage the disease....

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Cooperative assays for biological control of white mold in soybean crop - harvests from 2012 to 2015

Technical Report

Full-text available

Feb 2016

Mauricio Conrado Meyer · Hercules Diniz Campos · Claudia VIEIRA Godoy · Carlos Mitinori Utimada

This technical report summarizes the results of a nationwide network of uniform field trials on the biological control of white mold of soybean (*Sclerotinia sclerotiorum*) in Brazil. Tests were carried out from the 2012 to 2015 growing seasons. Several formulations of *Trichoderma* spp., *Bacillus* spp. and a plant resistance inducer (lignosulfonate) were tested. The main conclusion was the potential of some products to reduce th...

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Soybean rust: evaluation of lineage resistance, harvest 2015/2016

Chapter

Full-text available

Jan 2016

Soya: search results 2015/2016.

Leila Maria Costamilan · Paulo Fernando Bertagnoli · Claudia VIEIRA Godoy · [...] · Marcelo Oliveira

The release of soybean cultivars resistant to Asian rust is one of the objectives of Embrapa's breeding program. As the causal agent of Asian soybean rust, *Phakopsora pachyrhizi*, presents great variability, and geographically distant populations can differ in aggressiveness (Yamanaka et al., 2010; Kato and Yorinori, 2008), local analysis of effectiveness resistance genes and the behavior of soybean genotypes. Five major resistan...

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Automatic Classification of Soybean Diseases on Digital Images of Leaf Symptoms

Conference Paper

Full-text available

Oct 2015

https://www.researchgate.net/profile/Claudia_Godoy2

Conference Paper [Full-text available](#) Oct 2015 · X Brazilian Conference on Agrominformatics

Jayme Garcia Arnal Barbedo · Claudia VIEIRA Godoy

This paper presents an algorithm for automatic classification of diseases that produces symptoms in soybean leaves. The algorithm is based on digital image processing techniques and on a modified pairwise voting system that yields, at its output, the list of diseases with the respective likelihoods of being present in that leaf. Only color information is used, which is done by transforming the original RGB format into the HSV, L * ...

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Efficiency of fungicides for the control of the target spot, *Corynespora cassiicola*, in the 2014/15 crop: summary results of cooperative trials.Technical Report [Full-text available](#) Jul 2015

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TYPES OF RESISTANCE TO *Phytophthora sojae* IN SOY LINES OF EMBRAPA WHEATConference Paper [Full-text available](#) Jun 2015 · VII Brazilian Congress of Soy and Meiosur 2015

Leila Maria Costamilan · Paulo Bertagnoli · Cláudia Cristina Clebsch · [...] · Claudia VIEIRA Godoy

Phytophthora sojae root and stem rot in soybean, affects plants at any stage of development. The characteristic symptom is the darkening of the stem and lateral branches, from below upwards from the soil line, with yellowing and wilting of leaves and rotting of the root system. The most effective form of control is the use of resistant soybean cultivars. With complete resistance, ruled by host Rps genes, there is no onset of...

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Brazilian Soybean Pest Management and Threats to its Sustainability

Article [Full-text available](#) Jun 2015

Claudia VIEIRA Godoy · Adeney de Freitas Bueno · Dionisio Luiz Pisa Gazziero

Brazil is expected to harvest 198.65 million tons of grain in 2014/15 in an area of 57.08 million hectares and soybean stands out as the main crop sown. The cultivated soybean area has nearly tripled in the last 20 years, up from 11.6 million hectares in 1994/95 to 31.3 million hectares in 2014/15. One of the main factors for the success of soybean crop in Brazil was the development of cultivars adapted to low latitudes (up to 0°)...

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Photosynthesis of soybean leaves infected by *Corynespora cassiicola* and *Erysiphe diffusa*Article [Full-text available](#) Jun 2015

Sheila Ariana Xavier · Flávia Elis de Mello · Marcelo Giovanetti Canteri · Claudia VIEIRA Godoy

The visual estimate of the severity of plant diseases does not always correlate with the effect of this on the photosynthetic activity of the host. The objective of this work was to evaluate the interference of the fungi *Corynespora cassiicola* and *Erysiphe diffusa*, cause of the target spot and the powdery mildew in soybean, respectively, on the photosynthetic efficiency of infected leaves. Photosynthesis was related to the diseased le...

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Efficiency of fungicides for white mold control (*Sclerotinia sclerotiorum*) in soybean, in the 2013/2014 harvest - summary results of cooperative trials.Technical Report [Full-text available](#) Feb 2015

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Efficiency of fungicides for control of white mold (*Sclerotinia sclerotiorum*) in soybean, in the crop 2013/2014 - summary results of the cooperative trials Soybean is one of the main summer crops of Brazilian agricultural production that cultivates the second largest area of the oilseed, with 30.1 million hectares sown in the crop year 2013/2014 (CONAB, 2014). Soybean diseases represent an important restriction facto...

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Efficiency of fungicides for white mold control (*Sclerotinia sclerotiorum*) in soybean, in the 2013/2014 harvest - summary results of cooperative trials.Technical Report [Full-text available](#) Feb 2015

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Meta-Analysis of the Relationship Between Crop Yield and Soybean Rust Severity

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(Y, kg / ha) and rust severity (S, %) data from uniform fungicide trials (study, k) conducted over nine growing seasons in Brazil. For each selected study, correlation ($k = 231$) and regression analysis ($k = 210$) for the YS relationship were performed and three effects were obtained from these analyzes: Fisher's transformation of Pearson's correlation coefficient (Z_r) and the intercept (β_0) and slope (β_1) coefficients. These effects-sizes...

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Evaluation of virulence of *Phakopsora pachyrhizi* monourediniais isolates collected in Brazil

Conference Paper Full-text available Aug 2014 · 47 Brazilian Congress of Phytopathology

Luana Miekio Darben · A. Yokoyama · TR Parmezan · [...] · Francismar Marcelino Guimaraes

Phakopsora pachyrhizi monourediniais isolates collected in Brazil Asian soybean rust (ASR) caused by the obligate biotrophic fungus *Phakopsora pachyrhizi* is a major disease limiting soybean production in many producer areas in the world. Thus, knowledge of the spectrum of virulence in populations of *P. pachyrhizi* is essential for breeding programs can perform an efficient strategic planning against the fungus that causes ASR...

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Mycelial compatibility and aggressiveness of *Sclerotinia sclerotiorum* isolates from Brazil and the United States

Article Full-text available Apr 2014

Lucimara Junko Koga · CR Bowen · Claudia VIEIRA Godoy · [...] · GL Hartman

The objective of this work was to evaluate the genetic diversity among *Sclerotinia sclerotiorum* isolates from Brazil and the USA, assess their aggressiveness variability, and verify the existence of an isolate-cultivar interaction. Isolate variability was determined by mycelial compatibility grouping (MCG), and isolate aggressiveness by cut-stem inoculations of soybean cultivars. Two experiments for MCGs and two for...

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Cooperative assays of chemical control of white mold in the soybean crop: harvests 2009 to 2012.Book [Full-text available](#) Mar 2014 · Embrapa Soybean

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Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and prothioconazoleArticle [Full-text available](#) Oct 2013

Sheila Aiana Xavier · Marcelo Giovanetti Canteri · Daiane CM Barros · Claudia VIEIRA Godoy

The incidence of spot spot disease on soybean has increased in recent years in Brazil even with intensive use of fungicides, and fungal resistance has been reported in recent studies. The objective of this study was to determine the fungicide sensitivity to carbendazim and prothioconazole in a sample of 24 isolates of *Corynespora cassiicola* from soybean collected from 1996 to 2011 in the states of Paraná, Mato Grosso and São Pa...

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Pathogenic diversity of *Phytophthora sojae* pathotypes from BrazilArticle [Full-text available](#) Apr 2012

Leila Maria Costamilan · Cláudia Cristina Clebsch · Rafael Moreira Soares · [...] · Anne Dorrance

Phytophthora root and stem rot have developed in commercial soybean fields since 2006 in Brazil, and cultivars with resistance to this disease have not been targeted for this region. Thus, the *Phytophthora sojae* pathotypes are expected to have virulence to few if any of the Rps genes. The objectives of this study were to characterize the pathotype diversity of *P. sojae* in Brazil, determine the distribution of the pathogen and...

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Risk and management of fungicide resistance in the Asian soybean rust fungus *Phakopsora pachyrhizi*Article [Full-text available](#) Dec 2011

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Relations of cause and effect among environmental conditions, airborne urediniospores and asian soybean rust severity

Article Jul 2011

RM Mesquini · Rafael Augusto Vieira · Kátia Regina Freitas Schwan-Estrada · Claudia VIEIRA Godoy

The objective of this work was to study the relationships among environmental conditions, airborne urediniospores of *P. pachyrhizi* and the Asian soybean rust (ASR) severity. To quantify airborne urediniospores, collectors were installed in soybean fields in Itambé and Mandaguari, PR. Severity assessments were conducted and climatic data were collected in the two locations. The variables elaborated were: number of days wit...

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Cause-and-effect relationships between environmental conditions, urediniospores and soybean rust severity of soybean rust severityArticle [Full-text available](#) Jun 2011

RM Mesquini · Rafael Augusto Vieira · Kátia Regina Freitas Schwan-Estrada · Claudia VIEIRA Godoy

ABSTRACT: The objective of this work was to establish relationships between environmental conditions, the number of urediniospores of *Phakopsora pachyrhizi* and the severity of soybean-rust. To quantify *P. pachyrhizi* urediniospores, collectors were installed in soybean fields in Itambé and Mandaguari, PR. Severity assessments were performed and climatic data were collected at both sites. The variables elaborated wer...

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A new chitinase-like xylanase inhibitor protein (XIP) from coffee (*Coffea arabica*) affects Soybean Asian rust (*Phakopsora pachyrhizi*) spore germination

Article [Full-text available](#) Feb 2011

Erico AR Vasconcelos · Celso G Santana · Claudia VIEIRA Godoy · [...] · Maria Fátima Grossi-de-Sá

Asian rust (*Phakopsora pachyrhizi*) is a common disease in Brazilian soybean fields and is difficult to control. To identify a biochemical candidate with potential to combat this disease, a new chitinase-like xylanase inhibitor protein (XIP) from coffee (*Coffea arabica*) (CaClXIP) leaves was cloned into the pGAPZα-B vector for expression in *Pichia pastoris*. A cDNA encoding a chitinase-like xylanase inhibitor protein (XIP) from coffee...

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Development of sclerotium rolfii sclerotia in soybean, corn and wheat straw under different temperatures and soil moisture

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The objective of this work was to evaluate the effect of moisture and temperature on the development of *Sclerotium rolfii* on soybean, corn, and wheat straw. Wheat straw produced the lowest number of sclerotia. Intermediate soil moisture level (70% of field capacity), and temperatures ranging between 25-30 °C favored sclerotia development. In the sclerotia were formed at temperatures between 30-35 °C, on any type of straw.

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Role of Host-plant Resistance and Disease Development Stage on Leaf Photosynthetic Competence of Soybean Rust Infected Leaves

Article [Full-text available](#) Nov 2010

Saratha Kumudini · Claudia VIEIRA Godoy · B. Kennedy · [...] · Donald E Hershman

Host-plant resistance is known to reduce fungal growth of *Phakopsora pachyrhizi* Syd. & P. Syd., The causal agent of soybean rust (SBR) in soybean (*Glycine max* L. Merr.). This disease has been shown to reduce soybean leaf photosynthesis in susceptible soybean plants. Since resistant lines have reduced fungal growth, the resistance genes may protect these plants against injury to leaf photosynthesis. The objectives ...

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Relationships among disease intensity, canopy reflectance and grain yield in the Asian soybean rust pathosystem

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Marceli Hikishima · Marcelo Giovanetti Canteri · Claudia VIEIRA Godoy · [...] · J Da Silva

The objective of this study was to develop yield equations for the Asian soybean rust pathosystem and to verify the potential use of measures of reflectance to quantify yield due to this disease. The experiments were carried out in Londrina, PR, in the 2005/2006 and 2006/2007 growing seasons. The results were repeated at different sowing dates, in order to obtain variable disease intensity at different phenological stages of th...

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The objective of this work was to evaluate the effect of moisture and temperature on the development of *Sclerotium rolfii* on soybean, corn, and wheat straw. Wheat straw produced the lowest number of sclerotia. Intermediate soil moisture level (70% of field capacity), and temperatures ranging between 25-30°C favored sclerotia development. The objective of this work was to evaluate the effect of soil moisture and temperature...

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Influence of the luminosity and the epicuticular wax layer of soybean leaf surfaces on *Phakopsora pachyrhizi* infectionArticle [Full-text available](#) Oct 2009

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The Asian rust, caused by the fungus *Phakopsora pachyrhizi*, is one of the most serious phytosanitary problems of soybean cultivation in Brazil, mainly because there are no cultivars with satisfactory levels of resistance. The objective of this work was to study the influence of the luminosity and the wax layer of leaf surfaces on *P. pachyrhizi* infection of soybean leaves. The adaxial or abaxial surface of leaflets of the first plant trifolium...

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Diagrammatic scale for severity evaluation of soybean target spotArticle [Full-text available](#) Oct 2009

Rafael Moreira Soares · Claudia VIEIRA Godoy · Maria Cristina Neves De Oliveira

Soybean target spot, caused by *Corynespora cassiicola*, is found in practically all soybean growing regions in Brazil and it can cause economic losses. The objective of this work was to elaborate and validate a diagrammatic scale to evaluate soybean target spot severity. Soybean leaflets with disease symptoms were collected and severity was measured, determining the minimum and maximum limits and the intermediate levels...

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Influence of light and leaf epicuticular wax layer on *Phakopsora pachyrhizi* infection in soybeanArticle [Full-text available](#) Oct 2009

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Asian rust, caused by the fungus *Phakopsora pachyrhizi*, is one of the most serious phytosanitary problems of soybean in Brazil, especially because no cultivars with satisfactory resistance levels exist yet. The objective of this study was to evaluate the influence of luminosity and epicuticular leaf wax on the infection of soybean by *P. pachyrhizi*. The adaxial and abaxial leaflet surfaces of the first trifoliate leaf from cultivar...

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Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil

Article Sep 2009

H. Scherm · Rock SC Christiano · Paul Esker · [...] · Claudia VIEIRA Godoy

With the arrival of Asian soybean rust (caused by *Phakopsora pachyrhizi*) in the Western Hemisphere in 2001, field research to optimize chemical control of this important yield-limiting disease has proliferated. We present a meta-analytical synthesis of the results of 71 uniform fungicide trials containing 930 entries (specific fungicidal treatments) conducted in Brazil from 2003/2004 to 2006/2007. Our objectives were to: (1)...

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Soybean target spot, caused by *Corynespora cassiicola*, is found in practically all soybean growing regions in Brazil and it can cause economic losses. The objective of this work was to elaborate and validate a diagrammatic scale to evaluate soybean target spot severity. Soybean leaflets with disease symptoms were collected and severity was measured, determining the minimum and maximum limits and the intermediate levels ...

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Diagrammatic Scale for Assessment of *Septoria apiicola* and *Cercospora arracacina* in Arracacha

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Leaf spots, caused by the fungi *Septoria apiicola* and *Cercospora arracacina*, are among the most serious leaf diseases that affect arracacha. Since standardized methods for disease evaluation are necessary, this work proposes a diagrammatic scale for assessment of these diseases. Leaves presenting different severity levels were collected and the minimum and maximum severity limits were determined. The intermediate...

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Influence of soybean phenological stage and leaflets on infection by *Phakopsora pachyrhizi*

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This work was conducted to study the influence of soybean growth stage and leaf age on the infection of *Phakopsora pachyrhizi*, the soybean rust pathogen. Soybean plants (cv. BRS 154 and BRS 258) at the V3, R1 and R5 growth stages were inoculated with 1×10^5 urediniospores per mL suspension. After a period of 24 hours in dew chambers, all plants were removed from the chambers and placed under greenhouse conditio...

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Fungicides Associated with Two Adjuvant Formulations for Preventive and Curative Soybean Rust Control

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DC Ferguson · JR Yowell · Louise L. May-De Mio · [...] · SW Dean

This two-year study aimed to measure the effect of three different fungicide formulations in order to control soybean rust (*Phakopsora pachyrhizi*). This study also measured the effect of controlling soybean rust by adding a fixed concentration, 1% v/v of two adjuvants into the three fungicide formulations and then varying the concentration of the two adjuvants at 0.5, 1.0, and 1.5% into a tebuconazole formulation only...

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In order to study the efficiency of soybean rust control as a function of timing, trials were conducted in Londrina, PR, Brazil, during the 2005/06 and 2006/07 seasons. The mixture of 60 g azoxystrobin ha-1 + 24 g ciproconazole ha-1 was applied at different phenological stages, starting from R2 to R5.5, in single and sequential applications. The severity was estimated periodically for the calculation of the area under the disease...

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Asian soybean rust control efficacy as a function of application timing under epidemic conditions in Londrina, PR

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Fungicide trials were carried out in Londrina, PR, Brazil, during the 2005/06 and 2006/07 growing seasons with the objective of evaluating the efficacy of fungicide in Asian soybean rust control at different application times. The premix of 60 g azoxystrobin ha⁻¹ + 24 g cyproconazole ha⁻¹ was applied to different growth stages starting at R2 up to R5.5, and compared to two applications at R2 and R5.1. Following disease onset...

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Fungicide trials were carried out in Londrina, PR, Brazil, during the 2005/06 and 2006/07 growing seasons with the objective of evaluating the efficacy of fungicide in Asian soybean rust control at different application times. The premix of 60 g azoxystrobin ha⁻¹ + 24 g cyproconazole ha⁻¹ was applied to different growth stages starting at R2 up to R5.5, and compared to two applications at R2 and R5.1. Following disease onset...

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Influence of angular spot severity on photosynthetic efficiency of healthy leaf area of common bean under field conditions

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Marcelo Giovanetti Canteri · Claudia VIEIRA Godoy

This paper aims to show the relationship among leafy leaves, angular leaf spot severity and bean yield, under field conditions, indicating the influence of this relationship on photosynthetic efficiency of leaf tissue remaining on plants. Leaf area and severity of angular leaf spot [*Phaeoisariopsis griseola* (Sacc.) Ferraris] of bean were weekly assessed, beginning at 30 days after emergence (DAE). Different levels of severi...

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Relationship among measures of reflectance and leaf health, soybean rust severity and soybean yield Relationship between measures of reflectance and healthy leaf area, severity of Asian rust and soybean crop productivity

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The aim of this research was to verify if some measures of reflectance could detect variations in the healthy leaf area which could be used as parameter to estimate damages in the production due to soybean rust, and to determine the influence of the disease in photosynthetic efficiency of plants under field conditions. The experiment was lead in the experimental area of Embrapa, in Londrina, PR. The experimental design was...

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Mechanisms Involved in Soybean Rust-Induced Yield Reduction

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Saratha Kumudini · Claudia VIEIRA Godoy · James Board · [...] · Matthijs Tollenaar

Soybean rust (SBR; caused by *Phakopsora pachyrhizi* Syd. And P. Syd.) Leads to premature leaf loss and yield reduction. The objectives of this study were to assess the effects of SBR infection on soybean [*Glycine max* (L.) Merrill] yield and to identify causes for the yield reduction. Experiments were conducted in the 2005-2006 and 2006-2007 growing seasons at Londrina, Brazil. The five treatments were SBR infection...

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The objective of this work was to evaluate the effect of soybean seed treatment with fluquinconazole and foliar fungicide application on soybean rust management, in three sowing dates, during the 2007/2008 growing season, in Londrina, PR, Brazil. The experimental design was a randomized complete block, with a factorial arrangement of 2x5 (with two treatments of seeds with fluquinconazole, and five foliar fungicide...

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Genetic control of soybean (*Glycine max*) yield in the absence and presence of the Asian rust fungus (*Phakopsora pachyrhizi*)

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Aliny Simony Ribeiro · José Francisco Ferraz de Toledo · Carlos Arrabal Arias · [...] · Marcelo Oliveira

Soybean is one of the most important crops in Brazil and continuously generates demands for production technologies, such as cultivars resistant to diseases. In recent years, the Asian rust fungus (*Phakopsora pachyrhizi* Syd. & P. Syd 1914) has caused severe yield losses and the development of resistant cultivars is the best means of control. Understanding the genetic control and estimating parameters associated with...

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Chemical control of Asian soybean rust in southern Paraná

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Emerson Fabio dos Reis · Vismar da Costa Lima Neto · Claudia VIEIRA Godoy · [...] · Nilson Gonçalves Vicente

The Asian rust of soybean, caused by the fungus *Phakopsora pachyrhizi*, is a recent problem in Brazil, which can lead to premature loss of leaves and consequent reduction of productivity. In view of the inexistence of resistant cultivars, chemical control is currently the most important alternative for controlling the disease. The objective of this work was to test two active fungicides and five application times for the control of...

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Relationships between measures of reflectance and health leaf area, soybean rust severity and soybean yield

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The aim of this research was to verify if some measures of reflectance could detect variations in the healthy leaf area which could be used as parameter to estimate damages in the production due to soybean rust, and to determine the influence of the disease in photosynthetic efficiency of plants under field conditions. The experiment was lead in the experimental area of Embrapa, in Londrina, PR. The experimental design was...

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Distinct Biphasic mRNA Changes in Response to Asian Soybean Rust Infection

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Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is now established in all major soybean-producing countries. Currently, there is little information about the molecular basis of ASR-soybean interactions, which will be needed to assist future efforts to develop effective resistance. In the present study, it was found that soybean mRNAs were measured on a 7-day ASR infection time course in mock-inoculated and infected...

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Relation between measures of reflectance and healthy leaf area, severity of Asian rust and soybean crop productivity

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The aim of this research was to verify if some measures of reflectance could detect variations in the healthy leaf area which could be used as parameter to estimate damages in the production due to soybean rust, and to determine the influence of the disease in photosynthetic efficiency of plants under field conditions. The experiment was lead in the experimental area of Embrapa, in Londrina, PR. The experimental design was...

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Evaluation of monocyclic parameters and intensity of the Asian soybean rust (*Phakopsora pachyrhizi*) in both several soybean genotypes and canopy position

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Elisandra Batista Zambenedetti · Eduardo Alves · Edson Pozza · [...] · Claudia VIEIRA Godoy

Soybean rust caused by the fungus *Phakopsora pachyrhizi*, has been known to drastically reduce yields. Diverse studies are now in progress, to search information on the genetic resistance of cultivar currently planted. The objective of this study was to evaluate both incubation and latent period and the progress of soybean rust on several genotypes and three canopy position in greenhouse. The cultivars used were Uirapuru, BRS...

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Genetic control of Asian rust in soybean

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The objective of this study was to investigate the genetic mechanisms of soybean resistance to Asian rust (*Phakopsora pachyrhizi* Syd. & P. Syd). F2 and F3 generations from 15 diallel crosses involving six soybean cultivars, FT-2, EMBRAPA 48, BRS 154, BRS 184, BRS 214, BRS 231, were used to analyze the genetic control of Asian rust resistance in the soybean parents tested. Genetic models were fitted to means and variances of the...

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Models and Applications for Risk Assessment and Prediction of Asian Soybean Rust Epidemics

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Emerson M Del Ponte · Claudia VIEIRA Godoy · Marcelo Giovanetti Canteri · [...] · XB Yang

Asian rust of soybean [*Glycine max* (L.) Merrill] is one of the most important fungal diseases of this crop worldwide. The recent introduction of *Phakopsora pachyrhizi* Syd. & P. Syd in the Americas represents a major threat to soybean production in the main growing regions, and significant losses have already been reported. *P. pachyrhizi* is extremely aggressive under favorable weather conditions, causing rapid plant defoliation....

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Predicting Severity of Asian Soybean Rust Epidemics with Empirical Rainfall Models

Article Full-text available Jul 2006

Emerson M Del Ponte · Claudia VIEIRA Godoy · X. Li · XB Yang

ABSTRACT Although Asian soybean rust occurs in a broad range of environmental conditions, the most explosive and severe epidemics have been reported in seasons with warm temperatures and abundant moisture. Associations between weather and epidemics have been reported previously, but attempts to identify the major factors and model these relationships with field data have been limited to specific locations. Usin...

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Diagrammatic scale for assessment of soybean rust severity

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Claudia VIEIRA Godoy · Lucimara Koga · Marcelo Giovanetti Canteri

A diagrammatic scale to assess soybean (*Glycine max*) rust severity, caused by the fungus *Phakopsora pachyrhizi*, was developed in this study. Leaflets showing different severity levels were collected for determination of the minimum and maximum severity limits; intermediate levels were determined according to "Weber-Fechner's stimulus-response law". The proposed scale showed the levels of 0.6; 2; 7; 18; 42, and 78.5%. Scale...

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Evaluation of tolerance of soybean cultivars to Asian rust in western Bahia

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Ana Oliveira · Claudia VIEIRA Godoy · Monica [...] · MC Rating

The use of tolerance as a form of control for soybean rust can be a viable alternative, since qualitative resistance has been shown to be unstable in function of fungal variability. This trial was carried out in western Bahia in 2003/04, with the objective of assessing the tolerance of the main soybean (*Glycine max*) cultivars used in the area. Four early maturity cultivars (MG / BR 46 (Conquista), Emgopa 315, BRS Corisco and M-SOY...

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Tolerance assessment of soybean cultivars to Asian rust caused by *Phakopsora pachyrhizi* in western Bahia

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Epidemics of Soybean Rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003

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JT Yorinori · WM Paiva · RD Frederick · [...] · J. Jr. Nunes

In 5 March 2001, a severe rust outbreak was recorded at Pitapó, Paraguay, and the causal organism was determined to be *Phakopsora pachyrhizi* using polymerase chain reaction (PCR) and DNA sequence analysis. In May, rust surveys showed spread throughout most of Paraguay and into western and northern Paraná, Brazil. In the 2001-02 season, rust was widespread in Paraguay, but losses were reduced due to severe drought;...

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Influence of angular spot severity on the photosynthetic efficiency of healthy leaf area of common bean under field conditions

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Marcelo Giovanetti Canteri · Claudia VIEIRA Godoy

The objective of this work was to demonstrate the relationship between healthy leaf area, angular leaf spot severity and bean yield, in experimental plots under field conditions, indicating the influence of this relation on the photosynthetic efficiency of leaf tissue remaining in the plants. Foliar area and severity of angular leaf spot (*Phaeoisariopsis griseola* (Sacc. Ferraris) were evaluated weekly, beginning 30 days after...

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Effect of the severity of powdery mildew and leaf blight of cercospora on soybean yield

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plots Powdery mildew and cercospora blight were the predominant diseases in these trials. Disease severity at R6 (pods with full sized green beans) and R7 (pods yellowing, 50% of leaves yellowing) presented a significant negative linear correlation with yield. The relationship between the disease progression curve (AUDPC) and healthy leaf area duration (HAD) and yields were investigated. The AUDPC presented a negative...

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Field trials were carried out in 2002/03, with soybean (*Glycine max*) cultivars BRS 133 and BR 16, to evaluate the effect of foliar diseases on yield. The experimental design was complete randomized blocks with six treatments (different levels of disease) and four replications with plots of 18 m². Different times and numbers of applications of fungicide (difenoconazole + propiconazole) were used to generate a disease gradient...

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Soybean is one of the major items of agricultural production in Brazil, being the country the second largest producer of this grain. Due to the large increase in production in recent decades, soybean has become the focus of the main organs related to Brazilian agribusiness. With the introduction of Asian soybean rust in Brazil, a better management of this disease was necessary, therefore, created in September 2004, the...

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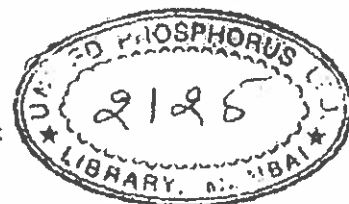


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Calculating Synergistic and Antagonistic Responses of Herbicide Combinations¹

S. R. COLBY²

Abstract. The responses of herbicides applied singly are used in calculating the "expected" response when they are combined. The expected response for a combination is obtained by taking the product of the percent-of-control values for herbicides applied alone and dividing by (100)ⁿ where n is the number of herbicides in the combination.

Despite the tremendous increase in testing of herbicide combinations, the words "synergistic" and "antagonistic" have been largely avoided in publication of results. Uncertainty in determining "expected" responses for herbicide combinations may be partially responsible for the failure of workers to report synergism and antagonism. Another difficulty frequently encountered is that the herbicides used in combination are not applied singly in the same study. When herbicides have not been applied singly, there is no basis for predicting the response when they are applied in combination.

Several mathematical methods are available for testing the selectivity of herbicide combinations (5, 6). This paper presents a method which facilitates calculating "expected" responses of herbicide combinations. The "expected" response for a given combination of two herbicides can be calculated as follows (3, 5):

If X = the percent inhibition of growth by herbicide A at p lb/A

and Y = the percent inhibition of growth by herbicide B at q lb/A

and E = the expected percent inhibition of growth by herbicides

A + B at $p + q$ lb/A

then, according to Gowing (3):

$$E = X + \frac{Y(100-X)}{100} \quad (I)$$

Algebraic manipulation of terms in equation I yields equation II, the form used by Limpel *et al.* (5):

$$E = X + Y - \frac{XY}{100} \quad (II)$$

¹Received for publication April 11, 1966. Contribution No. 3796 and Scientific Article No. A-1271 of the Maryland Agricultural Experiment Station, Department of Agronomy, University of Maryland, College Park.

²Assistant Professor, Agronomy Department, University of Maryland, College Park.

When the observed response is greater than expected, the combination is synergistic; when less than expected, it is antagonistic. If the observed and expected responses are equal, the combination is additive.

In the use of equation II, original units of data, such as weed counts or fresh or dry weights of plants, are converted to "percent inhibition" values. Once this is done, it is necessary to perform one addition, a subtraction, a multiplication, and one division to obtain each expected response (equation II).

If instead, we convert the original data to "percent-of-control" values, the number of arithmetic operations required to obtain "E" is reduced.

Let N_1 = growth as a percent-of-control with herbicide A at p lb/A

and Y_1 = growth as a percent-of-control with herbicide B at q lb/A

and E_1 = expected growth as a percent-of-control with herbicides

A + B at $p + q$ lb/A

then $E_1 = 100 - E$

$N_1 = 100 - X$

$Y_1 = 100 - Y$

hence $E_1 = 100 - (X + Y - \frac{XY}{100})$

and $E_1 = 100 - (100 - N_1) - (100 - Y_1) + \frac{(100 - N_1)(100 - Y_1)}{100}$

finally $E_1 = \frac{N_1 Y_1}{100} \quad (III)$

The use of formula III as compared with formula II eliminates the addition and subtraction, thus reducing the number of operations required to obtain an "expected" response.

Colby (2) extended formula I to apply to three-way combinations.

Thus, if Z = the percent inhibition of growth by herbicide

C at r lb/A

then $E = X + Y + Z - \frac{(XY + XZ + YZ)}{100}$

$$+ \frac{XYZ}{10,000} \quad (IV)$$

COLBY: CALCULATING HERBICIDAL RESPONSES

Now if Z_1 = growth as a percent-of-control with herbicide

$$\text{then } E_1 = \frac{C \text{ at } 1 \text{ lb/A} \times X_1 Y_1 Z_1}{10,000} \quad (V)$$

Obviously, the use of formula V instead of IV reduces the number of arithmetic operations required to obtain the expected response since the subtractions and additions are eliminated. In general, the expected response for any combination of herbicides may be obtained by taking the product of the percent-of-control values for herbicides applied alone and dividing by $(100)^{n-1}$ where n is the number of herbicides in the combination. Each herbicide must be applied singly at the same rate as used in combination.

Data published by Jagschitz and Skogley (4) are used to illustrate the calculation of expected responses for herbicide combinations. Four herbicides were applied singly and in certain combinations for the control of several weeds in turfgrass. The data as originally presented have been converted to percent-of-control values and are shown in Tables 1 and 2. Expected values for the combinations are shown in

Table 1. Dandelion control in fairway turf treated with various herbicides October 8, 1961.

Herbicide	lb/A	Dandelion response, % of control, 10-19-61
dandelion	0.125	55
	0.25	35
	0.5	43
meoprop	0.5	67
	1.0	81
	1.5	79
2,4-D	0.5	63
	1.0	54
	1.5	44
picloram	0.0625	40
	0.25	10
dandelion + meoprop	0.125 + 0.5	51 (53) +2
	0.125 + 1.0	33 (43) +10
dandelion + 2,4-D	0.125 + 0.5	51 (55) -4
	0.125 + 1.0	64 (60) +4
	0.25 + 1.0	58 (54) +4
	0.5 + 1.0	46 (43) +3
meoprop + 2,4-D	0.5 + 0.5	56 (63) -7
	0.5 + 1.0	47 (52) +5
	1.0 + 0.5	43 (51) +8
	1.0 + 1.0	52 (44) +8
	1.5 + 1.0	76 (43) +33
dandelion + meoprop + 2,4-D	0.125 + 0.5 + 0.5	63 (54) -9
	0.125 + 1.0 + 0.5	31 (28) +3
	0.125 + 0.5 + 1.0	52 (29) +23
	0.125 + 1.0 + 1.0	54 (24) +30
dandelion + meoprop + 2,4-D + picloram	0.125 + 0.5 + 0.5 + 0.0625	27 (13) +14

Adapted from the data of Jagschitz and Skogley (4). Expected responses for combinations are shown in parentheses following each observed response. The difference between observed and expected values are shown for a plus sign to indicate synergism and a minus, antagonism.

parentheses following each observed value. To the right of each expected value, the difference between observed and expected values is shown. A positive value is indicative of a synergistic response while a negative value is indicative of an antagonistic response. If the observed and expected values had been computed individually for each replication, then a chi-square test could have been used to de-

termine the statistical significance of the differences between observed and expected values. Even without the chi-square test, several conclusions seem probable from the data in Tables 1 and 2. First, the combinations appear antagonistic on dandelion. Furthermore, the antagonism seems to be greater with increasing combined rates, especially when the herbicides were applied in 1964. Possibly this antagonism is caused by greater contact injury or more plant tops being killed at higher rates resulting in less translocation of herbicide into the dandelion roots. It also appears from Table 2 that different weeds respond differently to the same

Table 2. Chickweed and dandelion control in fairway turf treated with various herbicides May 25, 1965.

Herbicide	lb/A	Chickweed response, % of control, 10-19-65	Dandelion response, % of control, 10-19-65
dandelion	0.125	40	66
	0.25	1	53
	0.5	0	42
meoprop	0.5	16	67
	1.0	0	52
	1.5	0	72
2,4-D	0.5	51	75
	1.0	32	64
	1.5	71	56
dandelion + meoprop	0.125 + 0.5	1 (6) +5	70 (57) -13
	0.125 + 1.0	0 (0)	28 (41) -13
dandelion + 2,4-D	0.125 + 0.5	7 (20) +13	67 (50) -17
	0.125 + 1.0	3 (13) +10	21 (42) -21
	0.25 + 1.0	0 (3) +3	41 (54) -13
	0.5 + 1.0	0 (0)	53 (37) -16
meoprop + 2,4-D	0.5 + 0.5	1 (6) +5	70 (55) -15
	0.5 + 1.0	1 (5) +4	68 (50) -18
	1.0 + 0.5	1 (6) +5	70 (47) -23
	1.0 + 1.0	1 (6) +5	55 (40) -15
	1.5 + 1.0	1 (6) +5	60 (46) -14
dandelion + meoprop + 2,4-D	0.125 + 0.5 + 0.5	1 (3) +2	32 (43) -11
	0.125 + 1.0 + 0.5	0 (0)	64 (37) -27
	0.125 + 0.5 + 1.0	0 (2) +2	34 (53) -19
	0.125 + 1.0 + 1.0	0 (0)	29 (25) -4

Adapted from the data of Jagschitz and Skogley (4). Expected responses for combinations are shown in parentheses following each observed response. The difference between observed and expected values are shown for a plus sign to indicate synergism and a minus, antagonism.

combination. Thus, combinations which were about additive or possibly synergistic on chickweed were antagonistic, in general, on dandelion.

The calculations involved in determining the expected response of one three-way combination from Table 1 illustrate the efficiency of formula V compared to formula IV. For example, using dandelion at 0.125 lb/A in combination with meoprop at 0.5 lb/A and 2,4-D at 0.5 lb/A the expected response is calculated as follows using formula IV and the data in terms of percent weed control as originally reported by Jagschitz *et al.* (4).

$$E = 45 \div 3 + 37 \div \frac{(45(3) + 45(37) + 3(37))}{100} + \frac{(45(3) + 37)}{10,000} + \frac{(115 + 1665 + 111)}{100} + \frac{4993}{10,000}$$

$$= 85$$

$$= 85$$

$$= 66.39\% \text{ weed control expected}$$



W E E D S

Using formula V and percent-of-control values, the computation is

$$F_1 = \frac{(55)(97)(63)}{10,000}$$

= 33.61 percent-of-control
and 33.61%-of-control is equal to 66.39% weed control.

Obviously, there are practical limitations in using mathematical formulas in predicting the responses for herbicide combinations, but they represent an improvement over no attempt to predict responses. The computations described should most effectively be applied to populations of single species although this would not seem to be an absolute requirement. Furthermore, the formulas are most accurate when values of X, Y, and Z are near the 50% level since

the dose-response curves deviate least from linearity at the 50% level.

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Seasonal Variation in Sprouting and Available Carbohydrate in Yellow Nutsedge Tubers¹

R. B. TAYLORSON²

Abstract. Two morphological types of tubers of yellow nutsedge (*Cyperus esculentus* L.) were collected over a 2 year period and were sprouted in the laboratory. Tuber dormancy occurred during late summer and early fall. Sprouting was highest during the winter and spring. Mechanical disturbance of the nutsedge stand increased tuber sprouting. Available carbohydrates followed a pattern similar to sprouting; minimum levels were found during late summer. The two types of tubers appeared to be similar in respect to the characteristics studied.

INTRODUCTION

DORMANCY commonly occurs in various organs and at different seasons of the year among species of higher plants (7). Generally, little is known of dormancy in subterranean organs of weeds, including tubers of yellow nutsedge (*Cyperus esculentus* L.). Tumbleson and Kommedahl (6) indicated that tubers were dormant when dug in September but would germinate in June. Breaking of tuber dormancy was thought to be associated with low temperature and leachable inhibitors. Other research has dealt mainly with methods of breaking dormancy of tubers by chemical techniques (2). Control of yellow nutsedge with postemergence herbicides is partially dependent on tuber dormancy, since emergence of shoots must be optimum when the herbicides are applied for maximum effects.

Another factor often related to effectiveness of herbicides in the control of perennial weed species is the level of reserve carbohydrates. However, studies attempting to

relate carbohydrate levels with herbicide acceptability have not been clearly successful (3, 5).

In these studies, I have attempted to characterize tuber dormancy and carbohydrate content and their possible relation to herbicide utilization.

METHODS AND MATERIALS

A dense stand of yellow nutsedge growing in a field of Tifton loamy sand was the source of plant material. Samples were collected at monthly intervals from July, 1962 to June, 1964. During July, 1962 to June, 1963, samples were randomly collected over the infested area. The stand was not disturbed mechanically except for an early spring plowing and harrowing. During the months of July to November, 1963, the area was subdivided into 25 by 50 ft plots. Three twice-replicated treatments were imposed. One treatment was a continuation of the mechanically undisturbed stand mentioned above. Other treatments were (a) mowing approximately 2 weeks prior to the next sampling date and (b) disk-harrowing approximately 2 weeks prior to the next sampling date. At the conclusion of this sampling period, further collections were made only from the mechanically undisturbed plots.

At each sampling date, duplicate lots of approximately 500 tubers were recovered by working the soil through a coarse screen. On several occasions during 1963, unwashed tubers were recovered from mechanically undisturbed plot samples by searching the soil samples and brushing off most of the adhering soil from the tubers.

Except for the unwashed lots, the tubers were subjectively graded into four types according to external color and morphology, and then counted. Type A tubers were black-skinned, shriveled, and usually dead; type B were black but turgid; type C were brown and turgid;

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²Plant Physiologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Tifton, Georgia. Present address is Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.



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Theory of synergistic effects: Hill-type response surfaces as 'null-interaction' models for mixtures

Michael Schindler

Institute of Theoretical Chemistry, Ruhr-University Bochum, Universitätsstr., Bochum, Germany

Michael Schindler, Email: Michael.Schindler@rub.de.

Corresponding author.

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Abstract

Background

The classification of effects caused by mixtures of agents as synergistic, antagonistic or additive depends critically on the reference model of 'null interaction'. Two main approaches are currently in use, the Additive Dose (ADM) or concentration addition (CA) and the Multiplicative Survival (MSM) or independent action (IA) models. We compare several response surface models to a newly developed Hill response surface, obtained by solving a logistic partial differential equation (PDE). Assuming that a mixture of chemicals with individual

Hill-type dose-response curves can be described by an n-dimensional logistic function. Hill's differential equation for pure agents is replaced by a PDE for mixtures whose solution provides Hill surfaces as 'null-interaction' models and relies neither on Bliss independence or Loewe additivity nor uses Chou's unified general theory.

Methods

An n-dimensional logistic PDE describing the Hill-type response of n-component mixtures is solved. Appropriate boundary conditions ensure the correct asymptotic behaviour. Mathematica 11 (Wolfram, Mathematica Version 11.0, 2016) is used for the mathematics and graphics presented in this article.

Results

The Hill response surface ansatz can be applied to mixtures of compounds with arbitrary Hill parameters. Restrictions which are required when deriving analytical expressions for response surfaces from other principles, are unnecessary. Many approaches based on Loewe additivity turn out to be special cases of the Hill approach whose increased flexibility permits a better description of 'null-effect' responses. Missing sham-compliance of Bliss IA, known as Colby's model in agrochemistry, leads to incompatibility with the Hill surface ansatz. Examples of binary and ternary mixtures illustrate the differences between the approaches. For Hill-slopes close to one and doses below the half-maximum effect doses MSM (Colby, Bliss, Finney, Abbott) predicts synergistic effects where the Hill model indicates 'null-interaction'. These differences increase considerably with increasing steepness of the individual dose-response curves.

Conclusion

The Hill response surface ansatz contains the Loewe additivity concept as a special case and is incompatible with Bliss independent action. Hence, when synergistic effects are claimed, those dose combinations deserve special attention where the differences between independent action approaches and Hill estimations are large.

Keywords: Synergistic effects, Hill response surface, Loewe additivity, Bliss independence, Colby's formula

Background

Quantifying the effect of an active substance upon application to its target organism is a central topic in life sciences. The description and prediction of dose-response curves of pure compounds and estimating the combined effect of simultaneous administration of several active ingredients (a.i.s) is a field of active research in pharmacology, anesthesiology, toxicology, environmental science, and agrochemistry. The detection of virtually any kind of interaction between drugs is, e.g., of utmost importance for risk assessment in the registration process of chemicals in general. While synergistic adverse effects present a challenge to say the least, synergistic effects in drug administration are

desirable as they might be used to reach the same goal with less effort. Disregarding the steadily growing area of possible interactions of chemicals with their environment, this article concentrates on interactions between a.i.s in the life-sciences. The terms 'agent', 'drug', 'chemical', and 'a.i.' are used interchangeably.

In 1989, Berenbaum [1] summarized the state of the art describing synergistic effects in pharmacology. Since then, numerous attempts to quantify non-additive effects have been published, mainly in the fields of chemotherapy [2], anesthesiology [3], toxicology [4], physiology [5], environmental science [6, 7], and pharmacology [8], to quote only a few reviews.

Generally, biological effects of an a.i. follow a dose-response relation, starting from a no-effect level (NOEL) and ending at the maximum effect corresponding to a saturation dose. In order to quantitatively describe interactions between substances, it is necessary to define a reference behaviour, namely the response of a system of compounds acting independently. Deviations from this ideal reference can then be classified as synergistic or antagonistic, being aware of the fact that these deviations can be caused by a multitude of physico-chemical and biochemical effects, especially in whole organisms (for a recent study see e.g. [9]).

Whenever the effect observed after applying a mixture exceeds the expectation, the action of the agents is called synergistic, and if it is smaller than expected it is antagonistic. This seemingly simple definition is not simple at all, as there is an ongoing debate since the beginning of the last century on how to correctly define this reference of 'no interaction'. In the literature, synergy is defined either phenomenologically or based on assumptions on the modes of action of the a.i.s involved. The observed effects like zero-interaction, synergism or antagonism, are often quantified by calculating interaction- [10] or combination-indices [11], however, often based on differing definitions of additivity [2, 4].

Mainly two types of reference models are currently in use [12, 13]. One class of models, the Additive Dose Model (ADM) pioneered by Loewe [14], assumes an additive behaviour of doses, similar modes of action and consequently parallel dose-response curves with identical slopes. Its basic concept is that of equipotent doses, which means that an arbitrary dose of a.i. A can be replaced by an isoeffective dose of a.i. B. The terms 'effect summation', 'dose addition', and 'concentration addition (CA)' are used in this context. As the joint action of the a.i.s is not independent in this model, the term 'mutually exclusive' [11] is more appropriate here. Implicitly the model assumes that the maximum possible effect achievable by the mixture is that of its most potent partner. A generalized concentration addition (GCA) model [6] extended the original ADM approach [10, 14–16] to mixtures with partially overlapping agonists.

The other class of models, called Multiplicative Survival Models (MSM), assumes that the effects caused by the two a.i.s are mutually non-exclusive and originate from different modes of action, and hence the asymptotically achievable effect is the sum of the individually possible ones. In agrochemical research, it is associated with the names of Colby [17] and Limpel [18], while in toxicology these models of independent action (IA) have been described by Bliss [19] and Finney [20] and in entomology by Abbott [21].

Chou's widely used combination indices [11] cover both classes, his formula for the mutually exclusive combination is of ADM type while his description of mutually non-exclusive mixtures is MSM like and reduces to Colby's formula for reaction orders of one. The approach presented here relies neither on Bliss independence or Loewe additivity nor uses Chou's unified general theory. It focuses on logistic (Hill) response surfaces as 'null-effect' models. For mixtures of n partners they result from solving an n -dimensional partial differential equation (PDE). Appropriate boundary conditions guarantee that the expressions describing the Hill-surfaces are asymptotically correct, i.e., lead to Hill's well-known dose-response curves in the one-dimensional case of pure a.i.s. In addition, the solutions are sham-compliant, meaning that a mixture consisting of combinations of a drug with itself shows no synergistic effect.

Sigmoid dose-response curves like Hill's equation [22, 23] are solutions of a class of ordinary differential equations (ODEs) which was originally used to describe population dynamics [24]. Examples of the phenomena described by this type of functions are titration curves in chemistry, dose-response curves in enzyme kinetics, and predator-prey models in biology.

Following a short recapitulation of the ODE leading to Hill-type dose-response curves for pure compounds, Hill response surfaces for binary and n -component mixtures are introduced as solutions of the corresponding PDEs. In the subsequent sections the properties of Hill surfaces are compared to other response surface models and applied to some literature examples, namely binary and ternary mixtures from various fields of life-science. A summary of our findings will be given in the last section.

Methods

While Hill's equation can be obtained by solving a first-order ODE, the logistic differential equation, its n -dimensional generalization results from solving a semilinear PDE with the appropriate boundary conditions. These are the requirements that in the limit of one dimension the original Hill equation results, and that the solution of the PDE is sham-compliant, meaning that an artificial partitioning of the one-dimensional problem into an n -dimensional one does not change the results.

Here an analytical expression of the n -dimensional extension of Hill's equation is provided. Verification is done by substituting the solution into the PDE by using Mathematica 11 [25]. Checking the fulfillment of the boundary conditions is achieved by performing the corresponding limiting processes.

Results

Logistic functions and the Hill response surface

Let A and B be active ingredients with dose-response curves $a(x)$ and $b(y)$ depending on the variables x and y and let U be a combination of A and B with the dose-response surface $u(x,y)$. Let us further assume that we can describe the effect z (being $a(x)$, $b(y)$ or $u(x,y)$), by a logistic function. Then z is characterized by 4 parameters, the minimum and maximum responses z_{min} and z_{max} , the position of the

inflection point and a slope parameter, i.e., the slope at the inflection point of the curve. All effects are limited by the no-effect- and the full-effect levels, $0 \leq x \leq x_{max}$ and $0 \leq y \leq y_{max}$.

The differential equations for the one- and two-dimensional cases, describing the variation of the effects $a(x)$, $b(y)$ and $u(x,y)$ with the variation of x and y , are

$$\frac{da(x)}{dx} = \alpha a(x) \left(1 - \frac{a(x)}{a_{max}} \right) \quad \frac{db(y)}{dy} = \beta b(y) \left(1 - \frac{b(y)}{b_{max}} \right) \quad 1$$

$$u_x + u_y = \gamma \left(x, y \right) u \left(x, y \right) \left(1 - \frac{u(x,y)}{u_{max}(x,y)} \right) \quad 2$$

where $u_x = \partial u(x,y) / \partial x$ and $u_y = \partial u(x,y) / \partial y$ denote the partial derivatives of u , and α , β and a_{max} , b_{max} are constants. In Eq. 2, γ and u_{max} are functions of x and y .

Denoting by x_{50} and y_{50} the positions of the half-maximum effects $a(x_{50}) = a_{max}/2$ and $b(y_{50}) = b_{max}/2$ the final forms of $a(x)$ and $b(y)$ are the logistic functions

$$a(x) = \frac{a_{max} e^{\alpha \Delta x}}{1 + e^{\alpha \Delta x}} = \frac{a_{max}}{1 + e^{-\alpha \Delta x}} \quad b(y) = \frac{b_{max} e^{\beta \Delta y}}{1 + e^{\beta \Delta y}} = \frac{b_{max}}{1 + e^{-\beta \Delta y}} \quad 3$$

with $\Delta x = x - x_{50}$, $\Delta y = y - y_{50}$ and α and β being the slopes at the inflection points x_{50} and y_{50} . They show an exponentially increasing effect at low doses, becoming linear close to $x = x_{50}$ and $y = y_{50}$ and finally an exponentially decreasing growth until the limiting effect z_{max} is reached. $a(x)$ and $b(y)$ are intimately connected to Hill's equation, providing a relation between the effect E and the dose C .

$$E = E_0 + \frac{E_{max} C^\alpha}{C^\alpha + EC_{50}^\alpha} = E_0 + \frac{E_{max}}{1 + \left(\frac{EC_{50}}{C} \right)^\alpha} \quad 4$$

Actually, x and y are the natural logarithms of doses with $-\infty \leq x, y \leq \infty$, whereas the doses themselves (i.e., e^x and e^y) are ≥ 0 . Hence, we can identify the effects E_0 , E_{max} and the shape parameter α with ε_{min} , ε_{max} and α or β , and the doses C and EC_{50} with e^x and $e^{x_{50}}$ or e^y and $e^{y_{50}}$ of Eq. 3, meaning that Hill's equation is the solution of a logistic ODE, subject to the appropriate boundary conditions.

Our approach to handle mixtures is completely analogous to that used for the pure compounds. The solution of the PDE describing the effect $u(x, y)$ of a binary mixture (Eq. 2) is the logistic Hill-surface as the response surface of 'null-interaction'.

$$u_{Hill}(x, y) = \frac{u_{max}(x, y)}{1 + [e^{\Delta x} + e^{\Delta y}]^{-\gamma(x, y)}} \quad 5$$

The boundary condition

$$u(x, -\infty) = a(x) \wedge u(-\infty, y) = b(y)$$

that in the limit of one vanishing agent the response due to the second one has to result, and the so-called sham-compliance requirement have to be fulfilled: If a dose d of one a.i. is artificially split into two contributions n d and $(1-n)d$ with $0 \leq n \leq 1$, the response due to this 'mixture' has to be identical to the response of the pure compound, i.e., $a(d) = u(n \times d, (1-n) \times d)$, irrespective of any possible interaction between different a.i.s. In our formalism a dose d is equal to e^x . Hence, sham partitioning means setting $e^x = n e^x + (1-n)e^x$, leading to the requirement $u(x + \ln n, x + \ln(1-n)) = a(x)$.

These conditions are satisfied by the ansatz

$$\begin{aligned} \gamma(x, y) &= \frac{\alpha e^{\Delta x} + \beta e^{\Delta y}}{e^{\Delta x} + e^{\Delta y}} \\ u_{max}(x, y) &= \frac{a_{max} e^{\Delta x} + b_{max} e^{\Delta y}}{e^{\Delta x} + e^{\Delta y}} \end{aligned} \quad 6$$

which provides smooth transitions between a and β and between a_{max} and b_{max} .

To facilitate a comparison with literature expressions for response surfaces, u_{Hill} can be re-written by substituting the doses d_a and d_b for e^x and e^y and using the doses scaled by their median effects $m_a = d_a/d_{a50} = e^{\Delta x}$ and $m_b = d_b/d_{b50} = e^{\Delta y}$. Then the 'null-interaction' reference response surface is

$$u_{Hill} = u_{max} \left(m_a, m_b \right) \times \frac{(m_a + m_b)^{\gamma(m_a, m_b)}}{1 + (m_a + m_b)^{\gamma(m_a, m_b)}} \quad 7$$

with

$$\begin{aligned} u_{max} &= \frac{\alpha_{max} m_a + \beta_{max} m_b}{m_a + m_b} \\ \gamma &= \frac{\alpha m_a + \beta m_b}{m_a + m_b} \end{aligned} \quad 8$$

As the ansatz presented here uses the known dose-response curves of the pure mixture partners to unambiguously define the 'null-interaction' surface for the binary mixture, there is no obvious way to introduce interaction parameters (and functions) which account for deviations from this reference [26–28]. At present the only way to detect synergism or antagonism is the direct comparison of experimental data with the Hill response surface, either visually or by statistical means like the root-mean squared error of prediction (RMSE).

A way out of this dilemma might be to obtain the necessary parameters directly from fitting the experimental mixture data to a logistic surface which could then be compared to the 'null-interaction' model. However, the functional form of u_{Hill} is not flexible enough for this purpose and some sort of perturbation theory might be more appropriate to handle deviations from the Hill surface.

The logistic PDE for mixtures of n components

The extension of the Hill formalism to mixtures of n agents A_i is straightforward. The corresponding semilinear logistic PDE [29] is

$$\sum_{i=1}^n u_{x_i} = \gamma(\vec{x}) u(\vec{x}) \left(1 - \frac{u(\vec{x})}{u_{max}(\vec{x})} \right) \quad 9$$

where $\vec{x} = (x_1, \dots, x_i, \dots, x_n)$ and $u_{x_i} = \partial u(\vec{x}) / \partial x_i$. Its solution

$$u(\vec{x}) = u_{max} \left(\vec{x} \right) \frac{[\sum_{i=1}^n e^{\Delta x_i}]^{\gamma(\vec{x})}}{1 + [\sum_{i=1}^n e^{\Delta x_i}]^{\gamma(\vec{x})}} \quad 10$$

with

$$\gamma(\vec{x}) = \frac{\sum_{i=1,n} \alpha_i e^{\Delta x_i}}{\sum_{i=1,n} e^{\Delta x_i}} \quad u_{max}(\vec{x}) = \frac{\sum_{i=1,n} a_{max,i} e^{\Delta x_i}}{\sum_{i=1,n} e^{\Delta x_i}}$$

describes an n-dimensional logistic surface. It satisfies the boundary conditions,

$$u(-\infty, \dots, x_i, \dots, -\infty) = a_i(x_i)$$

and the sham compliance condition, that any partition of the dose of a pure drug into various artificial new drugs must cause the same effect as the pure drug alone.

As an example, u_{Hill} for a ternary mixture of A, B and C satisfying the sham condition $a(d) = u((r-m) \times d, (1-n) \times d, m \times d)$ with $0 \leq r+m \leq 1$, is

$$u_{Hill} = u_{max} \times \frac{[m_a + m_b + m_c]^{\gamma}}{1 + [m_a + m_b + m_c]^{\gamma}}$$

where in a self-explaining notation

$$u_{max} = \frac{\alpha_{max} m_a + \delta_{max} m_b + \epsilon_{max} m_c}{m_a + m_b + m_c}$$

$$\gamma = \frac{\alpha m_a + \beta m_b + \delta m_c}{m_a + m_b + m_c}$$

Discussion

From a theoretical point of view it is an advantage of the Hill surface approach that it does not rest on assumptions on maximum effects or restrictions on specific parameter combinations of the mixture components. This distinguishes our approach from other response surface models as will be outlined in the next section. In practical applications, however, these differences become significant only if steep dose-response curves and/or strongly differing maximum-effect parameters of the individual agents are involved.

A Comparison of empirical response surface models

Many of the response surface models are based on two synergy approaches, the Bliss independence [19, 20] and Loewe additivity [14] models. The characteristics of some of them are listed in Table 1. There their functional forms describing the mixture effects are classified by their assumptions on maximum effects and slopes and by analyzing whether they are asymptotically correct and sham compliant. Apparently only a few authors [26, 28, 30] use the freedom to define the shape function $\gamma(\vec{x})$ and the maximum effect function $u_{max}(\vec{x})$ (Eq. 6).

Table 1

Characteristics of theoretical models describing mixtures

Approach		Assumptions		Properties	
	Eq. u	max	Slopes γ	Pure a.i. limit	Sham compliance
Bliss / Colby	13	Unequal	$\gamma_a \neq \gamma_b$	Ok	No
Loewe / CA	14	Equal	$\gamma_a = \gamma_b$	Ok	Yes
GCA	18	Unequal	$\gamma = 1$	Ok	Yes
Chou exc	19	Equal	$\gamma_a = \gamma_b$	Ok	Yes
Chou nonex	20	Equal	$\gamma_a \neq \gamma_b$	Ok	No
Greco	17	Equal	$\gamma_a \neq \gamma_b$ ^a	(Ok) ^b	(Yes) ^b
Minto / Fidler	21	Variable	Variable	(Ok) ^c	(Yes) ^c
Hill	5, 7	Variable	Variable	Ok	Yes

^aanalytical expression for the effect only for identical slopes

^bfor $\alpha=0$

^cif the polynomial expansions are truncated after the linear terms

Bliss independent joint action (IA) [3] can be formulated in terms of fractions of possible response unaffected f_u or fractions affected f_a , with $f_a + f_u = 1$. For a binary mixture $Fu_{ab} = f_u \times f_u_b$ and $Fa_{ab} = 1 - Fu_{ab}$. Hence

$$Fa_{ab} = 1 - (1 - fa_a) \times (1 - fa_b) = fa_a + fa_b - fa_a \times fa_b$$

11

It is related to the expression for the probability $P(A \cup B)$ of an event A or B if the basic events with probabilities $P(A)$ and $P(B)$ are independent. In crop science it is known as MSM [13] or Colby [17] model and is widely used to classify mixture effects [31]. Recently Colby's formula has been extended to multi-compound mixtures [32]. As the IA ansatz is not sham-compliant it is not compatible with the Hill approach.

$$u_{Colby}(x, y) = u_{Bliss}(x, y) = a(x) + b(y) - a(x) \times b(y) \quad 12$$

$$= \frac{a_{max}}{1+m_a^{-\alpha}} + \frac{b_{max}}{1+m_b^{-\beta}} - \frac{a_{max}b_{max}}{(1+m_a^{-\alpha})(1+m_b^{-\beta})} \quad 13$$

The ADM [13] or CA model of mutually exclusive action [1, 14] for two noninteracting isoactive drugs A and B is

$$1 = \frac{m_a}{f_a^{-1}} + \frac{m_b}{f_b^{-1}} \quad 14$$

where f_x^{-1} is the dose or concentration of compound x that causes the specified effect. If the agents are acting according to Hill dose-response functions with slopes γ_a and γ_b , it is given by

$$1 = \frac{m_a}{\left(\frac{u}{u_{max}-u}\right)^{1/\gamma_a}} + \frac{m_b}{\left(\frac{u}{u_{max}-u}\right)^{1/\gamma_b}} \quad 15$$

Adapting Berenbaum's approach, Greco derived a model for two-agent combined action by adding an interaction term, parameterized by a factor α . Assuming that the Hill-type dose-response curves of A and B differ only in the slope parameters, he gets [33]

$$1 = \frac{m_a}{\left(\frac{u}{u_{max}-u}\right)^{1/\gamma_a}} + \frac{m_b}{\left(\frac{u}{u_{max}-u}\right)^{1/\gamma_b}} + \alpha \frac{m_a m_b}{\left(\frac{u}{u_{max}-u}\right)^{(1/2\gamma_a-1/2\gamma_b)}} \quad 16$$

Although analytical expressions for u can be obtained from Eqs. 15 and 16 only under the restrictions of either a fixed maximum effect $a_{max}=b_{max}$ and identical slope parameters $\gamma_a=\gamma_b$ or of different maximum effects and identical slopes of unity $\gamma_a=\gamma_b=1$, they are the starting points for several response surface models, e.g., Greco's model from Eq. 16

$$u_{Greco} = u_{max} \frac{(m_a + m_b + \alpha \times m_a m_b)^\gamma}{1 + (m_a + m_b + \alpha \times m_a m_b)^\gamma} \quad 17$$

or the GCA expression [6] from Eq. 15. It permits different maximum effects but is limited to $\gamma=1$.

$$u_{GCA} = \frac{a_{max} m_a + b_{max} m_b}{1 + a_{max} m_a + b_{max} m_b} \quad 18$$

Hence, u_{Greco} (for $\alpha=0$) and u_{GCA} are special cases of u_{Hill} . The same holds true for Chou and Talalay's mutually exclusive model [11]. It was derived from the the median effect principle, assuming both a constant u_{max} and γ

$$u_{Chou_{ex}} = u_{max} \frac{(m_a + m_b)^\gamma}{1 + (m_a + m_b)^\gamma} \quad 19$$

Their mutually non-exclusive model [11] is an ad hoc extension of Eq. 19

$$u_{Chou_{nex}} = u_{max} \frac{(m_a + m_b + m_a m_b)^\gamma}{1 + (m_a + m_b + m_a m_b)^\gamma} \quad 20$$

Although it has been criticized by several authors [1, 5, 33] because of its questionable validity, it is one of the most often used models in the literature [34]. For $\gamma=1$ it becomes the Bliss IA expression (Eq. 13, with $\alpha=\beta=1$ and $u_{max}=a_{max}=b_{max}$). Chou's models are related to Greco's expression for identical slopes $\gamma_a=\gamma_b$ and identical u_{max} : i.e., $u_{Greco} = u_{Chou_{ex}}$ for $\alpha=0$ and $u_{Greco} = u_{Chou_{nex}}$ for $\alpha=1$.

Minto [30] proposed a model that solved the problem of the different denominators in Eq. 15 by expanding u_{max} and γ in polynomials in a parameter Θ . Fidler [28] extended Minto's approach by adding an interaction term. Their model is

$$\begin{aligned} u(\Theta_p) &= \frac{u_{max}(\Theta_p) [m_a + m_b + \alpha \times f \times \sqrt{m_a m_b}]^{\gamma(\Theta_p)}}{1 + [m_a + m_b + \alpha \times f \times \sqrt{m_a m_b}]^{\gamma(\Theta_p)}} \\ \theta_p &= \frac{m_a}{m_a + m_b} \end{aligned} \quad 21$$

where α indicates the type of interaction. Minto's model corresponds to $\alpha=0$, $\alpha>0$ means synergism and $\alpha<0$ antagonism. $f(s, w, \Theta_p)$ resembles a generalized Γ -distribution, and $u_{max}(\Theta_p)$ and $\gamma(\Theta_p)$ are functions of the potency fraction Θ_p and $f(s, w, \Theta_p)$. Θ_p ranges from 0 (drug A only) to 1 (drug B only).

Minto's model differs from the logistic Hill surface only in the functional forms of u_{max} and γ . By truncating their polynomial ansatz for u_{max} and γ after the linear terms in Θ_p , we have

$$\begin{aligned} \gamma(\Theta_p) &= \alpha\Theta_p + \beta(1 - \Theta_p) = \gamma_{Hill} \\ u_{max}(\Theta_p) &= a_{max}\Theta_p + b_{max}(1 - \Theta_p) = u_{max}(Hill) \end{aligned}$$

Thus by making $\gamma(\Theta_p)$ and $u_{max}(\Theta_p)$ symmetric with respect to m_a and m_b , u_{Minto} becomes identical to u_{Hill} . However, inclusion of higher powers of Θ_p leads to violations of the boundary conditions and the sham compliance requirement.

Examples

As identical theoretical concepts handling mixture effects have been developed by scientists from different disciplines, there is some confusion in the nomenclature used in the literature. We shall use the term 'Bliss independent action' in the subsequent discussions when referring to the probabilistic independent action ansatz, the exception being examples from agrochemistry, where we use the term 'Colby's formula'. This is justified because of its extensive use in the agrochemical literature.

In general, however, one has to keep in mind that a numerical evaluation of a model by comparison with experimental data is difficult because often the error bars of the experiments are large or unknown.

Crop protection agents

To demonstrate the applicability of the Hill model old mixture data from the crop protection area were chosen. All pesticides involved achieve an u_{max} of 100%. For the pairs of atrazine/alachlor [12] (herbicides), aldrin/dieldrin [35] (insecticides), and oxadixyl/mancozeb [36] (fungicides) the root mean square errors of prediction are shown in Table 2. The mixtures are equally well described by using variable slopes or slopes of 1. For all pairs of pesticides the original conclusions are confirmed: neither the herbicide- nor the insecticide-mixtures show deviations from the 'expected' response, whereas in the case of the fungicide mixtures the differences between expectation and experiment indicate the presence of synergism.

Table 2

Dependence of agrochemical mixture effect predictions on Hill parameters

Ligand	Slope $\gamma=1$		Slope γ variable	
	X_{50}^a	RMSE ^b	X_{50}^a	Slope RMSE ^b
Atrazine	0.057		0.059	0.96
Alachlor	0.082		0.076	1.45
Atrazine + Alachlor		9		9
Aldrin	0.008		0.010	2.20
Dieldrin	0.004		0.005	1.95
Aldrin + Dieldrin		10		8
Oxadixyl	18		19	1.37
Mancozeb	294		293	0.99
Oxadixyl + Mancozeb		25		26

All pesticides are assumed to achieve individual maximum effects of 100%. The magnitude of the deviations of mixture predictions from the experimental data is an indication of synergistic effects

^a LC_{50} (in kg/ha) for herbicides, LD_{50} (in g a.i./100 ml kerosene solution) for insecticides, EC_{50} (in mg/l) for fungicides;

^b root-mean-square error of efficacy prediction for mixture

Pyrethroids

Mixtures of pyrethroids can act both as synergists and antagonists. While permethrin/etofenprox and permethrin/cypermethrin show antagonism, cypermethrin/etofenprox act synergistically. These effects are only insufficiently described by percentages of 41, 58 and 332% [37] measuring the deviation from additivity. The full picture is given in Fig. 1, where Hill-, Bliss- and experimental dose-responses curves

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are overlaid with the Hill response surfaces. In this example the differences between the theoretical models are extraordinarily large. The contour plots of $u_{Hill} - u_{Colby}$ reveal that especially for $x \leq 50$ and $y \leq 50$ Colby's independent action formula predicts much lower effects than the Hill model. As compared with the latter, the IA approach underestimates antagonism and overestimates synergism. These large differences are caused by the extremely steep dose response curves of permethrin ($\lambda=25$), etofenprox ($\lambda=11$) and cypermethrin ($\lambda=106$), meaning that the effects vary strongly within very narrow dose-ranges.

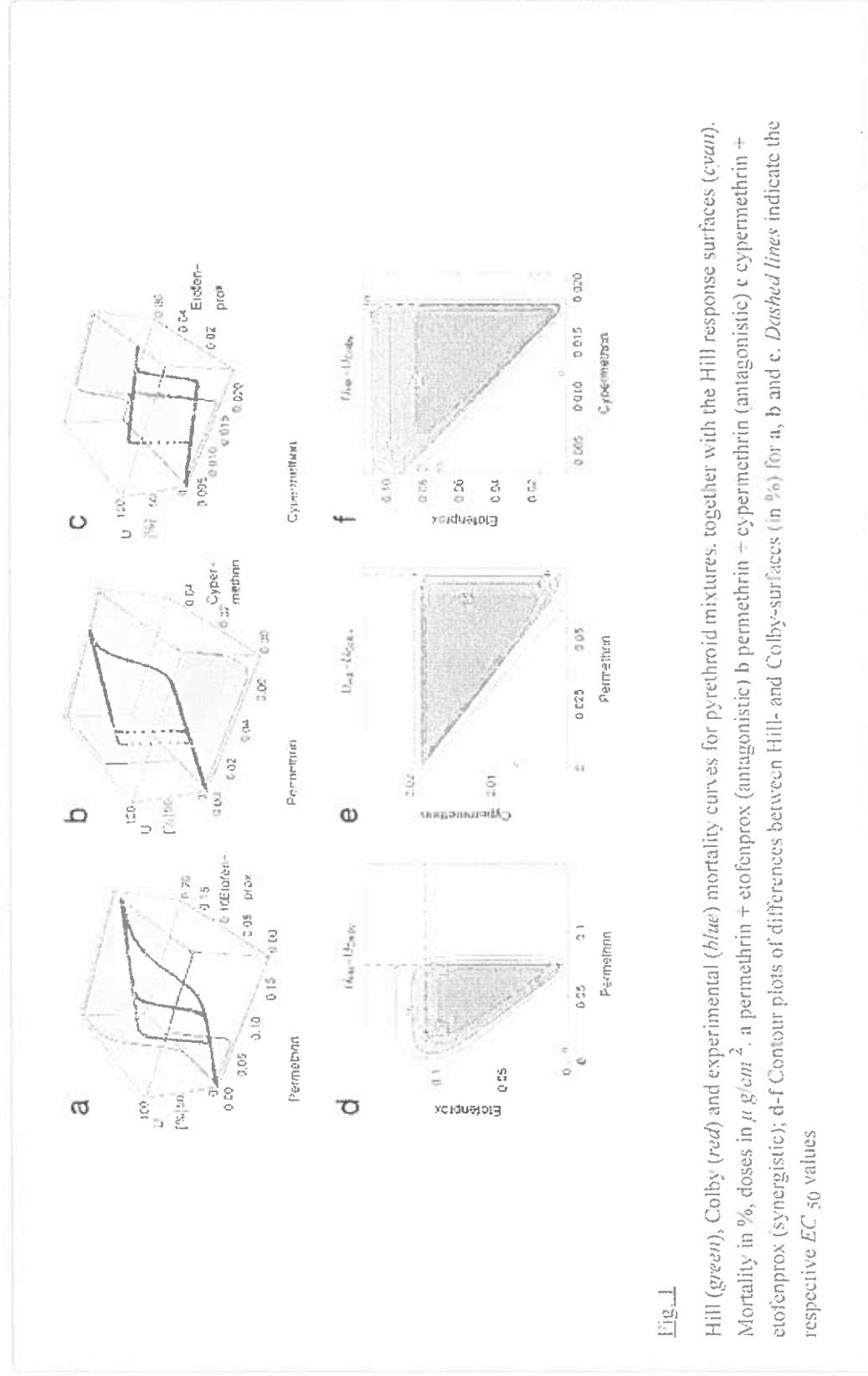


Fig. 1

Hill (green), Colby (red) and experimental (blue) mortality curves for pyrethroid mixtures, together with the Hill response surfaces (cyan). Mortality in %, doses in $\mu g/cm^2$. a permethrin + etofenprox (antagonistic) b permethrin + cypermethrin (antagonistic) c cypermethrin + etofenprox (synergistic); d-f Contour plots of differences between Hill- and Colby-surfaces (in %) for a, b and c. Dashed lines indicate the respective EC_{50} values

Dioxin-like chemicals

Aryl hydrogen receptor (AhR) ligands were used to compare the toxic equivalence factor (TEF) approach and the more general GCA ansatz by [38] in predicting the expected effect of mixtures containing partial agonists or competitive antagonists. From their supplementary material the Hill curves with variable γ were derived and used to predict α_{Hill} . GCA surfaces are Hill-surfaces with slopes of $\gamma=1$. Hence, one might expect that Hill-surfaces with variable γ exhibit slightly improved Mann-Whitney (MW) statistics. However, as the slope parameters from the fits are only marginally different from 1 (c.f. Table 2), the differences between the GCA and Hill in the E_{max} , EC_{50} -values and consequently in their response surfaces (not shown) are much smaller than the error bars of the experimental data, while the surface predictions are of comparable quality.

Table 3

AhR agonist parameters from GCA- and Hill-fits for mixture predictions

Ligand	GCA, $\gamma=1$ [38]		Hill, γ variable			
	$E_{max}(\%)$	$EC_{50}(M)$	MW ^a	$E_{max}(\%)$	$EC_{50}(M)$	γ MW ^a
TCDF ^b	100	2.9×10^{-11}		100	3.2×10^{-11}	0.88
PCB126 ^c	99	4.1×10^{-10}		100	4.5×10^{-10}	0.82
TCDF + PCB126			0.86			0.86
TCDD ^d	100	7.6×10^{-12}		100	6.3×10^{-12}	1.29
PCB105	61	1.4×10^{-6}		56	9.2×10^{-7}	1.45
TCDD + PCB105			0.63			0.78
TCDD	100	9.9×10^{-12}		100	8.5×10^{-12}	1.09
Galangin	30	4.1×10^{-6}		35	4.7×10^{-6}	0.79
TCDD + Galangin			0.79			0.93
TCDD	100	9.1×10^{-12}		100	6.5×10^{-12}	1.22
DIM ^e	8	6.6×10^{-6}		10	8.5×10^{-6}	1.62
TCDD + DIM			0.65			0.44

Parameters are slopes γ , maximum effects E_{max} and EC_{50} values of the agents

^aMW = Mann-Whitney test for mixture prediction;

^b2,3,7,8-tetrachlorodibenzofuran;

^c2,3,3',4,4'-pentachlorobiphenyl;

^d2,3,7,8-tetrachlorodibenz-p-dioxin;

^e3,3'-diindolylmethane

Response surfaces and isoboles

As the classification of mixture effects in terms of interaction indexes is not an adequate means to describe this complex phenomenon, an analysis of responses by looking at the form of iso-effect levels, isoboles, is certainly more appropriate [10, 14, 16]. Deviations from straight lines were used to classify the effects as (Loewe and Bliss) agonistic or antagonistic. From a response surface view [3, 33, 39] isoboles are cuts of the response surface at defined effect levels and the corresponding contour plots are the easiest way to get a full picture for the whole range of dose-combinations. This facilitates the understanding of the fact that linear isoboles are the exception and not the rule and are not a general means of detecting synergism. For critical discussions on the interpretation of the shape of isoboles see, e.g., [2, 40–42].

Here simulated dose-response surfaces are compared, based on two different drugs A and B, both acting according to Hill's formula. The following parameters were used: Maximum effects $a_{max}=0.7$, $b_{max}=1$, median effects $d_{a50}=100$, $d_{b50}=1$, (i.e., $x_{50}=2$, $y_{50}=0$), and slopes $\alpha=1$, $\beta=2$.

The comparison will be mainly between u_{Hill} and $u_{Bliss}=u_{Colby}$, as the GCA model u_{GCA} and Chou's mutually exclusive model $u_{Chou_{ex}}$ are special cases of the logistic surface u_{Hill} , and under special assumptions Chou's mutually non-exclusive model $u_{Chou_{nex}}$ reduces to Bliss IA.

In Fig. 2 differences between the two surfaces show up at high doses of both mixture partners. There IA would find large synergistic effects of up to 30% where the Hill model would indicate 'null-interaction'.

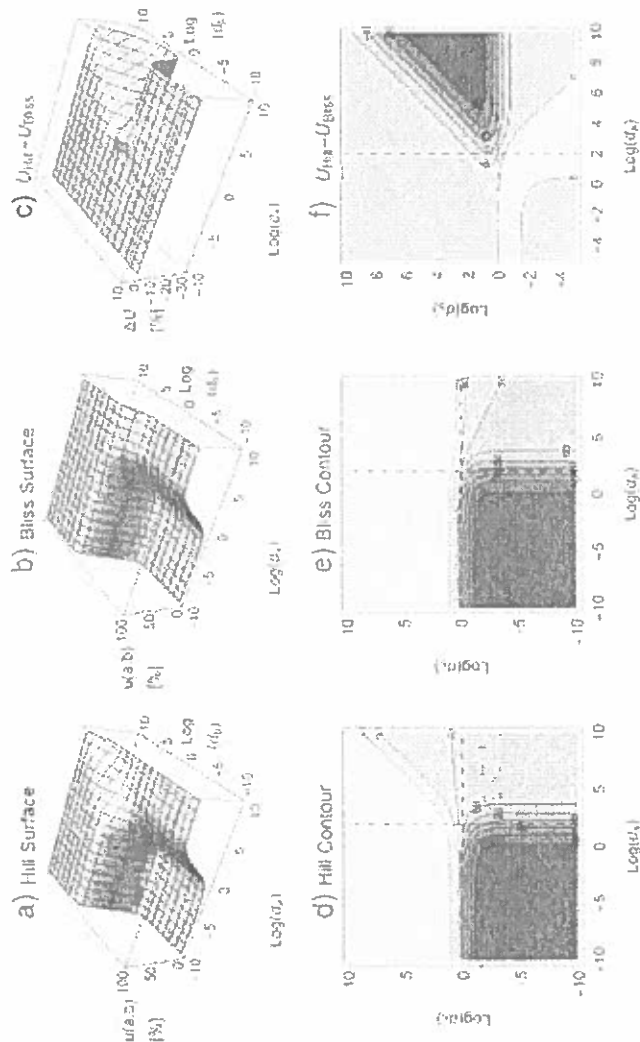


Fig. 2

Response surfaces (a–c) and isoboles (d–f) for binary mixtures. Effects are given in %. a, d Hill surface; b, e Bliss surface; c, f Difference plots $u_{Hill} - u_{Bliss}$ show that $u_{Bliss} \geq u_{Hill}$. (Parameters: $u_{min} = 0$, $u_{max} = 0.7$, $d_{d50} = 1.0$, $d_{b50} = 1$, $\beta = 2$). Dashed lines denote the respective d_{50} values

Some trends can be observed: For identical maximum effects and slopes, $u_{Hill} - u_{Bliss}$ is positive at doses below d_{50} and increases with increasing u_{max} and λ , and it is negative else, almost independent of the dose range. This means that Bliss independent action tends to overestimate synergism at doses smaller than their median effect doses and to underestimate synergism at doses above. At low doses the size of the difference increases with u_{max} and λ . If the maximum effects differ, the effect differences at higher doses increase with increasing $a_{max} - b_{max}$. Some of these findings are illustrated in Fig. 3 for binary mixtures of agents with identical parameters: maximum effects ($u_{min} = 1$ and $u_{max} = 0.4$) were combined with three different slope parameters ($\lambda = 0.5, 1.0, 5.0$). Using scaled doses $m = d/d_{50}$ for the axes simplifies the picture without obscuring the essentials.

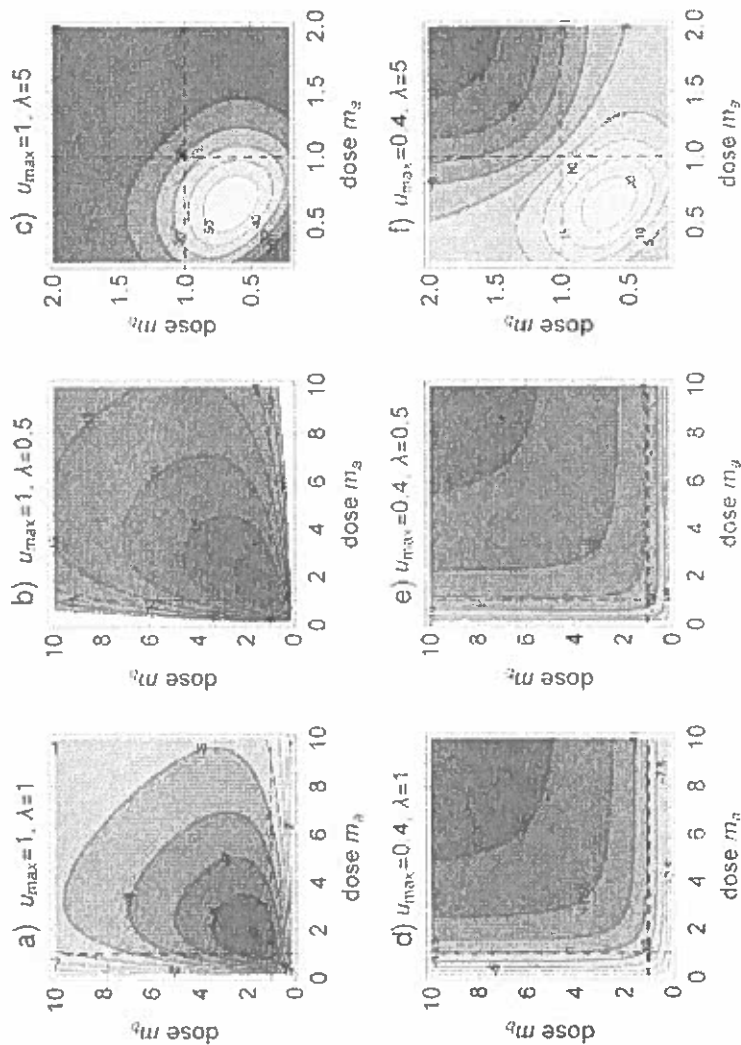


Fig. 3

Contour plots of differences $u_{\text{Hill}} - u_{\text{Bliss}}$ between Hill- and Bliss-response surfaces (in %). Shown for three slope parameters $\lambda=1, 0.5$ and 0.4 for agents A and B. a – c $u_{\max}=1$, d – f $u_{\max}=0.4$. Dashed lines indicate effects at normalized doses $m_1 = m_2 = 1$

Iso-surfaces

For ternary mixtures the pendants of isoboles are iso-surfaces, i.e., projections of the four-dimensional response surfaces to three-dimensional ones defined by triples of doses leading to a specified response. A visualization of effects resulting from more than three agents in one graphical object is hardly possible. As shown for some fictitious ternary mixtures in Fig. 4, planarity is achieved only if all mixture

components have identical slope parameters. In this case different maximum effects of the mixture partners do not affect planarity. Iso-surfaces of mixtures of agents having different slopes are non-planar.

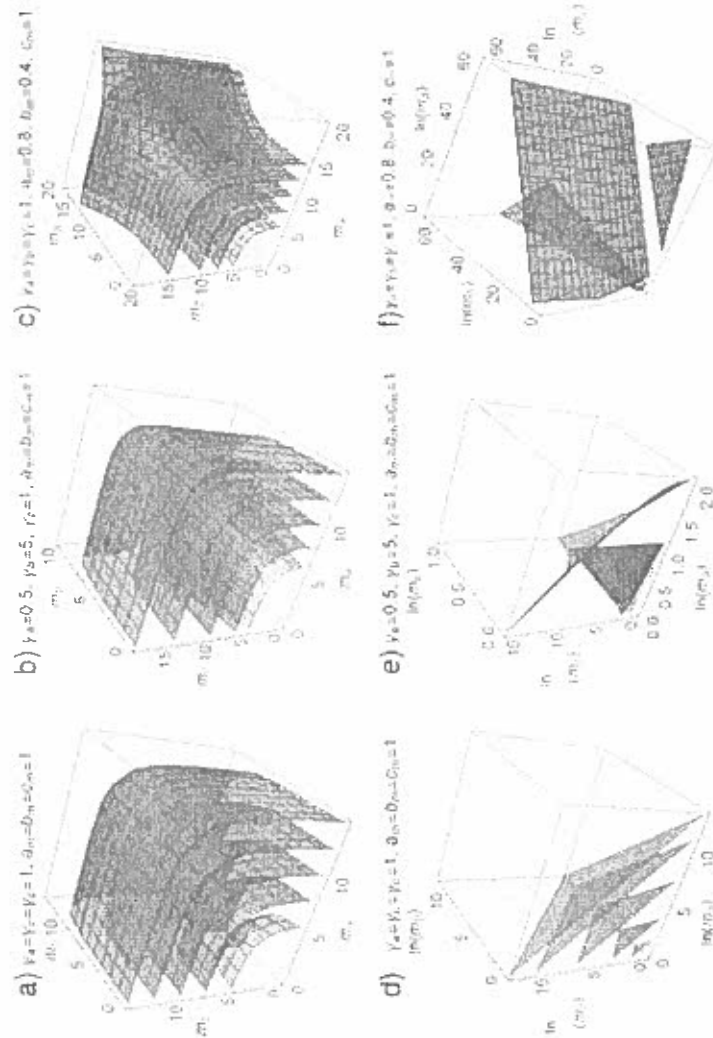


Fig. 1

Iso-surfaces for ternary mixtures at 10, 25, 50, 75 and 90% effect levels. Shown on logarithmic (a–c) and linear (d–f) dose scales. a, d sham combination, $\alpha=1$, $\mu_{max}=1$; b, e Mixture with variable slopes, $\alpha=0.5$, $\beta=5$, $\gamma=1$ and $\mu_{max}=1$; c, f Mixture with variable maximum effects, $\alpha_{max}=0.8$, $\beta_{max}=0.4$, $\gamma_{max}=0.1$ and slope $k=1$

This is shown for ternary anesthetic mixtures [43] as analyzed in great detail by [28] and [30]. For the present purpose the data for midazolam, propofol, and alfentanil were fitted to Hill dose-response curves assuming effect ranges from 0 (no hypnosis) to 1 (full hypnosis). The characteristics of fits and of predictions for the mixtures are summarized in Table 4. The corresponding iso-surfaces in Fig. 2 show slight deviations from planarity which result from different slopes (4.8–11.1) of the pure agents. As the Hill model provides only null-interaction surfaces, the size of the deviations of experimental data from the Hill prediction may give some hints on the presence of synergy. While the RMSEs of fit for the pure compounds are of the order of 5%, those for mixture prediction are of the order of 40%, indicating the presence synergistic effects of increasing magnitude for the binary mixtures of propofol/alfentanil, midazolam/propofol and midazolam/alfentanil, while the ternary mixture is comparable to the best binary one.

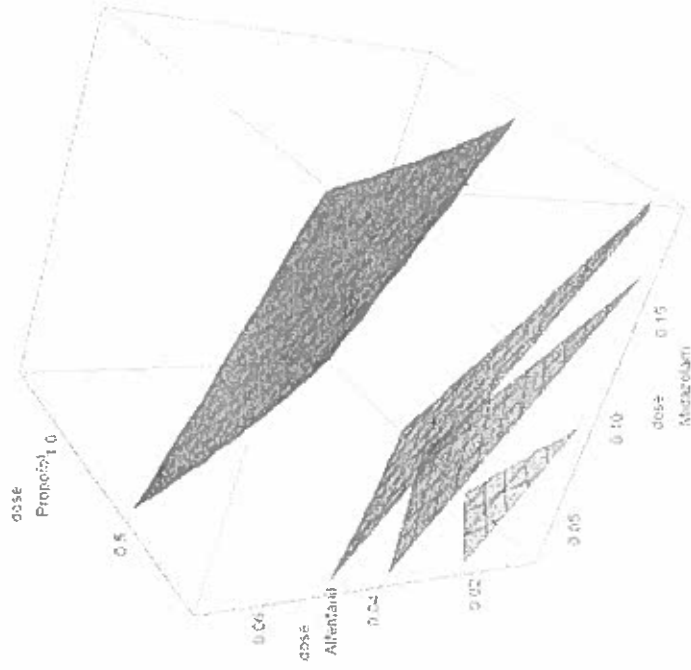


Fig. 5

Iso-surfaces (10, 50, 75, and 99%) for ternary mixtures of midazolam, propofol, and alfentanil. Doses in [mg.kg]. Deviation from planarity results from differing slopes of the individual anesthetics

Table 4

Fit parameters for anesthetics and their mixtures

Drug	$D_{50}(mg/kg)$	Slope	RMSE ^a
Midazolam	0.144	4.83	0.054
Propofol	1.078	11.14	0.035
Alfentanil	0.093	5.69	0.069
Midazolam/propofol			0.387
Midazolam/alfentanil			0.477
Propofol/alfentanil			0.228
Ternary mixture			0.461

^aRMSE = root-mean-square error with respect to expl. data. For pure compounds RMSE refers to fits, for mixtures it refers to predictions

Conclusions

Starting from a logistic PDE, analytical expressions for the response surfaces of n-component mixtures have been derived under the sole provision that each a.i. is described by a sigmoid dose-response curve. No further assumptions are required. The resulting 'null-interaction' surfaces, i.e., Hill-surfaces in the absence of synergistic or antagonistic effects, provide the 'expected' response for each dose combination. Deviations from this reference response in order to quantify synergism or antagonism should possibly be handled by some sort of perturbation theory.

The Hill approach provides a framework to classify several models describing mixtures like ADM (Loewe CA, GCA), MSM (Colby, Bliss, Finney, Abbott) or Chou's 'unified general theory' and various response surface models. It can be applied to mixtures of compounds having different maximum effects and differing slope-parameters. Many Loewe-additivity based approaches are found to be special cases of the Hill surface while Bliss IA is incompatible with the logistic ansatz.

The independent action model is frequently used, e.g., in patent applications to quantify synergistic effects [44]. Its outcome should be checked by comparison with the Hill response surface for any relevant mixture ratio. From the literature examples discussed two scenarios can be distinguished: For slopes of $\gamma \approx 1$ the response surfaces calculated from MSM formulae, deviate from the Hill surface by predicting synergism where the Hill model indicates 'null-interaction', especially at doses below the d_{50} -values of the components. In fact, under these conditions synergism postulated by MSM is an upper boundary for the synergism predicted by the Hill surface approach. However, if both mixture partners alone can cause effects of 100% or if the differences between their slopes are small either the deviations or the dose ranges affected are small.

For $\gamma \gg 1$ the differences between Hill- and MSM-surfaces become extremely large, but they are restricted to those small dose-ranges where the effects change rapidly.

As a consequence for claiming synergistic effects at doses below the respective d_{50} -values, special caution is required whenever the predictions from MSM and Hill approaches differ considerably.

An in-depth analysis of real mixtures would have to take into account not only errors in the models but also other sources of errors like the error statistics of experimental data.

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Abbreviations

ADM	Additive dose model
AhR	Aryl hydrogen receptor
CA	Concentration addition
EC_{50}	Effective concentration 50
GCA	Generalized Concentration addition
IA	Independent action
LC_{50}	Lethal concentration 50
LD_{50}	Lethal dose 50
MSM	Multiplicative survival model
MW	nonparametric Mann-Whitney U test
NOEL	No-effect level
ODE	Ordinary differential equation
PDE	Patial differential equation
RMSE	Root-mean-square error
TEF	Toxic equivalence factor
u_{Bliss}	Bliss independent action response
$u_{Chou_{nex}}$	Chou's mutually non-exclusive response
$u_{Chou_{ex}}$	Chou's mutually exclusive response
u_{Colby}	Bliss independent action response
u_{Greco}	Loewe additivity based response

$u_{GC,4}$ Generalized concentration addition response

u_{Hill} Hill response surface

$u(\mathcal{C}_p)$ Minto's and Fidler's polynomial response

Notes

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that he has no competing interests.

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Research article

Sensitivity of *Phakopsora pachyrhizi* towards Qo inhibitors and demethylation inhibitors, and corresponding resistance mechanisms[†]

Running title: Sensitivity and resistance mechanisms of *P. pachyrhizi* towards fungicides

Helena K Schmitz, Carlos-Antonio Medeiros, Ian R Craig and Gerd Stammler*

Address: BASF SE, Speyerer Strasse 2, 67117 Limburgerhof, Germany

*Corresponding author:

Gerd Stammler

BASF SE, Speyerer Strasse 2, 67117 Limburgerhof, Germany

gerd.stammler@basf.com

Phone: ++49 621 60 27299

Fax: ++49 621 60 27176

Keywords

demethylation-inhibitors; quinone-outside-inhibitors; overexpression; point mutation; sterol 14 α -demethylase; cytochrome *b*

Abstract

BACKGROUND: Since the invasion of *Phakopsora pachyrhizi* in Brazil, detrimental yield losses in soybeans have occurred. Disease management is mainly based on fungicide treatment with demethylation-inhibitors (DMIs) and quinone-outside-inhibitors (QoI) playing an essential role. The sensitivity of single *P. pachyrhizi* isolates towards different DMIs and QoIs was surveyed and the corresponding resistance mechanisms analyzed.

RESULTS: The QoI-response remained stable, while a loss of sensitivity towards DMIs occurred. Molecular analyses of *cytochrome b*, the target gene of QoIs, showed an intron after codon 143 which is reported to prevent the development of a G143A mutation. Analysis of *cyp51*, the target gene of DMIs, revealed that point mutations and overexpression are involved. Of the detected mutations, Y131F and Y131H, respectively, and K142R are likely homologous to mutations found in other pathogens. As suggested by modelling studies, these three mutations as well as additional mutations F120L, I145F and I475T correlate to increased ED₅₀-values towards all tested DMIs. Furthermore, an up-regulation of the *cyp51*-gene up to 10-fold was noticed in some of the DMI-adapted isolates, while all sensitive isolates responded as the wild type.

CONCLUSION: The G143A mutation is thought to result in significant resistance factors towards QoIs, while other mutations play only a minor role. Since G143A development is

43 prevented in *P. pachyhrizi*, a stable control of with Qols in future is rather likely. In
44 contrast, a shifting in sensitivity towards DMIs has been observed, which is due to
45 multiple independent mechanisms.

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1. INTRODUCTION

Soybean, *Glycine max* (L.) Merr., is one of the most important crops worldwide ranking only behind wheat, corn and rice in terms of total area under cultivation (FAOSTAT (<http://faostat.fao.org>)). Soybeans are not only used for human consumption but, more importantly, for livestock feeding and industrial purposes (ASA (<http://www.soystats.com>)). The USA is the most important producer of soybeans (35% of the world production), followed by Brazil (26%) and Argentina (20%) (FAOSTAT (<http://faostat.fao.org>)). In Brazil, soybeans account for 36% of cultivated area and are the most dominant crop being cultivated, followed by corn (20% of cultivated area) and sugarcane (14%; FAOSTAT (<http://faostat.fao.org>)).

Soybean yield is reduced by a number of biotical and abiotical factors. Diseases that can threat soybeans are *Cercospora kikuchii* (leaf bronzing and purple seed stain), *Cercospora sojina* (frog-eye leaf spot), *Corynespora cassiicola* (target spot), *Diaporthe phaseolorum* var. *sojae* (pod and stem blight), *Peronospora manshurica* (downy mildew), *Phakopsora pachyrhizi* (Asian soybean rust), *Phytophthora sojae* (phytophthora rot), *Pseudomonas syringae* pv. *glycinea* (bacterial blight), *Rhizoctonia solani* (aerial blight), *Sclerotinia sclerotiorum* (sclerotinia stem rot) or *Septoria glycines* (brown spot) (Kucharek, 2001; Ruhl, 2007; Carmona *et al.*, 2011; Hartman *et al.*, 2011). *P. pachyrhizi* originated in Asia leading to vast yield losses (Ono *et al.* 1992). The phytopathogen was first appeared in Brazil in 2001, leading to additional costs of US\$ 2 billion (Yorinori *et al.*, 2005). Cultivation practices such as late sowing, choice of soybean cultivar or elimination of secondary hosts have been applied to reduce the severity of the disease (Kawuki *et al.*, 2003; Corrêa da Silva *et al.*, 2011; Twizeyimana *et al.*, 2011; Hu and Wiatrak, 2012). Other attempts have included breeding for *P. pachyrhizi* resistance and

the introduction of a “free host period”, in which no soybeans are cultivated for 3 months (Hartman *et al.*, 2005; Kendrick *et al.* 2011; Godoy, 2009). However, the application of fungicides is the most employed and most efficient method for controlling *P. pachyrhizi*, and in particular demethylation inhibitors (DMIs) and Qo inhibitors (QoI) have provided the best and most consistent results (Ivancovich, 2005). Other phytopathogens developed adaptation against both DMIs and QoIs, which resulted in reduced efficiency. The most important resistance mechanism against QoIs are point mutations (F129L, G137R, G143A) within the *cytochrome b* gene, which have been found in *Alternaria* spp., *Blumeria graminis*, *Mycosphaerella* spp., *Plasmopara viticola*, *Pyrenophora* spp., *Pyricularia grisea* or *Venturia inaequalis* among others (Heaney *et al.*, 2000; Kim *et al.*, 2003; Grasso *et al.*, 2006; Sierotzki *et al.*, 2007). Resistance mechanisms against DMIs are variable and complex, involving point mutations, overexpression of the target gene *cyp51*, and up-regulation of efflux-transporter. A number of plant pathogens show a more or less significant adaptation to DMIs, such as *B. graminis*, *Mycosphaerella graminicola*, *Puccinia triticens* or *Uncinula necator* (Cools *et al.*, 2012; Délye *et al.*, 1997; Délye *et al.*, 1998; Leroux *et al.*, 2007; Stammler *et al.*, 2008; Stammler *et al.*, 2009; Leroux and Walker, 2010).

In this study, the sensitivity of *P. pachyrhizi* isolates towards DMIs and QoIs was analyzed by the detached leaf test. Subsequently, *cyp51* and *cytochrome b*, which are the respective target enzymes of DMIs and QoIs, were investigated using molecular genetic techniques.

2. MATERIAL AND METHODS

2.1 Origin of *P. pachyrhizi* isolates

P. pachyrhizi strains were isolated from soybean leaf samples from different regions of Brazil in 2009/2010. Dry infected leaves were hydrated in a humid chamber for ca. 20-24 hours. Spores from sporulating uredia were then either transferred to a Tween water (0.03%) drop on a six-days-old soybean leaf (BBCH scale 101; Munger et al., 1997) via inoculation needle or were suspended by shaking hydrated leaves in Tween water. Following this, suspensions were applied with an airbrush (0.3 mm nozzle) to the abaxial surfaces of unifoliate leaves, which had been placed on water agar (0.4%), which included 1H-benzimidazole (40 mg/l) and streptomycin sulphate (30 mg/l). Isolates were separated by transferring spores from only one uredium to a new soybean leaf via the inoculation needle. After inoculation, soybean leaves were incubated in a humid chamber in the dark for 20-24 h followed by 12 h light at 20°C. A total of 90 strains were isolated in this way and were transferred in three week intervals. *P. pachyrhizi* isolates were propagated for detached leaf tests in the greenhouse.

2.2 Detached leaf tests

Sensitivities of Brazilian *P. pachyrhizi* isolates (n = 38) and one strain originating from Taiwan were tested against different fungicides in detached leaf tests comparing to a reference strain isolated in 2004. Dilution series of cyproconazole (Alto®), epoxiconazole (Opus®), metconazole (Caramba ®) tebuconazole (Folicur®) azoxystrobin (Amistar ®) (all 0 / 0.01 / 0.1 / 1 / 10 mg L⁻¹) and pyraclostrobin (Comet ®) (0 / 0.03 / 0.3 / 3 / 30 mg L⁻¹) were applied on soybean plants (cultivar Dekalb 22-52) at growth stage 101 (BBCH scale) just before run off. Treated plants were placed in a growing chamber for drying at 22°C overnight, before leaves were harvested. The next day, leaves were cut and placed on water agar (0.4%), which included 1H-

benzimidazole (40 mg/l) and streptomycin sulphate (30 mg/l). Spore suspensions of *P. pachyrhizi* isolates in Tween water (0.03%) were prepared and applied with an airbrush (0.3 mm nozzle) to the abaxial surfaces of the unifoliate leaves. From each fungicide and concentration two soybean leaves were inoculated (approximately 0.5 ml spore suspension per leaf). Subsequently, plates were incubated in a humid chamber in the dark for 20-24 h followed by 12 h light at 20°C. Three weeks after inoculation, infected leaf area was evaluated by visual scoring, assessing thinly dispersed pustules in combination with total necrosis of the leaf as a disease severity of 100%. The effective doses of 50% (ED₅₀-value) were calculated.

2.3 Nucleic acid extraction and reverse transcription

DNA of *P. pachyrhizi* spores was extracted with the NucleoSpin DNA Plant II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the instructions of the manufacturer for SDS based DNA extraction.

Total RNA of *P. pachyrhizi* spores was extracted using the Spectrum Plant Total RNA Kit and on-column DNase digestion was performed using On-Column DNase I Digest Set (both Sigma Aldrich, St. Louis, USA) following the instructions of the manufacturer. RNA of each isolate was extracted at least twice at subsequent dates. RNA concentrations were measured on a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, USA) and RNA quality was assessed employing the Agilent RNA 6000 Nano Kit on the Agilent 2100 Expert Bioanalyzer (both Agilent Technologies, Santa Clara, USA) using the Plant RNA Nano Assay as described by the manufacturer. The RIN (RNA Integrity Number) obtained in this way varied between 6 and 8. RIN values above 5 are recommended for expression analysis (Feige und Pfaffl, 2006).

Each RNA (500 ng) was reverse transcribed to cDNA using the oligo-dT primer of the Verso cDNA Kit (Thermo Scientific Inc, Waltham, USA) as described by the manufacturers. Reverse transcription conditions were as follows: 42°C for 30 min, 45°C for 15 min, 50°C for 15 min and 95°C for 2 min.

2.4 Sequence analysis

Using Phusion Hot Start, High-Fidelity DNA Polymerase Master Mix (Finnzymes OY, Espoo, Finland) and primer pair KES 1098 fw (5' ATGTCTTCCAGCGTTATAATCG 3') and KES 1133 rv (5' TCGAGGGAAAGGAGTTGATC 3'), respectively, the *cyp51* gene (2155 bp) was amplified (primer end concentration 500nM). The following PCR program was applied for amplification: an initial heating for 15 min at 98°C was followed by 35 cycles at 98°C for 5 s, 63°C for 5 s and 72°C for 45 s, and a final amplification step at 72°C for 1 min. PCR products were gel electrophoretic separated, extracted using NucleoSpin Extract II (Macherey-Nagel GmbH & Co. KG) and cloned in XL-1 Blue Competent Cells (Agilent Technologies) using the CloneJET PCR Cloning Kit (Fermentas GmbH, St. Leon-Rot, Germany) following the instructions of the manufacturers. Clones were sequenced using primers pJet1.2 fw (5' CGACTCACTATAGGGAGAGCGGC 3'), pJet1.2 rv (5' AAGAACATCGATTTTCCATGGCAG 3') and KES 961 (5' TGGGAAGAGGGTATGATGTATTGG 3'). Sequences were analysed using the Lasergene Software package (DNASTAR, Madison, USA).

Cyt b (2598 bp) was cloned in the same manner, using the primer pair KES 1158 fw (5' CAGTAGCCTAAAGAAGGGTGTAA 3') and KES 1159 rv (5' CCCGTTGAATATCTTGACATCTTAC 3') for amplification and pJet1.2 fw, pJet1.2 rv,

169 KES 1175 fw (5' AATTCTACTAGATCCCCT 3') and KES 1176 fw
170 (5' AATTCTACTAGATCCCCT 3') for the sequencing reaction. The PCR program for
171 amplification was: initial heating at 98°C for 15 s, 35 cycles at 98 °C for 10 s, 64°C for
172 5 s. and 72°C for 45 s, followed by a final amplification step at 72°C for 1 min.
173 For point mutation studies of the cyp51 gene, pyrosequencing assays were developed
174 using the Pyrosequencing Assay Design Software (Qiagen, Hilden, Germany). Standard
175 PCR using Maxima Hot Start PCR Master Mix (Fermentas GmbH) and the primer pairs
176 listed in Table 1 were employed for amplification of all gene fragments under the
177 following conditions: initial heating for 15 min at 95°C, 40 cycles at 94°C for 15 s, 55°C
178 for 30 s and 72°C for 20 s, followed by a final amplification step at 72°C for 5 min. Every
179 template was applied in duplicate. Single strand preparation was performed by
180 immobilizing PCR products to Streptavidin Sepharose Beads (GE Healthcare,
181 Buckinghamshire, GB), and subsequently cleaned up with ethanol (70%), denaturated
182 with sodiumhydroxid (0,2M) and finally washed in tris-acetat (10mM) implementing the
183 Vacuum Prep Worktable (Qiagen) following the instructions of the manufacturer. After
184 transferring immobilized single strand samples to annealing buffer amended with the
185 according sequencing primer, the samples were heated at 80°C for 2 min in an
186 incubator. Samples, cooled to room temperature, were then pyrosequenced using
187 PyroMark Gold Q96 Reagents as described by the manufacturers on a PSQ 96MA (both
188 Qiagen) machine. Therefore, sequencing primers listed in Table 1 were used,
189 depending on the amino acids of interest. For assay validation, standard mixtures were
190 performed. Every run included standards of 100% wild type and mutation, respectively,
191 as well as 30/70%, 50/50% and 70/30% mixtures of wild type and mutation. While cDNA

of 48 isolates was studied for mutations, analysis of DNA was performed for all 90 Isolates.

2.5 Expression analysis

Expression studies were performed on a Rotor-Gene Q 2-Plex machine (Qiagen) and analyzed with the GenEx software (MultiD Analyses, Göteborg, Sweden) following MIQE guidelines (Bustin *et al.*, 2009). Six genes (actin, tubulin, GAPDH, SDHc, SDHd and cyp51) were validated as reference genes with geNorm and NormFinder (GenEx software), finding actin, tubulin and GAPDH to perform most stable (data not shown). QPCR efficacy was assessed by standard curves for all genes and was 0.97 for actin, 0.98 for tubulin, 0.94 for GAPDH and 1.04 for cyp51 (data not shown). All cDNA samples were used in duplicate. Threshold cycles (ct) were adjusted to the same level in each run. For qPCR performance, MESA FAST qPCR MasterMix Plus dTTP for SYBR Assay No ROX (Eurogentec, Seraing, Belgium) was used, together with the primer (200nM) listed in Table 1. Conditions for qPCR were as follows: initial heating to 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 20 s and a final step at 72°C for 20 s. Subsequently, specificity was verified by a melting curve. The fluorescence level was acquired during the elongation step. In a first approach *cyp51*-expression was measured from *P. pachyrhizi* spores of five DMI adapted isolates treated with epoxiconazole (3 mg L⁻¹) in a detached leaf test. In addition, expression of both DMI-adapted and sensitive isolates was analysed from the untreated control. In a second approach, expression analysis of non-treated spores was performed for 48 isolates. To prevent false positive results, the overexpression limit was set at expression 3-fold higher than the reference strain.

216

217 2.6 Modelling studies

218 A structural model of the CYP51 protein from the *P. pachyrhizi* reference strain was
219 constructed using the homology modelling tool in MOE (Version 2010.1, Chemical
220 Computing Group Inc., Montreal, Canada). The template for this modelling was the
221 crystallographically determined structure of human CYP51 (PDB ID: 3LD6), which has a
222 sequence identity with the fungal CYP51 of 38%. The homology model was built using
223 the software's default parameter settings. The resistant mutations identified in this work
224 were then manually implemented into the three-dimensional structure of the reference
225 strain protein.

226 To investigate the effect of the resistant mutations on DMI binding, the following four
227 fungicide compounds were docked into the homology model: cyproconazole,
228 epoxiconazole, metconazole and tebuconazole. The docking was performed using Glide
229 (Version 5.7, Schrödinger, LLC, New York, NY, 2011) following ligand preparation with
230 LigPrep (Version 2.5, Schrödinger, LLC, New York, NY, 2011). Default parameter
231 settings were used for both steps. No constraints were applied during the docking run,
232 but of the resulting poses only those with an azole-heme binding distance less than 2.8
233 Å were retained.

234

235 3. RESULTS

236 3.1 Sensitivity of *P. pachyrhizi* to QoI and DMI fungicides

237 In detached leaf tests, all isolates were highly controlled by QoI, showing narrow ranges
238 of ED₅₀-values, ranging from 0.14 mg L⁻¹ to 2.47 mg L⁻¹ for azoxystrobin (median:
239 0.41 mg L⁻¹) and 0.43 mg L⁻¹ to 1.53 mg L⁻¹ for pyraclostrobin (median:0.95 mg L⁻¹),

respectively (Fig. 1). The sensitivity of the reference strain was within these ranges, showing ED₅₀-values of 0.24 and 1.11 mg L⁻¹, respectively.

The sensitivity of the different *P. pachyrhizi* strains towards DMIs covered a broader range. While the reference strain was controlled by 0.01 mg L⁻¹ for all DMIs, most Brazilian isolates needed higher concentrations to be completely controlled, depending on the isolate and fungicide. Most isolates were controlled by fungicide concentrations as high as 4 mg L⁻¹ (Fig. 3). The ranges for all DMIs were 0.01 to 10 mg L⁻¹ with medians of 2.2 mg L⁻¹ for cyproconazole and metconazole, 2.3 mg L⁻¹ for epoxiconazole, and 2.5 mg L⁻¹ for tebuconazole. While a number of isolates are not controlled to 50% at the highest fungicide rate tested in this study by cyproconazole, metconazole and tebuconazole, all isolates, with the exception of two, are controlled to 50% at 10 mg L⁻¹ by epoxiconazole (Fig. 3).

3.2 Analysis of the *cytochrome b* gene sequence

Sequencing DNA and cDNA of the *P. pachyrhizi* reference strain revealed that the *cytochrome b* gene sized 2524 bp, containing one intron of 1336 bp. The intron was located directly after the amino acid at position 143 (Fig. 2). Amino acids known to lead to reduced sensitivity against QoI exhibited wild type codons, i.e. F129, G137 and G143. Identity of the pronounced sequence was verified by BLAST search.

3.3 Analysis of the *cyp51* gene sequence

Amplification of *P. pachyrhizi* with KES 1098 fw and KES 1133 rv resulted in a 2155 bp fragment, of which 2107 bp encode the corresponding CYP51 protein. The *cyp51* gene was disrupted by 5 introns of different size (Fig. 2). Sequencing of the reference strain

revealed some variation due to several amino acid substitutions. Clearly, these substitutions did not constitute mutations leading to reduced sensitivity towards DMIs, because the reference strain is highly sensitive. Isolates which showed reduced sensitivity were also sequenced. Amino acid substitutions at positions 120 (F120L), 131 (Y131H / F), 142 (K142R), 145 (I145F) and 475 (I475T) could be linked to increased ED_{50} -values (Fig. 3). Several *cyp51*-clones of sequenced isolates did not only show one identical *cyp51*-haplotype with amino acid substitutions, but also other haplotypes including the wild type, suggesting that more than one gene copy is present in a single isolate (data not shown). With the exception of I145F, all mutations occur in combinations (i.e. Y131H + F120L, Y131F + K142R, Y131F + I475T). Despite this, substitutions are not necessarily coupled within one haplotype since haplotypes with only one substitution can be found. Pyrosequencing never showed 100% mutation in *P. pachyrhizi* DNA but values of approximately 33% or 50%, respectively. The same mutations as in DNA were detected in *P. pachyrhizi* cDNA; however, mutation frequency in cDNA was usually higher than in DNA (Table 2).

The occurrence of I145F is related to ED_{50} -values between 0.3 mg L⁻¹ and 4 mg L⁻¹ for the fungicides used in this study. A threshold of 1.4 and 3 mg L⁻¹ accounts for substitutions of Y131F + I475T and Y131F + K142R, respectively. The widest range of ED_{50} -values is observed in the presence of Y131H + F120L, ranging from 0.06 mg L⁻¹ to 10 mg L⁻¹.

The mutations found in the current study were widely distributed within Brazil (Fig. 5). With exception of K142R, which was located in the central region around Senador Canedo, the mutations were not restricted to specific regions. Combinations of Y131H + F120L and Y131F + I475T appeared as the most frequent mutations, though Y131F +

I475T was only found in sites where more than 10 isolates originated from. Y131H + F120L occurred as the most widely distributed mutation, whereas I145F was the least observed mutation and was mainly located in Santo Antônio de Posse. Isolates with the wild type sequence were present at all sites.

3.4 Expression of *cyp51*

In a first approach, *cyp51* expression was analyzed from *P. pachyrhizi* spores of DMI-sensitive and -adapted isolates treated with 3 mg L⁻¹ of epoxiconazole in a detached leaf test. Expression of *cyp51* using *actin*, α -*tubulin* and *gapdh* as reference genes was 4- to 8-fold increased for adapted isolates, in comparison to the reference isolate (Fig. 4). Though DMI-adapted isolates differ in their expression, three isolates out of five being overexpressed, all sensitive isolates were normally expressed. *Cyp51* expression was increased regardless whether the isolates were treated with epoxiconazole.

In a second approach, *cyp51* expression of all fungicide-tested isolates was studied (Table 2). Expression ranges from 3- to 10-fold increase in comparison to the reference strain. Overexpression was found in isolates with wild type *cyp51* gene as well as isolates showing mutations in the *cyp51* gene. A combination of overexpression and mutation correlates with increased ED₅₀-values towards DMIs (Fig. 3).

3.5 Modelling of *cyp51*

The homology model of *P. pachyrhizi* CYP51 was used to identify the locations of the resistance mutations F120L, Y131F/H, K142R, I145F, and I475T in the three-dimensional structure of the protein. The amino acids at positions 120 and 131 are located within the binding site of the enzyme (Fig. 6). Residue K142 makes a salt bridge

with one of the carboxylate groups of the heme, while I145 and I475 are respectively located near enough to the edge and the lower face of the heme to make non-directional van-der-Waals interactions (Fig. 7).

The docking of four DMIs (cyproconazole, epoxiconazole, metconazole and tebuconazole) suggests that F120 and Y131 are in van-der-Waals contact with these inhibitors, with minimum heavy-atom to heavy-atom distances between 3.6 Å and 4.1 Å. Amino-acids K142, I145 and I475 are not in contact with the DMIs considered here.

4. DISCUSSION

As observed by other groups (Miles *et al.*, 2007; Mueller *et al.*, 2007; Scherm *et al.*, 2009), no loss of QoI sensitivity was detected in our study. Results obtained by detached leaf test were confirmed by molecular biological analyses. The *cytochrome b* gene was sequenced, demonstrating that an intron of 1336 bp occurred just after codon 143 of the 2524 bp nucleotide sequence. As proposed by Grasso *et al.* (2006), an intron after codon 143 results in deficient splicing of the mRNA, which would be lethal in the case of a G143A mutation. A number of pathogens were analyzed for G143A, but it was never found adjacent to the subsequent intron (Grasso *et al.*, 2006). Since an intron is found in rusts directly after codon 143, the G143A mutation has never been found (Grasso *et al.*, 2006). Nevertheless, there is a possibility that other mutations may evolve such as F129L or G137R as found in other plant pathogens (Kim *et al.*, 2003; Sierotzki *et al.*, 2007). While the G137R mutation is responsible for reduction in fitness, both F129L and G137R mutations can be controlled by field application rates (Kim *et al.*, 2003; Semar *et al.*, 2007; Sierotzki *et al.*, 2007). Thus, should F129L or G137R develop in *P. pachyhrizi*, the impact of these mutations is suggested to be low. However, neither

of these mutations have been detected in monitoring studies in any plant pathogenic rust species so far.

For the DMIs used in this study, a broad range of ED₅₀-values between the reference strain and the Brazilian isolates have been detected, with resistance factors up to 1000. Sensitivity monitoring in Brazil revealed a lower range of ED₅₀-values than we observed, since a slightly different methodology was applied following FRAC recommendations with populations of *P. pachyrhizi* (FRAC (<http://www.frac.info>); Godoy, 2009). Due to a mixture of sensitive and resistant strains, fungicides show improved performance in fungi populations than in single isolates. Single strains represent both best and worst cases for managing pathogens in the field. Detached leaf tests are appropriate for discriminating the sensitivity of different isolates towards DMIs. However, the growth conditions of the isolate are optimized in detached leaf tests and are different to conditions in the field. Natural defense mechanisms such as hypersensitive response (HR) and systemic acquired resistance (SAR) of the plant against the pathogen might be missing or significantly reduced in a detached leaf. Furthermore, the mechanical barrier of detached leaves might be reduced due to a poor cuticle, which makes it easier for the pathogen to penetrate and infect. Thus, the pronounced ED₅₀-values and resistance factors in this study may differ to a certain extent in the field. Nevertheless, the recent study emphasizes that a significant sensitivity loss of *P. pachyrhizi* towards DMIs developed. Other studies have analyzed disease severity and yield rather than ED₅₀-values to assess the efficiency of fungicide treatments (compounds, quantity, timing) in the field (Miles *et al.*, 2007; Mueller *et al.*, 2009; Scherm *et al.*, 2009). DMI- and QoI-fungicides, or a combination of these, performed the best resulting in adequate control of *P. pachyrhizi*.

360 The *P. pachyrhizi* reference strain revealed several amino acid substitutions from which
 361 some were located within introns. These variations were also found in Brazilian isolates
 362 and the Taiwan strain. Since there was a difference in sensitivity of most Brazilian
 363 isolates and the reference strain in detached leaf tests, these substitutions do not
 364 account for adaptation towards DMIs. Sequence analysis of sensitive and adapted *P.*
 365 *pachyrhizi* isolates revealed mutations at codons 120, 131, 142, 145 and 475. Since
 366 mutated and non-mutated wild type *cyp51* was found in single isolates it could be
 367 concluded that more than one *cyp51* gene copy exists in the genome of *P. pachyrhizi*.
 368 Multiple *cyp51* copies are also described for other fungal pathogens, e. g. *Aspergillus* sp.
 369 or *Fusarium* sp. (Mellado *et al.*, 2001; Yin *et al.*, 2009). The comparison of the frequency
 370 of mutations in DNA (i. e. in the genome) and cDNA (i. e. the expressed mRNA) showed
 371 that mutated genes were always more expressed than the wild type. When we assume
 372 that mutated *cyp51* is less sensitive to DMI inhibition, the selective (over-) expression of
 373 the mutated *cyp51* allele leads to a synergistic effect on the adaptation to DMIs.
 374 Alignment of *P. pachyrhizi cyp51* indicated that the Y131F mutation is homologous to
 375 Y134F in *P. triticina*, Y136F in *U. necator* and *B. graminis* f. sp. *hordei* or Y137F in *M.*
 376 *graminicola* (Délye *et al.*, 1997, Délye *et al.*, 1998; Leroux *et al.*, 2007; Stammeler *et al.*,
 377 2009). In addition, Y131F in *P. pachyrhizi* is homologous to Y132H in *C. albicans* and
 378 K142R is homologous to K143R/E in *C. albicans* and K147Q in *B. graminis* (Sanglard *et*
 379 *al.*, 1998; Wyand and Brown, 2005; Marichal *et al.*, 1999). To our knowledge, no
 380 homologous mutations in other fungi are reported for *P. pachyrhizi* mutations F120L,
 381 I145F and I475T.
 382 In *B. graminis* f. sp. *hordei*, *M. graminicola* and *P. triticina* it is known that Y136F, Y137F
 383 and Y134F, respectively, confer sensitivity losses to DMIs to a greater or lesser extent

depending on the DMI (Wyand and Brown, 2005; Stammier *et al.*, 2009; Mullins *et al.*, 2011). High resistance factors against triadimenol were associated with the corresponding mutation in *M. graminicola* and *U. necator* (Délye *et al.*, 1997; Leroux *et al.*, 2007).

The binding site of CYP51 is buried in the core of the protein, such that the substrate must pass along an access channel that is formed by movement of an α -helix (Boscott and Grant, 1994). The K147Q mutation in *B. graminis*, which is located on the α -helix, alters the interaction between the protein and the inhibitor, thus preventing the DMI from accessing the binding site of the protein (Wyand und Brown, 2005). Similarly, substitution of Y136F in *B. graminis* and Y137F in *M. graminicola* prevents DMI-binding due to a change in polarity (Mullins *et al.*, 2011). In other organisms mutations corresponding to *P. pachyrhizi* F120L and Y131H/F are located in one of the highly conserved substrate recognition sites, suggesting the same function (Leroux und Walker, 2010). The modelling of *P. pachyrhizi* CYP51 in this study suggests that Y131 and F120 can make non-directional van-der-Waals interactions with DMIs. Since these mutations result in smaller amino-acid side-chains, they may affect DMI binding by increasing the size of this part of the binding site, therefore weakening the van-der-Waals contacts. The mutation Y131F/H also represents a change in polarity that could influence inhibitor binding (Mullins *et al.*, 2011). In contrast, mutations K142R, I145F, and I475T make no direct interaction with the DMIs considered here, but may indirectly affect DMI binding by altering the position of the heme. In particular, the change in volume associated with I475T may enforce a change in protein conformation that is propagated to the heme via the adjacent C474.

Besides mutations within the *cyp51* gene, other mechanisms causing sensitivity changes to DMIs have been reported, such as *cyp51*-overexpression (Hamamoto *et al.*, 2000; Schnabel and Jones, 2001; Luo and Schnabel, 2008; Stammer *et al.*, 2009; Cools *et al.*, 2012). Overexpression of *cyp51* has also been found in isolates of *P. pachyrhizi* in this study. All highly sensitive isolates did not show any overexpression of *cyp51*, while in about half of the strains with lower sensitivity to all DMIs an up-regulation up to 10-fold was noticed. Most of the isolates overexpressing *cyp51* responded to DMI treatment with highest ED₅₀-values; in particular 2 strains (isolates 37 and 72) showed high ED₅₀ values to all 4 DMIs. Interestingly, overexpression was never found in isolates containing the Y131F + I475T haplotype, but in all cases with Y131H + F120L haplotype. Whether this overexpression is connected with the mutation or if these two resistance mechanisms are independent acquired was not fully elucidated in this study. Overexpression mechanisms may include insertions within the promotor region, up-regulation of transcription factors or gene duplication (Hamamoto *et al.*, 2000; Schnabel und Jones, 2001; Dunkel *et al.*, 2008; Marichal *et al.*, 1997).

The sensitivity response of some of the strains might not only be related to alterations of the target site *cyp51* in the form of mutations or overexpression. The sensitivity loss of these isolates might be caused by other additional mechanisms such as enhanced efflux. This has been described for some pathogens as a mechanism for DMI adaptation (Sanglard *et al.*, 1995; Leroux and Walker 2010; Stammer and Semar, 2011).

Taking our data together, it can be stated that the Brazilian population of *P. pachyrhizi* is sensitive to QoI fungicides and that the risk of QoI resistance development in future is rather low. However, the population has been adapted to DMIs and mutations in *cyp51* and overexpression of the mutated allele have been identified as important mechanisms

for the acquired sensitivity loss, though additional and so far unrecognized mechanisms might also play a role in the sensitivity response.

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 563
 564

565 Table 1

566 Oligonucleotide primers used in this study and their purposes.

Name	Sequence 5' – 3'	Purpose
KES 498 fw	GGACACTAGTATGGGCGATTG	Pyrosequencing of <i>cyt b</i> , 124 bp fragment with KES 499 rv
KES 499 rv ^{a, b}	CATGTGAGGCGGTCTCATT	Pyrosequencing of <i>cyt b</i> , 124 bp fragment with KES 498 rv
KES 500 fw	TTGTAATAATAGCGACAGC	Pyrosequencing of <i>cyt b</i> (F129L), sequencing primer
KES 501 fw	TATGGACAGATATCACTATG	Pyrosequencing of <i>cyt b</i> (G143A), sequencing primer
KES 1132 fw	TAGGTTATGTACTACCGTAT	Pyrosequencing of <i>cyt b</i> (G137R), sequencing primer
KES 1496 fw ^{a, b}	GTTTTAACTTTTTGATGGCCTGAA	Pyrosequencing of <i>cyp51</i> (Y131F, Y131H), 101 bp fragment with KES 1493 rv
KES 1493 rv	CTGCTGCATTAGGATCGAGTTG	Pyrosequencing of <i>cyp51</i> (Y131F, Y131H), 101 bp fragment with KES 1496 fw
KES 1494 rv	CGAGTTGGGTACATCR	Pyrosequencing of <i>cyp51</i> (Y131F, Y131H), sequencing primer
KES 1564 fw ^{a, b}	ACCCTTTGTTGATGAGAAGTGTC	Pyrosequencing <i>cyp51</i> (K142R, I145F), 396 bp fragment with KES 1563 rv
KES 1563 rv	ACGTCCTTAGTGACAGATTTTACATTT	Pyrosequencing of <i>cyp51</i> (K142R, I145F), 396 bp fragment with KES 1564 fw
KES 1562 rv	GTAAGGCCAGCCTTG	Pyrosequencing of <i>cyp51</i> (K142R,

		I145F), sequencing primer
KES 1595 fw	AGGAAGCATGGAAATGTGTTTAC	Pyrosequencing of <i>cyp51</i> (F120L), 273 bp fragment with KES 1596 rv
KES 1596 rv ^{a, b}	TCCTTACCAAAGACAGGTGTAGC	Pyrosequencing of <i>cyp51</i> (F120L), 273 bp fragment with KES 1595 fw
KES 1594 fw	GAAGAAGCTTACACCCAT	Pyrosequencing of <i>cyp51</i> (F120L), sequencing primer
KES 1599 fw ^{a, b}	GGTCAATGCCGAAGAAGCTTA	Pyrosequencing of <i>cyp51</i> cDNA, 233 bp fragment KES 1600 fw
KES 1600 rv	CATGGACGTCCTTAGTGACAGAT	Pyrosequencing of <i>cyp51</i> cDNA, 233 bp fragment with KES 1599 fw
KES 1601 rv	TTACCAAAGACAGGTGTAG	Pyrosequencing of <i>cyp51</i> (F120L, cDNA), sequencing primer
KES 1461 fw	ACAGTTTCACCACAACCGCC	Expression analysis reference gene, <i>aktin</i>
KES 1462 rv	TGACCGTCGGGAAGTTCG	Expression analysis reference gene, <i>aktin</i>
KES 1216 fw	TGATAGACTGAGGCGTGAACAGG	Expression analysis, <i>cyp51</i>
KES 1178 rv	AATAGAGGTGTTTGGAGTCGATCGTAATC	Expression analysis, <i>cyp51</i>
KES 1465 fw	GGTATGGCTTTCCGAGTTCCA	Expression analysis reference gene, <i>gapdh</i>
KES 1466 rv	TCAGTTGATACCAAATCATCCTCAG	Expression analysis reference gene, <i>gapdh</i>
KES 1463 fw	CTGCGAACAACCTATGCTCGTG	Expression analysis reference gene, α - <i>tubulin</i>
KES 1464 rv	CACGAAGAAGCCTTGGAGTCC	Expression analysis reference gene, α - <i>tubulin</i>

567 a) Synthesis by Eurogentec, (Seraing, Belgium)

568 b) 5' Biotin labelling

569

570

571 Table 2

572 Frequency of mutations in DNA and cDNA, and relative expression of *cyp51*, using *actin*,
 573 *α -tubulin* and *gapdh* as reference genes.

574

Isolate	Frequency of mutations [%]												Expression <i>cyp51</i> [-fold]
	F120L		Y131H		Y131F		K142R		I145F		I475T		
	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	
Ref.	-	-	-	-	-	-	-	-	-	-	-	-	1,0
T	-	-	-	-	-	-	-	-	-	-	-	-	0,9
G-54	-	-	-	-	-	-	-	-	47	79	-	-	3,0
1	-	-	-	-	-	-	-	-	50	66	-	-	1,9
3	33	88	32	93	-	-	-	-	-	-	-	-	5,9
4	35	89	34	97	-	-	-	-	-	-	-	-	6,9
5	29	83	35	99	-	-	-	-	-	-	-	-	4,0
6	34	94	31	87	-	-	-	-	-	-	-	-	8,2
7	-	-	-	-	-	-	-	-	-	-	-	-	0,5
8	-	-	-	-	-	-	-	-	-	-	-	-	0,5
9	-	-	-	-	34	73	36	79	-	-	-	-	3,8
12	-	-	-	-	34	64	36	68	-	-	-	-	2,9
13	33	89	33	98	-	-	-	-	-	-	-	-	9,0
24	-	-	-	-	-	-	-	-	48	66	-	-	2,8
26	-	-	-	-	55	42	-	-	-	-	50	40	0,7
27	-	-	-	-	55	78	-	-	-	-	54	72	1,0
28	-	-	-	-	51	85	-	-	-	-	52	82	1,7
34	-	-	-	-	32	88	35	81	-	-	-	-	2,5
35	34	91	33	95	-	-	-	-	-	-	-	-	7,2
37	36	91	32	98	-	-	-	-	-	-	-	-	8,4
48	34	89	33	95	-	-	-	-	-	-	-	-	5,8
49	-	-	-	-	-	-	-	-	50	65	-	-	8,3
50	-	-	-	-	-	-	-	-	50	57	-	-	2,7
53	-	-	-	-	-	-	-	-	2	0	-	-	3,1
54	-	-	-	-	-	-	-	-	47	71	-	-	5,3
56	-	-	-	-	-	-	-	-	-	-	-	-	2,1
57	-	-	-	-	-	-	-	-	-	-	-	-	8,6

58	-	-	-	-	-	-	-	-	-	-	-	4,8
59	-	-	-	-	-	-	-	-	-	-	-	10,0
60	-	-	-	-	-	-	-	-	-	-	-	9,5
62	-	-	-	-	33	66	37	59	-	-	-	2,2
63	-	-	-	-	33	72	34	77	-	-	-	4,4
66	-	-	-	-	-	-	34	69	-	-	-	3,7
67	-	-	-	-	43	81	-	-	-	-	45 80	1,3
71	32	88	34	95	-	-	-	-	-	-	-	5,9
72	31	89	30	96	-	-	-	-	-	-	-	7,4
73	32	89	32	98	-	-	-	-	-	-	-	10,1
77	-	-	-	-	-	-	-	-	47	65	-	1,5
78	-	-	-	-	-	-	-	-	48	64	-	1,6
79	-	-	-	-	-	-	-	-	-	-	-	1,9
80	-	-	-	-	-	-	-	-	-	-	-	10,3
81	-	-	-	-	-	-	-	-	-	-	-	0,6
82	-	-	-	-	52	70	-	-	-	-	49 61	2,0
83	-	-	-	-	52	74	-	-	-	-	47 76	1,2
84	34	88	32	100	-	-	-	-	-	-	-	8,0

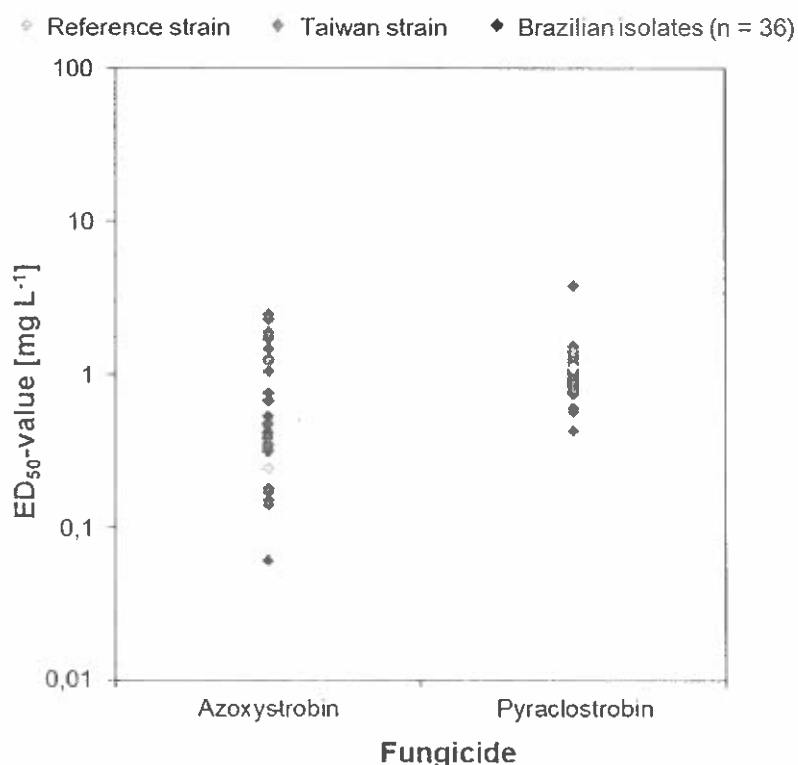
575

576

577 Fig. 1:

578 Sensitivity of *P. pachyrhizi* towards azoxystrobin and pyraclostrobin. ED₅₀-values from
 579 all tested isolates ranged from 0.14 mg L⁻¹ to 2.47 mg L⁻¹ (azoxystrobin) and 0.43 mg L⁻¹
 580 to 1.53 mg L⁻¹ (pyraclostrobin). The reference strain and the Taiwan strain did not differ
 581 in their sensitivity.

582



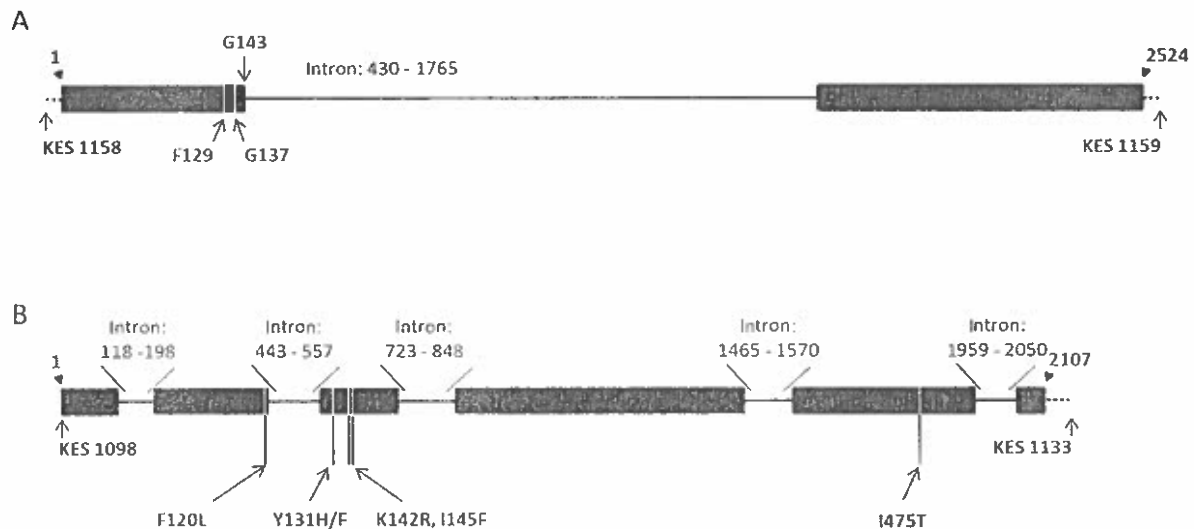
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Fig. 2:

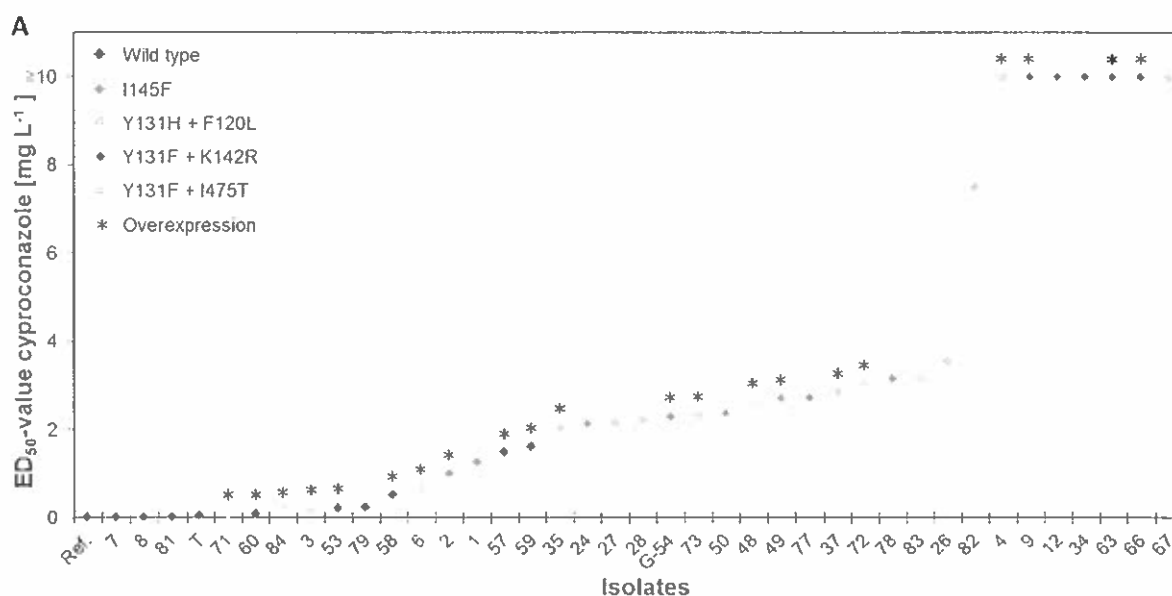
Schematic gene structure of *P. pachyrhizi* cytochrome *b* (A) and *cyp51* (B). Cytochrome *b* DNA is 2524 bp in size and interrupted by a 1336 bp intron which is located directly after codon 143. Location of used primers in this study and codons of most important mutations (i.e. F129L, G137R, G143A) in phytopathogens are indicated. The 2107 bp sized *cyp51* DNA is interrupted by 5 introns of different size (81 bp, 115 bp, 126 bp, 105 bp, 92 bp). Mutations found in this study (F120L, Y131H/F, K142R, I145F, I475T) are marked.



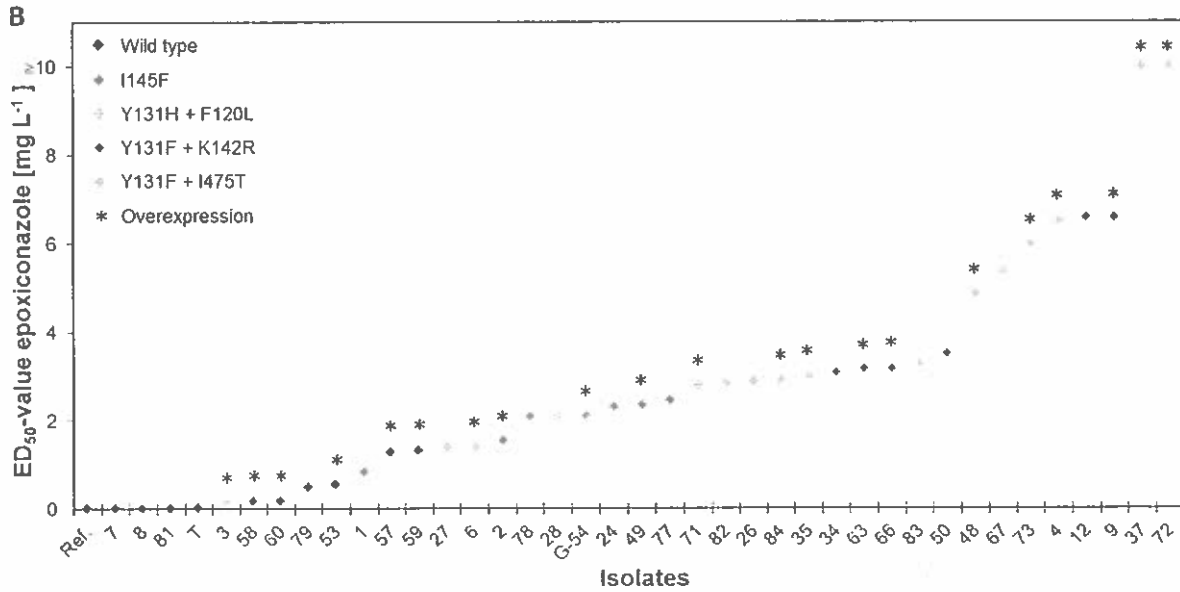
598 Fig. 3:

599 Sensitivity of *P. pachyrhizi* towards different triazoles and corresponding resistance
 600 mechanism. Isolates are sorted by rising ED_{50} -values of cyproconazole (A),
 601 epoxiconazole (B), metconazole (C) and tebuconazole (D). Colours represent different
 602 amino acid substitutions in the *cyp51* gene, while overexpressed *cyp51* is highlighted by
 603 stars. Threshold for overexpression is determined as 3-fold higher expression compared
 604 to *cyp51* expression of the reference strain (Ref.).

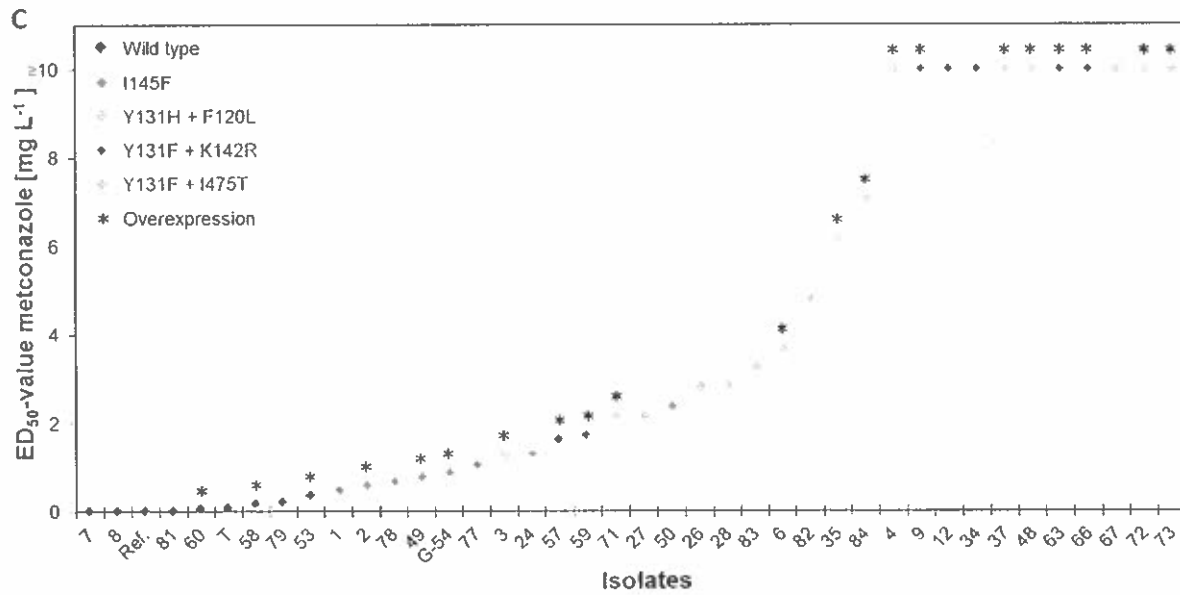
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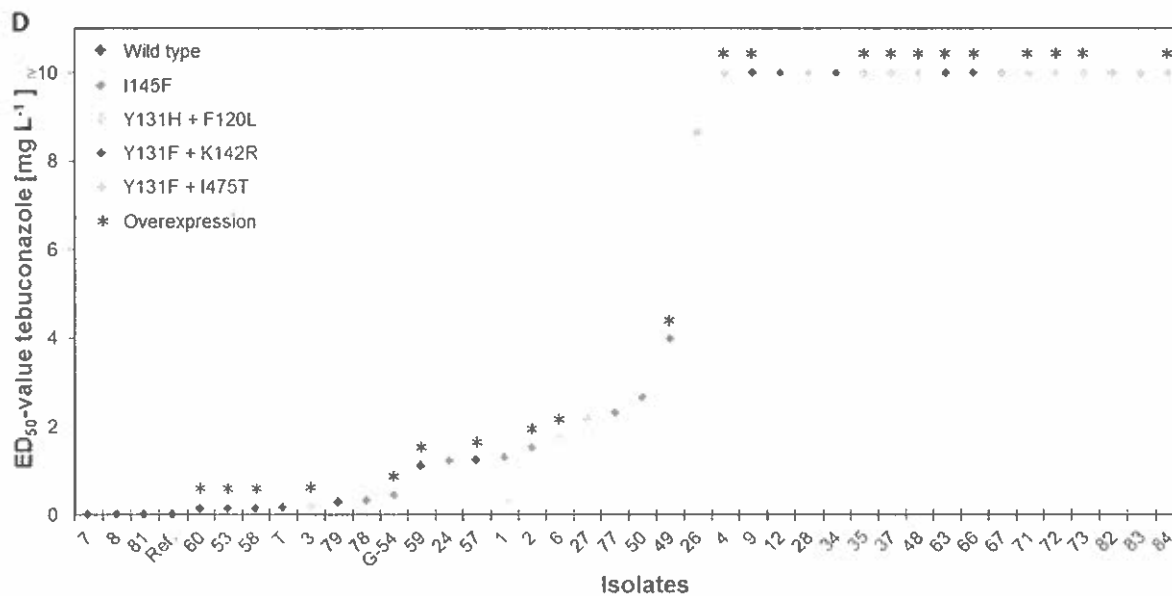


Fig. 4:

Relative expression of *cyp51*, using *actin*, *tubulin* and *gapdh* as reference genes.

The threshold of overexpression, indicated by a dash line, was set at 3-fold. *Cyp51*

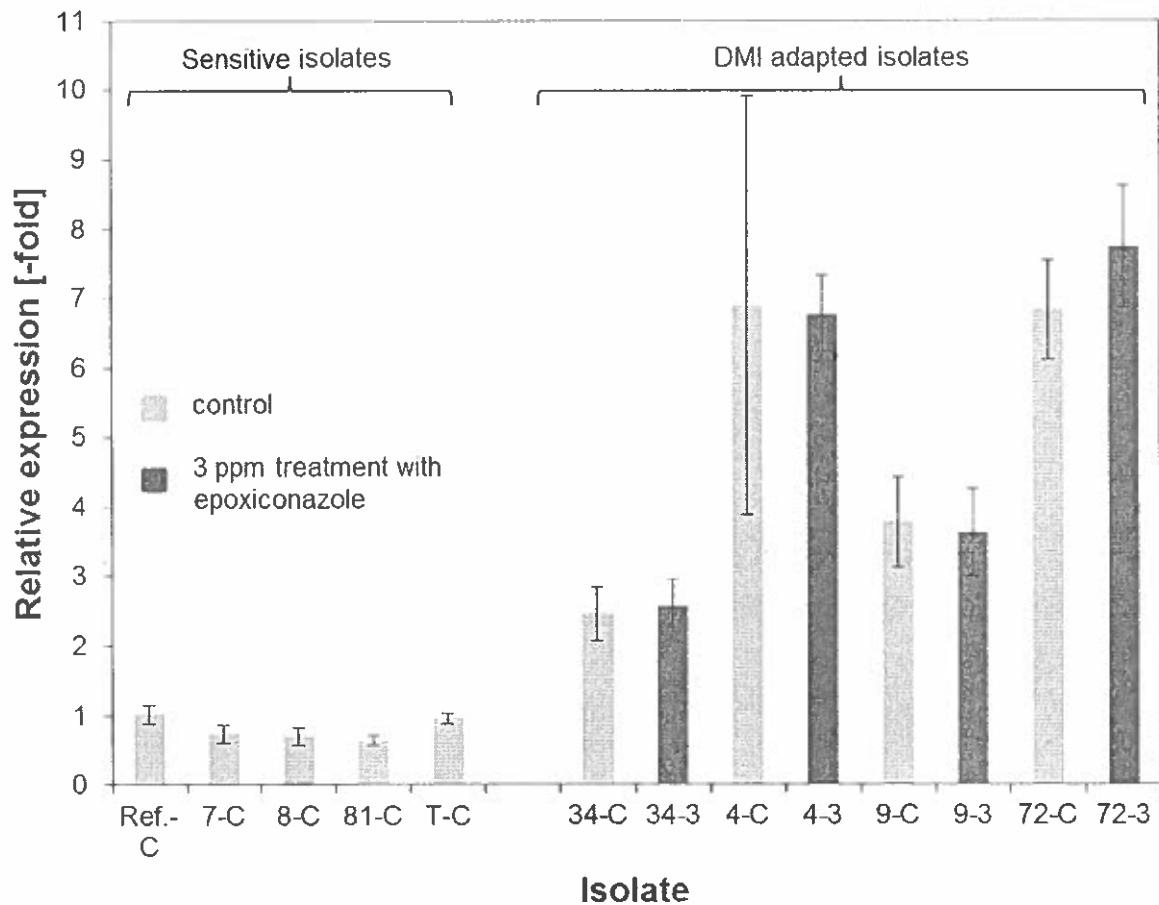
expression of triazole-sensitive isolates (reference strain, isolates 7, 8, 81 and Taiwan

strain) and triazole-adapted isolates were analysed from an untreated control (-C) and a

3 mg L⁻¹ treatment with epoxiconazole (-3). Expression of *cyp51* is increased to a

different extent in some of the adapted isolates (i. e. isolates 4, 9 and 72).

Overexpression is not induced by treatment with 3 mg L⁻¹ epoxiconazole.



625 Fig. 5:

626 Origin of *P. pachyrhizi* isolates in Brazil, classified by *cyp51* amino acid substitutions and
 627 *cyp51* expression. While colours represent amino acid substitutions, different shapes
 628 correspond to wild type expression (circle), overexpression (square) or not analysed
 629 (triangle).

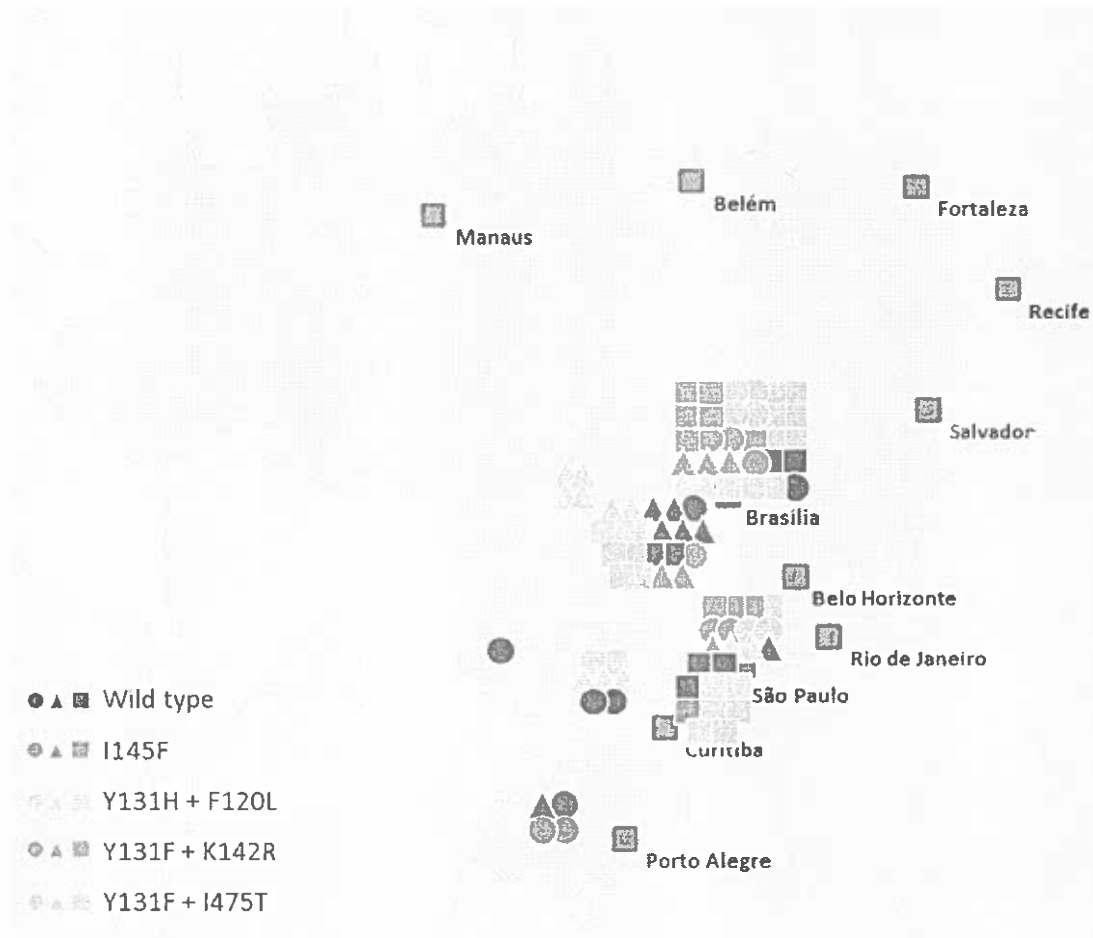
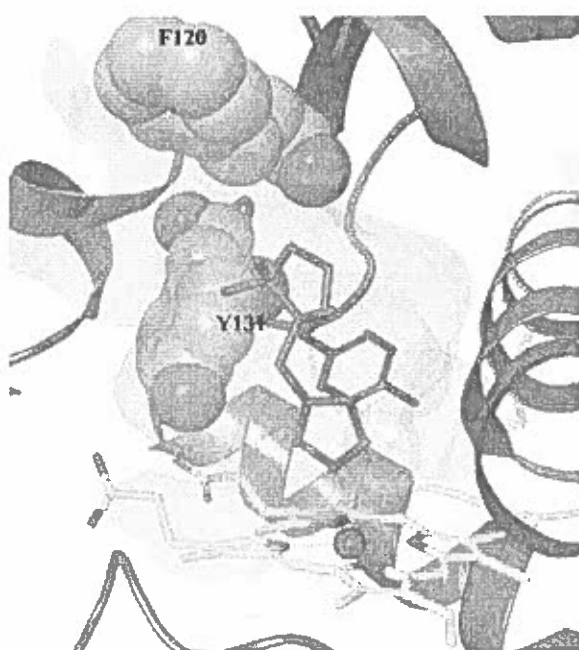
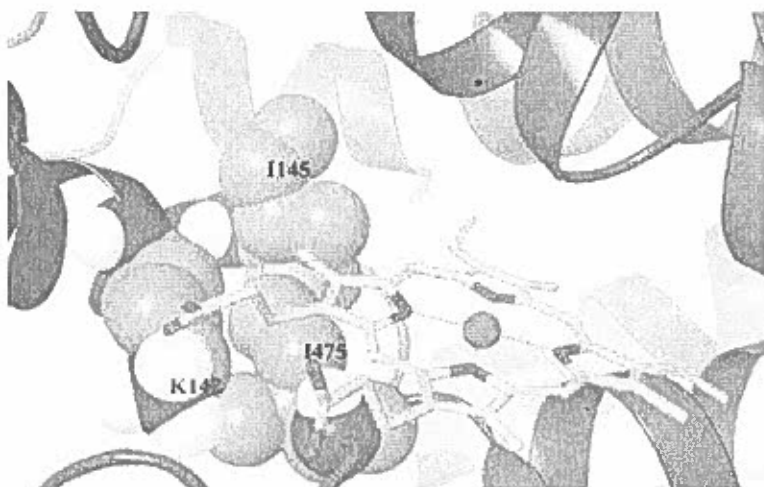


Fig. 6:

Location of F120 and Y131 in the binding site of the *P. pachyrhizi* CYP51 homology model. Amino-acids F120 and Y131 are depicted as space-filling spheres with carbon atoms coloured grey. The protein backbone is represented in grey ribbon, and the heme in green tube with the central iron atom coloured red. A docked pose of metconazole is shown in dark green tube for reference.



643 Fig. 7:
644 Location of K142, I145, and I475 adjacent to the heme in the *P. pachyrhizi* CYP51
645 homology model. Amino-acids K142, I145, and I475 are depicted as space-filling
646 spheres with carbon atoms coloured grey. The protein backbone is represented in grey
647 ribbon, and the heme in green tube with the central iron atom coloured red.



Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi*

Ana C Klosowski,^{a*} Louise L May De Mio,^a Simone Miessner,^b Ronaldo Rodrigues^c and Gerd Stammler^b

Abstract

BACKGROUND: Asian soybean rust, caused by *Phakopsora pachyrhizi*, is mostly controlled by demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides. Mutations in the cytochrome *b* (CYTB) gene can lead to pathogen resistance to QoIs. The occurrence of the mutations in codons 129, 137 and 143 in the CYTB gene was investigated, and a pyrosequencing assay was developed for rapid and quantitative detection of the F129L mutation.

RESULTS: Molecular analysis of the CYTB gene showed the presence of the F129L mutation in field samples and monouredinal isolates, while other mutations (G143A and G137R) were not found. The pyrosequencing was an effective method for quantitative detection of the F129L mutation, and many of the *P. pachyrhizi* samples showed high frequency of F129L.

CONCLUSION: This is the first report of the occurrence of the F129L mutation in *P. pachyrhizi*. The practical relevance of this mutation for field efficacy of QoIs needs further investigation.

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Keywords: Asian soybean rust; QoI fungicides; fungicide resistance; cytochrome *b*; point mutation

1 INTRODUCTION

Asian soybean rust, caused by *Phakopsora pachyrhizi* Syd. & P. Syd., is one of the most important economic threats to soybean growers in South America owing to its potential to damage the crop yield. Damage of up to 80% of yield production has been reported as a result of the poor pod filling^{1,2} associated with the early defoliation caused by the pathogen in the absence of measures to control the disease.³

Both sterol demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides represent the most important classes of fungicides currently used for Asian soybean rust control.^{4,5}

The QoI fungicides inhibit mitochondrial respiration by binding to the Qo site of the cytochrome *bc*₁ complex (complex III), preventing the transfer of electrons and consequently ATP production, leading to an energy deficiency in fungal cells.⁶

Mutations in the target cytochrome *b* (CYTB) gene may result in reduced sensitivity of pathogens to QoI fungicides because of reduction in fungicide binding affinity owing to the changes in the amino acid sequence.⁷

Three mutations, located at positions 129, 137 and 143, have been detected in the CYTB gene of several phytopathogenic fungi and oomycetes that are less sensitive to QoIs. The substitution from glycine to alanine at position 143 (G143A) is the most common mutation in QoI-resistant pathogens and is associated with high levels of resistance to QoI.^{8–10} The F129L (change of phenylalanine to leucine) and the G137R (substitution of glycine to arginine) mutations are reported to be associated with low to moderate levels of sensitivity decrease.¹⁰

Sequence analyses of the CYTB gene have revealed that rusts, including *P. pachyrhizi*, have a type I intron after codon 143, and

the nucleotide substitution in this codon would prevent splicing of the intron, being lethal for the fungus.¹¹ Meanwhile, other species have been identified with such an intron sequence directly after codon 143.¹² As QoI resistance via G143A is not possible for such 'intron pathogens', some of these pathogens (e.g. *Alternaria solani*, *Pyrenophora teres*) have taken the alternative, but less effective way of adaption, by the F129L mutation.

The aims of the present work were to monitor mutations in the CYTB gene, especially at the codons 129, 137 and 143, in *P. pachyrhizi* isolates from Brazil and to develop a pyrosequencing assay that provides a rapid and quantitative detection of the F129L mutation in field samples.

2 MATERIALS AND METHODS

2.1 Isolation of monouredinal *P. pachyrhizi* strains

Leaves infected with *P. pachyrhizi* were collected from Brazilian soybean fields in the 2012–2013 and 2013–2014 seasons. A total of 41 strains of *P. pachyrhizi* were isolated from the states of Paraná and Mato Grosso by transferring spores from single uredia to healthy unifoliate leaves of soybean plants grown in the greenhouse with an inoculation needle.

* Correspondence to: Ana C Klosowski, Universidade Federal do Paraná, Rua dos Funcionários 1540, 80035-050 Curitiba, Brazil. E-mail: ana_clk@yahoo.com.br

a Universidade Federal do Paraná, Curitiba, Brazil

b BASF SE, Limburgerhof, Germany

c BASF SA, Estação Experimental Agrícola, Santo Antônio de Posse, Brazil

The inoculum of monouredinial isolates was multiplied by transferring the spores to new leaves every 3 weeks by inoculation of spore suspension with Tween water (0.01%) to the abaxial surfaces of unifoliate leaves, using an airbrush (0.3 mm nozzle).

The leaves were kept in Petri dishes with water agar medium (1%) including streptomycin sulphate (30 mg L⁻¹) and kinetin (0.2 mg L⁻¹). Soybean leaves were incubated in the dark during the first 24 h after inoculation at room temperature and afterwards at 23 °C with a photoperiod of 12 h.

2.2 Collection of field samples

Samples from various soybean trial sites in Brazil (Table 1) were taken in the form of 20 leaves with soybean rust disease symptoms. The leaves were dried and subsequently processed and analysed as described in the next section.

2.3 DNA and RNA extraction

Molecular analyses were conducted for populations of *P. pachyrhizi* from the 2013–2014 season and monouredinial isolates from the 2012–2013 and 2013–2014 seasons.

For field samples, DNA of *P. pachyrhizi* was extracted from soybean leaves by cutting out typical rust lesions and homogenising these with a bead mill, using the NucleoSpin DNA Plant II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the instructions of the manufacturer for CTAB-based DNA extraction.

For monouredinial isolates, DNA of *P. pachyrhizi* spores was extracted using the NucleoSpin DNA Plant II kit (Macherey-Nagel) following the instructions of the manufacturer for CTAB-based DNA extraction.

Total RNA of *P. pachyrhizi* spores was extracted and reverse transcribed to cDNA following the procedures described by Schmitz *et al.*⁵

2.4 Cytochrome *b* sequencing

The whole cytochrome *b* gene was sequenced according to the method described by Schmitz *et al.*⁵ using the primer pair KES 1158 fw (5' CAGTAGCCTAAAGAAGGGTGTAA 3') and KES 1159 rv (5' CCCGTTGAATATCTTGACATCTTAC 3') for amplification of the whole gene with the following PCR programme for amplification: initial heating at 98 °C for 15 s, 35 cycles at 98 °C for 10 s, 64 °C for 5 s and 72 °C for 45 s, followed by a final amplification step at 72 °C for 1 min.

2.5 Pyrosequencing for F129L

A pyrosequencing assay was developed using the Pyrosequencing Assay Design software (Qiagen, Hilden, Germany). In a first step, the CYTB gene fragment, which contained the target sequence, was amplified in PCR reaction using 12.5 µL of 2× Maxima Mastermix (Fermentas GmbH St., Leon-Rot, Germany), 7.5 µL of bidest water, 2.5 µL of DNA and 1.25 µL of each primer: KES 498 (5' GGACACTAGTATGGGCGATTG 3') and KES 499 (5' Biotin – CATGTGAGGCGGTCTCATT 3'), under the following conditions: an initial heating step for 4 min at 95 °C, followed by 40 cycles with 15 s at 94 °C, 30 s at 55 °C and 15 s at 72 °C and a final step of 5 min at 72 °C. Every template was applied in duplicate.

The subsequent pyrosequencing reaction of codon 129 was performed using the specific sequencing primer KES 500 (5' TTGTAATAATAGCGACAGC 3'). For single strand preparation, PCR products were immobilised on Streptavidin Sepharose beads (GE Healthcare, Little Chalfont, Bucks, UK) and cleaned up with ethanol (70%), denaturated with sodium hydroxide (0.2 M), washed in

Table 1. Origin of Brazilian populations of *Phakopsora pachyrhizi* and the frequency of the F129L mutation in the cytochrome *b* gene

Origin/state	Season	F129L (%)
Goiás	2013–2014	97
Goiás	2013–2014	95
Goiás	2013–2014	97
Goiás	2013–2014	95
Goiás	2013–2014	94
Goiás	2013–2014	100
Goiás	2013–2014	96
Goiás	2013–2014	96
Goiás	2013–2014	99
Goiás	2013–2014	99
Mato Grosso	2013–2014	50
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	99
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	52
Mato Grosso	2013–2014	95
Mato Grosso	2013–2014	96
Mato Grosso	2013–2014	99
Mato Grosso	2013–2014	97
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	99
Mato Grosso	2013–2014	78
Mato Grosso	2013–2014	88
Mato Grosso	2013–2014	94
Mato Grosso	2013–2014	95
Mato Grosso	2013–2014	93
Mato Grosso	2013–2014	95
Mato Grosso	2013–2014	91
Mato Grosso	2013–2014	100
Mato Grosso	2013–2014	89
Mato Grosso	2013–2014	85
Mato Grosso	2013–2014	94
Mato Grosso	2013–2014	93
Mato Grosso	2013–2014	98
Mato Grosso	2013–2014	95
Mato Grosso	2013–2014	98
Mato Grosso	2013–2014	54
Mato Grosso	2013–2014	95
Mato Grosso	2013–2014	96
Mato Grosso	2013–2014	93
Mato Grosso	2013–2014	91
Mato Grosso	2013–2014	95
Mato Grosso do Sul	2013–2014	99
Mato Grosso do Sul	2013–2014	98
Mato Grosso do Sul	2013–2014	93
Mato Grosso do Sul	2013–2014	94

Table 1. Continued

Origin/state	Season	F129L (%)
Mato Grosso do Sul	2013–2014	76
Minas Gerais	2013–2014	52
Minas Gerais	2013–2014	52
São Paulo	2013–2014	52
São Paulo	2013–2014	58
São Paulo	2013–2014	30
São Paulo	2013–2014	47
São Paulo	2013–2014	57
São Paulo	2013–2014	57
São Paulo	2013–2014	58
São Paulo	2013–2014	59
São Paulo	2013–2014	81
São Paulo	2013–2014	70
São Paulo	2013–2014	91
São Paulo	2013–2014	11
São Paulo	2013–2014	14
São Paulo	2013–2014	49
São Paulo	2013–2014	31
São Paulo	2013–2014	56
Paraná	2013–2014	95
Paraná	2013–2014	78
Paraná	2013–2014	86
Paraná	2013–2014	94
Rio Grande do Sul	2013–2014	87
Rio Grande do Sul	2013–2014	96
Rio Grande do Sul	2013–2014	89

tris-acetate (10 mM) and released into a mixture of annealing buffer with the sequencing primer, implementing the Vacuum Prep Worktable (Qiagen), following the instructions of the manufacturer. Then, the samples were heated at 80 °C for 3 min in an incubator and cooled to room temperature. Pyrosequencing was performed for sequence determination and allele quantification using PyroMark Gold Q96 reagents (Qiagen) on a PSQ 96MA (Qiagen) machine as described by the manufacturer.

This method was used for quantitative detection of the F129L mutation in field samples and monouredinial isolates of *P. pachyrhizi*.

3 RESULTS

The F129L mutation was found in the CYTB gene sequencing in some isolates (Table 2), while no other mutations were detected, including G143A and G137R. The change of phenylalanine to leucine at position 129 in all isolates and samples tested results from the substitution of the first nucleotide of codon 129 'T' to 'C' (TFT to CTT). The intron after codon 143 was present in all isolates tested (Fig. 1).

The pyrosequencing assay developed is an efficient tool for quantitative detection of F129L in *P. pachyrhizi* field samples. Different proportions of F129L mutation were detected, and representative pyrograms with different frequencies of the F129L mutation are given in Fig. 2. All samples from the 2013/14 season from different regions of Brazil showed the F129L mutation, but in different frequencies. Many samples (65%) showed a high frequency ($\geq 90\%$) of F129L, 28% of them had between 50 and 90% of F129L and 7% of samples had frequencies less than 50% of F129L (Table 1).

Table 2. Origin of Brazilian monouredinial isolates of *Phakopsora pachyrhizi* and the presence of the F129L mutation in the cytochrome b gene

Isolate	Origin	Season	Genotype	F129L (%)
3	South-west of Paraná	2012–2013	Wild type ^a	0
7	South-west of Paraná	2012–2013	F129L	99
11	South-west of Paraná	2012–2013	Wild type	0
12	South-west of Paraná	2012–2013	Wild type	0
14	South-west of Paraná	2012–2013	Wild type	0
52	Centre-east of Paraná	2012–2013	Wild type	0
54	Centre-east of Paraná	2012–2013	Wild type	0
57	Centre-east of Paraná	2012–2013	Wild type	0
61	Centre-east of Paraná	2012–2013	Wild type	0
62	Centre-east of Paraná	2012–2013	Wild type	0
63	Centre-east of Paraná	2012–2013	Wild type	0
21	South-west of Paraná	2013–2014	F129L	98
22	South-west of Paraná	2013–2014	F129L	99
23	South-west of Paraná	2013–2014	Wild type	0
28	South-west of Paraná	2013–2014	F129L	96
29	South-west of Paraná	2013–2014	F129L	100
40	South-west of Paraná	2013–2014	F129L	100
41	South-west of Paraná	2013–2014	Wild type	0
91	Centre-east of Paraná	2013–2014	Wild type	0
92	Centre-east of Paraná	2013–2014	Wild type	0
93	Centre-east of Paraná	2013–2014	F129L	96
94	Centre-east of Paraná	2013–2014	F129L	96
95	Centre-east of Paraná	2013–2014	F129L	90
96	Centre-east of Paraná	2013–2014	Wild type	0
97	Centre-east of Paraná	2013–2014	Wild type	0
98	Centre-east of Paraná	2013–2014	Wild type	0
99	Centre-east of Paraná	2013–2014	Wild type	0
100	Centre-east of Paraná	2013–2014	Wild type	0
71	Centre-south of Mato Grosso	2013–2014	F129L	95
72	Centre-south of Mato Grosso	2013–2014	F129L	97
73	Centre-south of Mato Grosso	2013–2014	F129L	98
76	Centre-south of Mato Grosso	2013–2014	F129L	99
77	Centre-south of Mato Grosso	2013–2014	F129L	97
78	Centre-south of Mato Grosso	2013–2014	Wild type	0
81	Centre of Mato Grosso	2013–2014	F129L	99
82	Centre of Mato Grosso	2013–2014	F129L	100
84	Centre of Mato Grosso	2013–2014	F129L	93
85	Centre of Mato Grosso	2013–2014	F129L	98
86	Centre of Mato Grosso	2013–2014	F129L	99
87	Centre of Mato Grosso	2013–2014	F129L	100
88	Centre of Mato Grosso	2013–2014	F129L	100

^a F129L mutation not detected.

Monouredinial isolates showed 0% (wild type) or approximately 100% F129L mutation (F129L mutated isolate) (Table 2). Overall, the F129L mutation was detected in 21 of 41 isolates (51%) (Table 2). From the state of Mato Grosso, 92% of strains carried the mutation (all from the 2013–2014 season), and 32% from the state of Paraná. From season 2012–2013 to season 2013–2014, the frequency of F129L strains in Paraná increased from 9 to 47%.

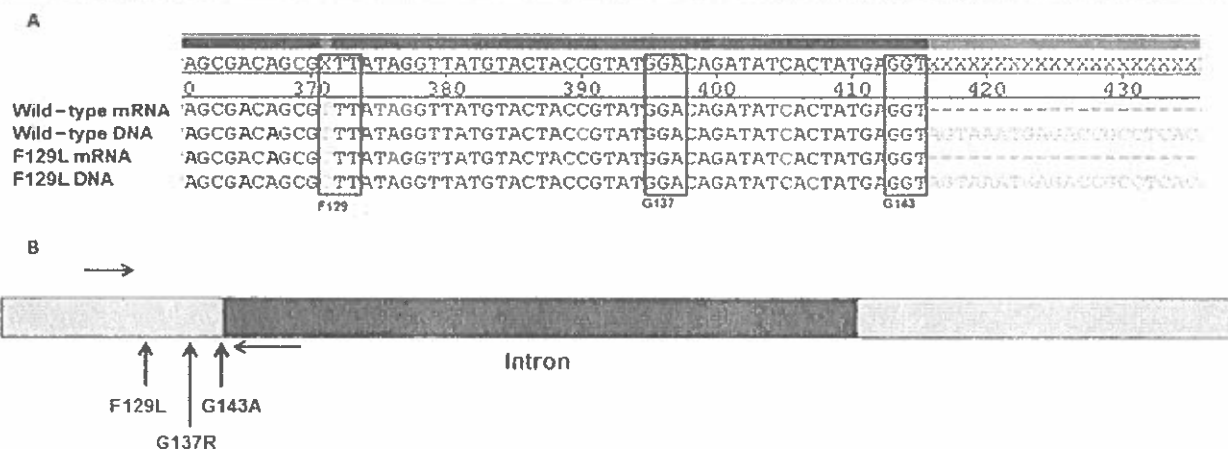


Figure 1. Gene sequencing of *Phakopsora pachyrhizi* cytochrome *b* and detection of the F129L mutation (A) and a schematic structure showing the position of codons 129, 137 and 143 and the intron (B). (A) The nucleotide sequences are alignments of cDNA and DNA of wild type and isolates with F129L. The alignment shows fragments of the sequence containing codons at positions 129, 137 and 143. Only the mutation F129L was detected, and no other, e.g. at 137 or 143. The intron starts directly after codon 143 and was present in all sequenced isolates. (B) The schematic structure shows the cytochrome *b* gene (total of 2524 bp) and the interruption by the intron (1336 bp), which is located directly after codon 143. Arrows indicate where the primer was designed for the PCR amplification step of the pyrosequencing analysis.

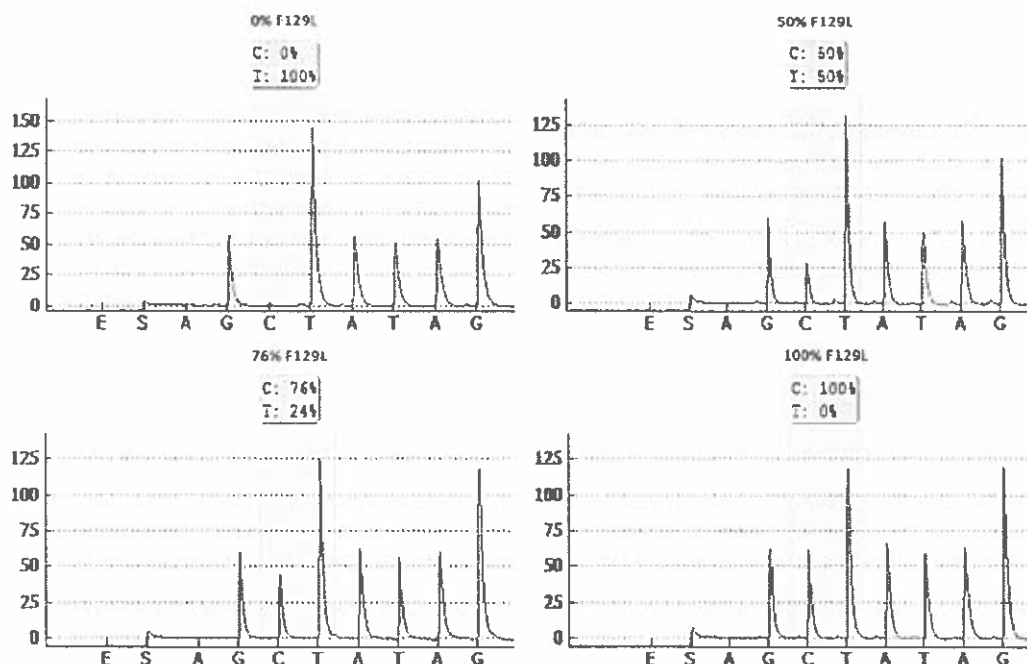


Figure 2. Pyrograms showing the quantitative detection of the F129L mutation in the cytochrome *b* gene of *Phakopsora pachyrhizi* samples from Brazil. The quantitative assessment programme calculated the percentage of the allelic bases C and T. The enzyme (E) and substrate (S) mixture containing APS and luciferin are added before the first addition of nucleotide.

4 DISCUSSION AND CONCLUSIONS

The findings of this work revealed the occurrence of F129L mutation in the CYTB gene in *P. pachyrhizi* from soybean fields in Brazil. This is the first report of mutations in the CYTB gene related to less sensitivity to Qol fungicides, not only in *P. pachyrhizi* but in any rust species. In an earlier study on Qol sensitivity of *P. pachyrhizi*, with isolates from 2009 and 2010, no F129L or any other mutation in the CYTB gene was found.⁵

The F129L mutation has been reported in different plant-pathogenic fungi, such as *A. solani*,¹³ *Pyricularia grisea*,¹⁴ *P. teres*^{15–17} and *P. tritici-repentis*,^{16,17} as a mechanism for decreased

Qol sensitivity. In species where isolates with F129L and G143A have been found (e.g. *P. tritici-repentis*, *P. grisea*), a direct comparison of such mutants on the effect on Qol sensitivity showed that the F129L mutation leads to a much lower sensitivity decrease than the G143A mutation.^{14,17} Glasshouse and field studies with *P. teres* have shown that the effect of the F129L mutation on the field performance of pyraclostrobin is rather limited, and that efficacy was good even in fields with a high frequency of F129L in the population.^{15,17} A stronger decrease in efficacy of Qols has been described for F129L mutants of *A. solani*.¹⁸ The effect of the F129L mutation on the sensitivity of *P. pachyrhizi* and on the efficacy of Qols needs further investigation.

Our data show that the pyrosequencing assay is an appropriate method for rapid and quantitative detection of F129L mutation in field samples and monouredinal isolates. While different frequencies of the F129L mutation were detected in populations of *P. pachyrhizi* from field samples, all monouredinal isolates had an F129L frequency of either 0 or approximately 100%. A heteroplasmy of the CYTB gene, as has been reported in single cases for *Botrytis cinerea*,¹⁹ has so far not been found in *P. pachyrhizi*. This shows that no or all CYTB genes in a monouredinal isolate are mutated. As CYTB is mitochondrial inherited and therefore present in multiple copies in a cell, this finding is of importance for the interpretation of quantitative results of F129L detection in field samples (populations). In fact, this means that in a sample with 60% of F129L, 60% of the strains making up this sample population carry the F129L, and 40% are wild type.

The relatively high frequency of F129L mutated monouredinal isolates and of the F129L in field samples (populations) indicates that the continued use of Qols to control soybean rust has led to the selection of F129L mutated individuals. Frequency of mutated (resistant) strains in a population is positively influenced by selection pressure by the fungicides on the one hand and negatively influenced by any possible fitness costs caused by the mutation conferring resistance on the other hand. Therefore, the question as to whether the F129L mutation is accompanied with fitness penalties is of great interest.

The dynamic of the F129L mutation in *P. pachyrhizi* populations in Brazil and the impact of this mutation on field efficacy of Qol fungicides should be monitored in future seasons.

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08/03/2017

INFORMATIVO 01/2017

INFORMAÇÃO SOBRE CARBOXAMIDAS EM FERRUGEM DA SOJA
FRAC INTERNACIONAL
SDHI- WORKING GROUP

Cumpre ao FRAC-BR através do presente informar que durante o encontro do Working Group de SDHI do FRAC Internacional, realizado no dia 7 de março de 2017, foram discutidas informações sobre a Ferrugem Asiática da Soja no Brasil, as quais foram compartilhadas com o FRAC Brasil através de uma mensagem enviada pela Sra. Kristin Klappach, líder do Working Group.

Segue abaixo o conteúdo dessa mensagem.

"Durante a reunião do working group do FRAC de SDHI (SDHI FRAC WG), foram discutidas informações sobre a sensibilidade de ferrugem da soja no Brasil.

Este texto será incluído nas minutas oficiais do SDHI FRAC WG.



Amostras de Ferrugem asiática da soja vem sendo testadas quanto a sua sensibilidade para SDHIs desde 2007. A performance no campo dos fungicidas contendo SDHI permanece em geral boa, mas pela primeira vez na safra 2015/16 e particularmente na safra 2016/17, em áreas com um histórico intensivo do uso de SDHIs e em condições de alta pressão de doença, foram detectados casos de redução de performance das mesmas. Em adição, a caracterização inicial das populações coletadas em 2015/2016 indicaram uma mutação na subunidade C na posição 186F. A relevância e distribuição de tal mutação para a redução da sensibilidade a SDHIs, parcialmente também observadas nas amostras de 2016/2017, estão sendo investigadas. Programas intensivos de monitoramento estão sendo conduzidos para investigar a magnitude e relevância destas descobertas.

SDHI FRAC WG irá se reunir quando novos dados de monitoramento estiverem disponíveis (Maio/Junho) e as recomendações para a próxima safra 2017/2018 serão revisadas.

Espécies de fungos podem apresentar mutações diferentes que afetam os SDHIs. Algumas mutações podem levar a diferentes níveis de sensibilidade, dependendo da estrutura química do ingrediente ativo. Como todos os SDHIs apresentam resistência cruzada, o gerenciamento da resistência deve ser o mesmo para todos os SDHIs. Todo o monitoramento e recomendação aqui relatados referem-se a todo o grupo dos SDHIs.

O SDHI FRAC WG deseja trabalhar em conjunto com o FRAC Brasil neste importante tema.



Vemos a forte necessidade de alinhar as recomendações futuras e esperamos que as recomendações para o uso de SDHIs em soja permaneçam as mesmas até que novos dados de sensibilidade estejam disponíveis e o grupo de trabalho se reúna novamente para analisar os dados de monitoramento”.

Kristin Klappach

(Chairwoman of the FRAC SDHI Working Group)

Isto posto, considerando a relação existente entre o FRAC-BR e o FRAC Internacional, bem como da comunicação recebida, resta finalizado o informativo que ora vai publicado.

Atenciosamente.

FRAC - BRASIL

Londrina, PR
Julho, 2007

Autores

Cláudia V. Godoy
Eng^a Agrônoma, Dra.

Embrapa Soja
Cx. Postal 231
86001-970, Londrina, PR
godoy@cnpso.embrapa.br

Cláudia B. Pimenta
Eng^a Agrônoma
Agenciarrural

R. Jornalista Geraldo Vale, 331
74130-012, Goiânia, GO
claudia@ctpa.com.br

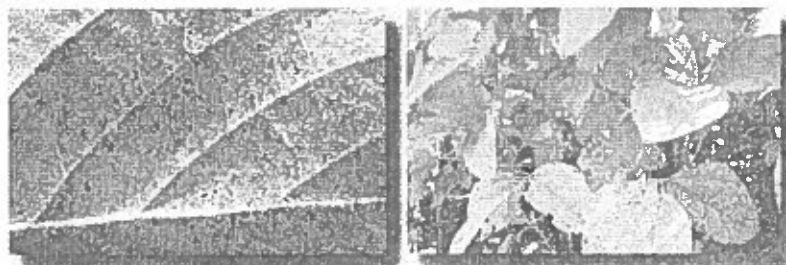
**Dulândula S. Miguel-
Wruck**

Eng^a Agrônoma, Dra.
Epamig
Cx. Postal 351
38001-970, Uberaba, MG
dmiguel@epamiguberaba.com.br

Edison U. Ramos Junior
Eng^a Agrônomo, Dr.
Apta Regional do Sudoeste
Paulista/ DDD
Cx. Postal 22



Eficiência de fungicidas para controle da ferrugem asiática da soja, *Phakopsora pachyrhizi*, na safra 2006/07. Resultados sumarizados dos ensaios em rede



As doenças que incidem na cultura da soja [*Glycine max* (L.) Merr.] têm assumido papel importante na definição da produtividade da cultura, safra após safra. As perdas anuais de produção por doenças são estimadas em cerca de 15% a 20% (Tecnologias, 2006). A utilização de fungicidas para controle de doenças na cultura é uma prática recente, tendo iniciado os estudos com o surto epidêmico de oídio [*Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takam], na safra 1996/97. Posteriormente, o aumento da incidência das doenças de final de ciclo [*Septoria glycines* Hemmi e *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner], principalmente em função do cultivo intensivo e da ausência de rotação de culturas, também demandaram o registro de fungicidas. Com o surgimento da ferrugem asiática (*Phakopsora pachyrhizi* Syd. & P. Syd.), no Brasil, em 2001 (Yorinori et al., 2005), e a ausência de cultivares resistentes para controle dessa doença, novos produtos foram registrados. O uso de fungicidas foi intensificado por ser a única ferramenta que evita reduções de produtividade na presença da ferrugem. Informações sobre eficiência de fungicidas para controle das diferentes doenças são cada vez mais necessárias para orientar a sua correta utilização no campo.

A rede de ensaios para controle de doenças na cultura da soja surgiu durante a XXV Reunião de Pesquisa de Soja da Região Central do Brasil, realizada no ano de 2003, em Uberaba (MG), com o objetivo de fornecer resultados de pesquisa que auxiliem a assistência técnica na escolha do fungicida para controle das diferentes doenças que incidem na cultura. O delineamento dos ensaios em rede não tem como objetivo avaliar o momento da aplicação e o residual dos diferentes produtos, sendo o único objetivo a comparação dos produtos, em uma mesma situação. Ensaios comparando os diferentes produtos registrados, e em fase de registro, são realizados por instituições de pesquisa públicas e privadas, fundações, universidades e cooperativas (Godoy, 2005a,b). Na safra 2006/2007, 18 instituições participaram da rede de ensaios, nas diferentes regiões produtoras: Embrapa Soja, Agenciarrural/CTPA; Instituto Biológico; Embrapa Cerrados; MClamamoto; Fundação MS para Pesquisa e Difusão de Tecnologias Agropecuárias; Fundação Mato Grosso; Instituto Agronômico de Campinas; APTA Regional do Sudoeste Paulista / DDD; EPAMIG; Universidade Federal do Tocantins; Universidade Federal Grande Dourados; Fundação de Apoio à Pesquisa e Desenvolvimento do Oeste Baiano; Agência Estadual de Defesa Agropecuária da Bahia; Fundação Agrária de Pesquisa Agropecuária; Universidade Tecnológica Federal do Paraná e Universidade Federal de Santa Maria.

Durante a safra 2006/2007, seguindo os protocolos propostos para os "ensaios em rede", foram conduzidos ensaios para avaliar a eficiência dos produtos registrados, e em fase de registro, para controle da ferrugem asiática da soja. Em função do número de produtos, os tratamentos foram separados em dois ensaios, de acordo com o grupo dos fungicidas, contemplando os triazóis em uma lista (Tabela 1) e as estrobilurinas, as misturas de triazóis com estrobilurinas e as misturas de triazóis com benzimidazóis em outra lista (Tabela 2). O

SYNGENTA EXHIBIT 1038

SYNGENTA v. UPL, PGR2023-00017

18300-970, Capão Bonito, SP
edisonramosjr@aptaregional
sp.gov.br

Fabiano V. Siqueri

Engº Agrônomo
Fundação MT
Cx. Postal 79
Rondonópolis, MT
fabianosiqueri@fundacaomt.
com.br

Heraldo R. Feksa

Engº Agrônomo, MS.c
FAPA
Praça Nova Pátria, s/n
85139-400, Guarapuava, PR
heraldo@agraria.com.br

Idalmir dos Santos

Engº Agrônomo, Dr.
UTFPR
Cx. Postal 571
85503-390, Pato Branco, PR
idalmir@utfpr.edu.br

Ivani O. N. Lopes

Matemática, MS.c
Embrapa Soja
Cx. Postal 231
86001-970, Londrina, PR
negrao@cnpso.embrapa.br

José Nunes Junior

Engº Agrônomo, Dr.
CTPA
Av. Assis Chateaubriand, 1491
74130-012, Goiânia, GO
nunes@ctpa.com.br

Márcio A. Ito

Engº Agrônomo, MS.c
Apt Regional do Sudoeste
Paulista - DDD
Cx. Postal 33
15270-000, Tatui, SP
akira@aptaregional.sp.gov.br

Marcos M. Iamamoto

Engº Agrônomo, Dr.
MCIamamoto
Rua Prof. José Augusto
Assumpção, 137
14883-218, Jaboticabal, SP
iamamoto@asbyte.com.br

Margarida F. Ito

Bióloga, Dra.
IAC
Cx. Postal 28
13001-970, Campinas, SP
mfito@iac.sp.gov.br

Maurício C. Meyer

Engº Agrônomo, Dr.
Embrapa Soja
Cx. Postal 131
65800-000, Balsas, MA

tratamento com tebuconazole (Folicur®) foi comum nas duas listas. As listas de tratamentos, o delineamento experimental e as avaliações foram padronizados para sumarização conjunta ao final da safra, sendo realizados de acordo com as normas para avaliação e recomendação de fungicidas para a cultura da soja (Reunião, 2006). De maneira geral, em cada local, os ensaios (Tabela 1 e 2) foram realizados na mesma área experimental, e instalados no mesmo dia ou no dia seguinte com a intenção de realizar a análise conjunta de todos os tratamentos. No entanto, isso não foi possível uma vez que foi observada diferença nos tratamentos em comum nas duas listas (testemunha sem controle e Folicur®), provavelmente devido à variação das condições climáticas no momento da aplicação.

O delineamento experimental foi o de blocos ao acaso com quatro repetições, sendo cada repetição constituída de parcelas de 6 linhas x 5 metros, com variações entre locais. Foram realizadas

Tabela 1. Tratamentos com fungicidas do grupo dos triazóis para controle da ferrugem asiática da soja.

Ingrediente ativo	Produto comercial	Dose (L p.c.ha ⁻¹)
1. Testemunha		
2. ciproconazole	Alto 100	0,30
3. ciproconazole + propiconazole	Artea	0,30
4. difenoconazole	Score	0,20
5. epoxiconazole	Soprano	0,40
6. epoxiconazole	Virtue	0,40
7. fluquinconazole	Palisade ¹	0,25
8. flutriafol	Impact ²	0,50
9. miclobutanil	Systhane	0,40
10. propiconazole	Juno	0,50
11. tebuconazole	Rival	0,50
12. tebuconazole	Folicur	0,50
13. tebuconazole	Orius	0,40
14. tebuconazole	Tebuconazole Nortox	0,50
15. tetraconazole	Eminent	0,40
16. tetraconazole	Domark	0,50
17. metconazole	Caramba	0,60
18. prothioconazole	Proline ³	0,30

¹adicionado Áureo 250 mL ha⁻¹; ²adicionado Oppa 1% v/v; ³produto em fase de registro no MAPA

Tabela 2. Tratamentos com fungicidas do grupo das estrobilurinas, das misturas de triazóis com estrobilurinas e das misturas de triazóis com benzimidazóis.

Ingrediente ativo	Produto comercial	Dose (L p.c.ha ⁻¹)
1. Testemunha		
2. azoxistrobina	Priori ¹	0,20
3. picoxistrobina	Aproach ¹	0,20
4. ciproconazole + azoxistrobina	Priori Xtra ¹	0,30
5. ciproconazole + trifloxistrobina	Sphere ²	0,30
6. epoxiconazole + piraclostrobina	Opera	0,50
7. propiconazole + trifloxistrobina	Stratego ²	0,40
8. tebuconazole + trifloxistrobina	Nativo ³	0,50
9. flutriafol + tiofanato metílico	Impact Duo ⁴	0,60
10. flutriafol + tiofanato metílico	Celeiro ⁵	0,60
11. flutriafol + carbendazin	Battle ⁴	0,60
12. ciproconazole + picoxistrobina	Alto 100 + Aproach ¹	0,24 + 0,24
13. tebuconazole	Folicur	0,50

¹adicionado Nimbus 0,5% v/v; ²adicionado Áureo 250 mL ha⁻¹; ³adicionado Áureo 0,25% v/v; ⁴adicionado Oppa 1% v/v; ⁵adicionado Itharol 1% v/v

mauricio@embrapabalsas.
com.br

Moab D. Dias
Engº Agrônomo, MS.c
UFT
Cx. Postal 66
77402-970, Gurupi, TO
moab@uft.edu.br

Mônica C. Martins
Engº Agrônomo, Dra.
Fundação Bahia
Av. Ahylon Macedo, 11
47806-180, Barreiras, BA
soja@fundacaoba.com.br

Nailton S. Almeida
Engº Agrônomo, MS.c
ADAB
Av. Ahylon Macedo, 196
47806-180, Barreiras, BA
nailton_nap@aiba.org.br

Newton S. Andrade
Engº Agrônomo, especialista
em fiscalização
ADAB
Av. Ahylon Macedo, 196
47806-180, Barreiras, BA
newandrade@uol.com.br

Paulino J. M. Andrade
Engº Agrônomo, MS.c
Embrapa Soja
Cx Postal 39
79560-000, Chapadão do Sul,
MS
paulino@fundacaochapadao.
com.br

Plínio I. M. Souza
Engº Agrônomo, Ph.D.
Embrapa Cerrados
Cx. Postal 08223
73301-970, Planaltina, DF
plinio@cpac.embrapa.br

Ricardo S. Balardin
Engº Agrônomo, Ph.D.
UFSM
Cx. Postal 5025
97111-970, Santa Maria, RS
balardin@balardin.com

Ricardo Barros
Engº Agrônomo, MS.c
Fundação MS
Cx. Postal 105
79150-000, Maracaju, MS
ricardobarros@cooagri.coop.br

Sergio A. da Silva
Biólogo
Embrapa Cerrados
Cx. Postal 08223
73301-970, Planaltina, DF
abud@cpac.embrapa.br

aplicações nos estádios R1/R2 (florescimento/ florescimento pleno) e reaplicações em R5.1 (início da formação de grãos) ou 21 dias após a primeira aplicação. Para aplicação dos produtos, foi utilizado pulverizador costal pressurizado com CO₂ e volume de aplicação de 150 a 200 L. ha⁻¹.

Foram realizadas avaliações da severidade da ferrugem no momento da aplicação dos produtos e semanalmente, da severidade de outras doenças, da desfolha quando a testemunha apresentava ao redor de 80%, da produtividade, nos 5m² centrais de cada parcela e do peso de 1000 sementes. As avaliações de severidade foram realizadas com auxílio de escalas diagramáticas (Martins et al., 2004; Godoy et al., 2006) para diminuir a variação da estimativa entre os locais. Para análise conjunta foram utilizadas as avaliações de severidade, realizada próximo a R6 (vagens com 100% de granação), e produtividade.

Foram realizados 19 ensaios com os produtos da Tabela 1 e 20 ensaios com os produtos da Tabela 2, nas principais regiões produtoras, por diferentes instituições (Tabela 3). A redução de produtividade, em porcentagem, foi calculada comparando a testemunha sem controle ao melhor tratamento de cada ensaio, sendo observada maior redução para os ensaios aplicados com sintomas, no momento da primeira aplicação, quando comparado com os ensaios aplicados sem sintomas, indicando maior agressividade da doença. Esse fato já era esperado uma vez que a ferrugem é uma doença policíclica (vários ciclos do fungo em um único ciclo do hospedeiro) e quanto mais cedo ocorre sua incidência na cultura maior a probabilidade de ocorrer redução de produtividade, caso as condições climáticas favoreçam a evolução da doença.

As maiores reduções de produtividade foram observadas nos ensaios realizados pela Embrapa Cerrados, em Planaltina, DF, e as menores nos ensaios realizados pela Universidade Federal de Tocantins, em Alvorada, TO (Tabela 3).

Os ensaios para a sumarização conjunta foram selecionados de acordo com critérios estatísticos e técnicos. Foram realizadas análises de variância exploratória, para cada local. Nas análises individuais foram verificadas a significância do efeito de blocos, o quadrado médio residual, o coeficiente de variação, a assimetria, a curtose, a normalidade da distribuição de resíduos (Shapiro & Wilk, 1965), a aditividade do modelo estatístico (Tukey, 1949) e a distribuição de variâncias, por meio do teste de Burr & Foster (1972).

Além dos critérios estatísticos, características técnicas como severidade da doença no tratamento testemunha em R6, correlações entre as variáveis severidade e produtividade e diferença estatística entre os tratamentos também auxiliaram na seleção dos ensaios. Para a lista de tratamentos com triazóis (Tabela 1) foram utilizados 13 ensaios na análise conjunta (locais 1, 2, 3, 6, 7, 8, 9, 10, 11, 13, 14, 17, 18), sendo sete com sintomas na primeira aplicação e seis sem sintomas na primeira aplicação. Para a lista de tratamentos com estrobilurinas e misturas (Tabela 2) foram utilizados 10 ensaios na sumarização conjunta (locais 1, 2, 3, 6, 7, 8, 9, 10, 11, 18) sendo cinco sem sintomas na primeira aplicação e cinco com sintomas. É importante ressaltar que mesmo uma aplicação sem sintomas pode ter sido realizada de forma curativa, uma vez que a ferrugem apresenta um período de incubação no qual não são observados sintomas, embora já esteja ocorrendo o processo de colonização do fungo na folha.

As análises exploratórias para cada local e as análises conjuntas dos resultados dos ensaios foram realizadas utilizando o teste de comparação múltiplas de médias Tukey ($p \leq 0,05$), no programa SAS/STAT® (2001).

Como os locais não foram os mesmos na análise dos dois grupos (Tabela 1 e 2) os resultados não podem ser comparados como um ensaio único, sendo a apresentação dos resultados e discussão realizada separadamente.

Triazóis

Na análise conjunta dos 13 ensaios, todos os tratamentos apresentaram severidade estatisticamente inferior à testemunha sem controle (Tabela 4). Os tratamentos que apresentaram a

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Silvania H. Furlan
Eng^a Agrônoma, Dra.
Instituto Biológico
Cx. Postal 70
13001-970, Campinas, SP
silvania@biologico.sp.gov.br

Walber L. Gavassoni
Eng^a Agrônomo, Ph.D.
UFGD
Cx Postal 533
79804-970, Dourados, MS
walber.gavassoni@ufgd.edu.br

Tabela 3. Instituições, locais, incidência de ferrugem na primeira aplicação, severidade e redução de produtividade nos ensaios com produtos da Tabela 1 e Tabela 2, para controle de ferrugem da soja. Safra 2006/07.

Instituição	Local	Primeira aplicação sem (0) e com sintomas (1)	Severidade média na primeira aplicação (%)	Redução de produtividade. Ensaio com produtos da Tabela 1 (%)*	Redução de produtividade. Ensaio com produtos da Tabela 2 (%)*
1. Embrapa Soja	Tamarana, PR	0	0	34	39
2. Embrapa Soja	Londrina, PR	1	1,2	68	72
3. Instituto Biológico	Paulinia, SP	1	0,5	32	24
4. Embrapa Cerrados	Planaltina, DF	1	4	82	74
5. MClamamoto	Ipameri, GO	1	2	48	37
6. Fundação MS	São Gabriel do Oeste, MS	0	0	30	38
7. Fundação MT	Campo Verde, MT	0	0	32	40
8. IAC/DDD	Capão Bonito, SP	1	2	61	64
9. Epamig	Uberaba, MG	1	0,1	43	40
10. CTPA	Senador Canedo, GO	1	3,18	56	69
11. CTPA	Goiânia, GO	0	0	34	39
12. UFT	Alvorada, TO	0	0	16	14
13. Embrapa Soja	Chapadão do Sul, MS	1	2	42	40
14. Embrapa Soja	Chapadão do Sul, MS	1	2	66	68
15. Embrapa Soja	Chapadão do Sul, MS	1	1	-	55
16. UFGD	Dourados, MS	1	0,5	24	22
17. Fundação BA	LEM, BA	0	0	43	31
18. Embrapa Soja	Riachão, MA	0	0	22	25
19. FAPA	Guarapuava, PR	1	0,1	51	52
20. UTFPR	Pato Branco, PR	1	0,1	42	54

* comparação entre a testemunha sem controle e o melhor tratamento.

Tabela 4. Severidade da ferrugem, próxima ao estágio R6, para os diferentes tratamentos (p.c. = produto comercial). Todos os locais (média de 13 ensaios), locais com a primeira aplicação realizada sem sintomas (média de seis ensaios) e locais com a primeira aplicação realizada com sintomas (média de sete ensaios). Safra 2006/07.

Tratamento (p.c.)	Ingrediente ativo e dose (L p.c. ha ⁻¹)	Severidade (%)		
		Todos locais	Sem sintomas	Com sintomas
1. Testemunha		59 A	54 A	63 A
2. Alto 100	ciproconazole	0,3	33 H	33 DEFG
3. Artea	ciproconazole + propiconazole	0,3	28 I	27 GHIJ
4. Score	difenoconazole	0,2	47 BC	43 BC
5. Soprano	epoxiconazole	0,4	43 CD	39 BCD
6. Virtue	epoxiconazole	0,4	38 EFG	28 FGHI
7. Palisade ¹	fluquinconazole	0,25	48 B	45 B
8. Impact ²	flutriafol	0,5	34 GH	31 EFGH
9. Systane	miclobutanil	0,4	47 BC	42 BC
10. Juno	propiconazole	0,5	40 DE	35 DEF
11. Rival	tebuconazole	0,5	24 IJ	22 IJK
12. Folicur	tebuconazole	0,5	23 J	22 IJK
13. Orius	tebuconazole	0,4	23 J	21 JK
14. Tebuconazole Nortox	tebuconazole	0,5	22 J	18 K
15. Eminent	tetraconazole	0,4	35 FGH	31 EFGH
16. Domark	tetraconazole	0,5	39 DEF	37 CDE
17. Caramba	metconazole	0,6	27 IJ	23 IJK
18. Proline ³	prothioconazole	0,3	23 J	24 HIJK
C.V.		18	20	17

Médias seguidas de mesma letra na coluna não diferem entre si pelo teste de Tukey ($p \leq 0,05$).

¹adicionado Aureo 250 mL.ha⁻¹; ²adicionado Oppa 1% v/v; ³produto em fase de registro no MAPA

maior severidade foram fluquinconazole (T7), difenoconazole (T4) e miclobutanil (T9). Os tratamentos com a menor severidade foram prothioconazole (T18), os quatro tratamentos com produtos comerciais à base de tebuconazole (T11, T12, T13 e T14) e metconazole (T17). A eficiência de controle para o melhor tratamento foi de 62%, sendo considerada baixa. A baixa eficiência de controle ocorreu devido ao número fixo de duas aplicações, estabelecidas no protocolo inicial, e longo intervalo para a realização da segunda aplicação, que foi realizada em R5 ou 21 dias após a primeira aplicação. As condições climáticas da safra 2006/07 favoreceram a evolução da doença na maioria dos locais, contribuindo para a diferenciação entre os produtos. A severidade apresentou alta correlação com a produtividade ($r = -0,95$) na análise utilizando os 13 locais.

Na análise, separando os ensaios com e sem sintomas no momento da aplicação, de maneira geral, os tratamentos mais e menos eficientes mantiveram-se no mesmo grupo estatístico da análise conjunta com os 13 ensaios, sendo observadas pequenas mudanças na ordem dos tratamentos. Mesmo nos ensaios sem sintomas na primeira aplicação, a evolução da doença foi suficiente para observar diferença de eficiência entre os tratamentos.

As maiores produtividades, na análise dos 13 ensaios, foram observadas para os tratamentos com prothioconazole (T18) e com tebuconazole (T12, T13 e T14) (Tabela 5). A

produtividade dos tratamentos com fluquinconazole (T7), difenoconazole (T4) e miclobutanil (T9) não diferiu estatisticamente da testemunha sem controle. A redução de produtividade, comparando a testemunha e o melhor tratamento, foi de 36%. Para os ensaios sem sintomas na aplicação a redução de produtividade foi de 27% e para os ensaios aplicados com sintomas, 47%. Para os ensaios realizados sem sintomas na primeira aplicação, as maiores produtividades foram observadas para os tratamentos com prothioconazole (T18), tebuconazole (T11, T12, T13 e T14) e ciproconazole + propiconazole (T3). Para os ensaios aplicados com sintomas, além dos tratamentos descritos anteriormente, ciproconazole (T2) e metconazole (T17) apresentaram as maiores produtividades.

A média de produtividade do tratamento testemunha nos ensaios aplicados sem sintomas foi de 2131 kg.ha⁻¹, sendo superior a média do tratamento testemunha nos ensaios aplicados com sintomas (1075 kg.ha⁻¹), evidenciando novamente a maior severidade da doença em função da ocorrência mais cedo em relação ao ciclo da cultura. As produtividades máximas observadas nos ensaios aplicados sem e com sintomas foram de 2936 e 2013 kg.ha⁻¹, respectivamente, evidenciando a necessidade de um maior número de aplicações para controle eficiente da doença. No entanto, o limitado número de aplicações favoreceu a diferenciação entre os produtos, sendo esse o objetivo dos ensaios em rede.

Tabela 5. Produtividade para os diferentes tratamentos (p.c. = produto comercial). Todos os locais (média de 13 ensaios), locais com a primeira aplicação realizada sem sintomas (média de seis ensaios) e locais com a primeira aplicação realizada com sintomas (média de sete ensaios). Safra 2006/07.

Tratamento (p.c.)	Ingrediente ativo e dose (L p.c. ha ⁻¹)	Produtividade (kg.ha ⁻¹)		
		Todos locais	Sem sintomas	Com sintomas
1. Testemunha		1562 I	2131 H	1075 H
2. Alto 100	ciproconazole 0,3	2163 CD	2600 CDEF	1788 ABC
3. Artea	ciproconazole + propiconazole 0,3	2211 BCD	2695 ABC	1796 ABC
4. Score	difenoconazole 0,2	1706 GHI	2256 GH	1235 GH
5. Soprano	epoxiconazole 0,4	1857 FG	2396 DEFGH	1396 EFG
6. Virtue	epoxiconazole 0,4	1823 FGH	2354 EFGH	1520 DEF
7. Palisade ¹	fluquinconazole 0,25	1643 HI	2164 H	1271 FGH
8. Impact ²	flutriafol 0,5	2120 DE	2632 BCDE	1682 BCD
9. Systane	miclobutanil 0,4	1739 GHI	2235 H	1441 DEFG
10. Juno	propiconazole 0,5	1892 FG	2310 FGH	1557 CDE
11. Rival	tebuconazole 0,5	2220 BCD	2668 ABCD	1900 AB
12. Folicur	tebuconazole 0,5	2382 AB	2936 A	1908 AB
13. Orius	tebuconazole 0,4	2319 ABC	2812 ABC	1897 AB
14. Tebuconazole Nortox	tebuconazole 0,5	2254 ABCD	2800 ABC	1865 AB
15. Eminent	tetraconazole 0,4	1855 FG	2384 DEFGH	1553 CDE
16. Domark	tetraconazole 0,5	1967 EF	2548 CDEFG	1469 DEFG
17. Caramba	metconazole 0,6	2092 DE	2632 BCDE	1784 ABC
18. Proline ³	prothioconazole 0,3	2430 A	2917 AB	2013 A
C.V. (%)		13	11	16

Médias seguidas de mesma letra na coluna não diferem entre si pelo teste de Tukey ($p \leq 0,05$)

¹adicionado Aureo 250 mL.ha⁻¹; ²adicionado Oppa 1% v/v; ³produto em fase de registro no MAPA

Estrobilurinas e misturas

Na análise conjunta dos 10 ensaios com estrobilurinas e misturas de triazóis com estrobilurinas e triazóis com benzimidazóis, todos os tratamentos apresentaram severidade estatisticamente inferior à testemunha sem controle (Tabela 6). Os tratamentos que apresentaram a maior severidade foram azoxistrobina (T2), picoxistrobina (T3), propiconazole + trifloxistrobina (T7) e flutriafol + tiofanato metílico (T9). Os tratamentos com a menor severidade, sendo estatisticamente diferentes dos demais, foram tebuconazole + trifloxistrobina (T8), ciproconazole + azoxistrobina (T4), ciproconazole + trifloxistrobina (T5), ciproconazole + picoxistrobina (T12) e o triazol tebuconazole (T13). A maior eficiência de controle foi de 70%, sendo considerada baixa. A baixa eficiência de controle ocorreu devido ao número fixo de duas aplicações, estabelecidas no protocolo inicial, e longo intervalo para a realização da segunda aplicação, que foi realizada em R5.1 ou 21 dias após a primeira aplicação. A avaliação de severidade apresentou alta correlação com produtividade ($r = -0,94$).

Na análise separando os ensaios sem e com sintomas no momento da aplicação, os tratamentos com a menor severidade foram tebuconazole + trifloxistrobina (T8), tebuconazole (T13) e ciproconazole + trifloxistrobina (T5), para os ensaios aplicados sem sintomas, e ciproconazole + azoxistrobina (T4), ciproconazole + trifloxistrobina (T5), ciproconazole + picoxistrobina (T12) e tebuconazole + trifloxistrobina (T8),

para os ensaios com sintomas na primeira aplicação. A análise separando os ensaios de acordo com a incidência no momento da aplicação foi semelhante à análise usando todos os ensaios, sendo observada somente inversão na ordem dos tratamentos com as menores severidades.

Todos os tratamentos apresentaram produtividade estatisticamente superior ao tratamento testemunha (Tabela 7), sendo as maiores produtividades observadas para os tratamentos com azoxistrobina + ciproconazole (T4), picoxistrobina + ciproconazole (T12), ciproconazole + trifloxistrobina (T5) e tebuconazole (T13), considerando a análise com todos os 10 locais. Para os ensaios aplicados sem sintomas os tratamentos com epoxiconazole + piraclostrobina (T6) e tebuconazole + trifloxistrobina (T8) também apresentaram as maiores produtividades, juntamente com os produtos descritos anteriormente. Na análise dos ensaios aplicados com sintomas, as maiores produtividades foram observadas para os tratamentos com ciproconazole + azoxistrobina (T4), picoxistrobina + ciproconazole (T12), tebuconazole + trifloxistrobina (T8), tebuconazole (T13), ciproconazole + trifloxistrobina (T5), picoxistrobina (T3).

De maneira semelhante à observada nos ensaios com triazóis (Tabela 5), para os ensaios com estrobilurinas e misturas, a média de produtividade do tratamento testemunha nos ensaios aplicados sem sintomas foi superior a média do tratamento testemunha nos ensaios aplicados com sintomas, sendo de

Tabela 6. Severidade da ferrugem, próxima ao estágio R6, para os diferentes tratamentos (p.c. = produto comercial). Todos os locais (média de 10 ensaios), locais com a primeira aplicação realizada sem sintomas (média de cinco ensaios) e locais com a primeira aplicação realizada com sintomas (média de cinco ensaios). Safra 2006/07.

Tratamento (p.c.)	Ingrediente ativo e dose (L p.c. ha ⁻¹)	Severidade (%)		
		Todos locais	Sem sintomas	Com sintomas
1. Testemunha		61 A	62 A	60 A
2. Piori ¹	azoxistrobina 0,2	32 B	36 B	28 BC
3. Aproach ¹	picoxistrobina 0,2	29 BCD	33 BC	25 CD
4. Piori Xtra ¹	ciproconazole + azoxistrobina 0,3	19 E	22 EF	15 G
5. Sphere ²	ciproconazole + trifloxistrobina 0,3	19 E	22 FG	17 FG
6. Opera	epoxiconazole + piraclostrobina 0,5	26 CD	29 CD	24 CDE
7. Stratego ²	propiconazole + trifloxistrobina 0,4	29 BC	25 DEF	32 B
8. Nativo ³	tebuconazole + trifloxistrobina 0,5	18 E	16 G	19 EFG
9. Impact Duo ⁴	flutriafol + tiofanato metílico 0,6	29 BCD	33 BC	25 CD
10. Celeiro ⁵	flutriafol + tiofanato metílico 0,6	28 CD	33 BC	22 DE
11. Battle ⁴	flutriafol + carbendazim 0,6	25 D	28 CDE	22 DE
12. Alto 100 + Aproach ¹	ciproconazole + picoxistrobina 0,24 + 0,24	20 E	22 EF	17 FG
13. Folicur	tebuconazole 0,5	21 E	21 FG	21 DEF
CV		19	20	17

Médias seguidas de mesma letra na coluna não diferem entre si pelo teste de Tukey (ps0,05).

¹adicionado Nimbus 0,5% v/v; ²adicionado Áureo 250 mL.ha⁻¹; ³adicionado Áureo 0,25% v/v; ⁴adicionado Oppa 1% v/v; ⁵adicionado Iharol 1% v/v

2238 kg.ha⁻¹ e 1227 kg.ha⁻¹, respectivamente, evidenciando novamente a maior agressividade da doença em função da incidência mais cedo em relação ao ciclo da cultura.

A amplitude de diferença de produtividade entre os tratamentos com triazóis (Tabela 5) foi maior quando comparada com os tratamentos com estrobilurinas e misturas (Tabela 7). Considerando somente as análises com todos locais, para os triazóis (Tabela 5), a diferença entre a produtividade do tratamento mais eficiente (prothioconazole - T18) e menos eficiente (fluquinconazole - T7) foi de 787 kg.ha⁻¹, sem considerar a testemunha sem aplicação de fungicida. Para os tratamentos da Tabela 2, a diferença entre a produtividade do tratamento mais eficiente (ciproconazole + azoxistrobina - T4) e menos eficiente (propiconazole + trifloxistrobina - T7) foi de 575 kg.ha⁻¹ (Tabela 7).

As análises conjuntas foram separadas em sem e com sintomas na primeira aplicação, mas é importante ressaltar que mesmo uma aplicação sem sintomas pode ter sido curativa, considerando o período de incubação (infecção até a manifestação dos sintomas) do fungo e que a doença pode ocorrer em qualquer estágio fenológico da cultura.

Embora os resultados obtidos para os tratamentos das Tabelas 1 e 2 não sejam comparáveis, devido aos locais envolvidos na análise serem diferentes, pode-se observar que o tratamento com tebuconazole (Folicur®), comum nas duas listas, ficou no melhor agrupamento nos dois ensaios, permitindo comparar o agrupamento com a maior eficiência de controle das duas listas. Desta forma, os tratamentos com as maiores eficiências de controle, de acordo com os ensaios em rede realizados na safra 2006/07, foram prothioconazole (T18), os quatro tratamentos com produtos comerciais à base de

tebuconazole (T11, T12, T13 e T14) e metconazole (T17) da Tabela 1 e tebuconazole + trifloxistrobina (T8), ciproconazole + azoxistrobina (T4), ciproconazole + trifloxistrobina (T5), ciproconazole + picoxistrobina (T12) e o triazol tebuconazole (T13), da Tabela 2, considerando a variável severidade na análise conjunta dos 13 e 10 ensaios. A variável severidade apresentou alta correlação com produtividade ($r=-0,95$ para os ensaios com triazóis e $r=-0,94$ para os ensaios com estrobilurinas e misturas), indicando que a ferrugem foi o principal fator que causou redução da produtividade, nos ensaios analisados.

Sintomas de fitotoxicidade, do tipo clorose internerval ("folha carijó"), foram observados em alguns locais para os tratamentos com tebuconazole, prothioconazole e metconazole, o que, aparentemente, não comprometeu a produtividade nos ensaios, uma vez que os tratamentos com tebuconazole e prothioconazole apresentaram as maiores produtividades.

Embora ocorram diferentes grupos de eficiência na análise conjunta dos resultados, é importante salientar que os produtos podem ter a mesma eficiência no campo, em condições de baixa pressão da doença. Essa diferença na eficiência dos produtos é mais fácil de ser observada em situações onde a ferrugem ocorre de forma mais severa. O delineamento dos ensaios em rede e a seleção dos ensaios para a sumarização conjunta têm como objetivo selecionar os locais onde a doença foi mais agressiva para verificar a diferença entre produtos nas situações mais críticas. Apesar da maior eficiência de controle observada para alguns produtos, esse fato não implica em flexibilidade de aplicação. Os produtos devem ser utilizados nos sintomas iniciais (traços da doença) ou preventivamente, levando em conta os fatores necessários ao aparecimento da ferrugem (presença do fungo na

Tabela 7. Produtividade para os diferentes tratamentos (p.c. = produto comercial). Todos os locais (média de 10 ensaios), locais com a primeira aplicação realizada sem sintomas (média de cinco ensaios) e locais com a primeira aplicação realizada com sintomas (média de cinco ensaios). Safra 2006/07.

Tratamento (p.c.)	Ingrediente ativo e dose (L p.c. ha ⁻¹)	Produtividade (kg.ha ⁻¹)		
		Todos locais	Sem sintomas	Com sintomas
1. Testemunha		1733 G	2238 F	1227 G
2. Priori ¹	azoxistrobina 0,2	2431 EF	2973 DE	1889 DEF
3. Aproach ¹	picoxistrobina 0,2	2511 CDE	2999 CDE	2022 ABCDE
4. Priori Xtra ¹	ciproconazole + azoxistrobina 0,3	2843 A	3441 A	2245 A
5. Sphere ²	ciproconazole + trifloxistrobina 0,3	2684 ABC	3286 ABCD	2082 ABCD
6. Opera	epoxiconazole + piraclostrobina 0,5	2595 CDE	3225 ABCDE	1966 BCDEF
7. Stratego ²	propiconazole + trifloxistrobina 0,4	2268 F	2915 E	1750 F
8. Nativo ³	tebuconazole + trifloxistrobina 0,5	2606 BCDE	3171 ABCDE	2155 ABC
9. Impact Duo ⁴	flutriafol + tiofanato metílico 0,6	2473 DE	3097 BCDE	1848 DEF
10. Celeiro ⁵	flutriafol + tiofanato metílico 0,6	2441 EF	3053 CDE	1828 EF
11. Battle ⁴	flutriafol + carbendazin 0,6	2502 CDE	3073 BCDE	1931 CDEF
12. Alto 100 + Aproach ¹	ciproconazole + picoxistrobina 0,24 + 0,24	2803 AB	3403 AB	2203 AB
13. Folicur	tebuconazole 0,5	2668 ABCD	3313 ABC	2152 ABC
CV		10	11	12

Médias seguidas de mesma letra na coluna não diferem entre si pelo teste de Tukey (p<0,05).

¹adicionado Nimbus 0,5% v/v; ²adicionado Áureo 250 mL.ha⁻¹; ³adicionado Áureo 0,25% v/v; ⁴adicionado Oppa 1% v/v; ⁵adicionado Itharol 1% v/v

região, idade das plantas e condição climática favorável), a logística de aplicação (disponibilidade de equipamentos e tamanho da propriedade), a presença de outras doenças e o custo do controle. As reaplicações dos produtos devem levar em conta o monitoramento da doença na lavoura, uma vez que o residual pode variar entre produtos e em função das condições climáticas.

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SYNGENTA EXHIBIT 1038

SYNGENTA v. UPL, PGR2023-00017

APLICAÇÕES DE FUNGICIDAS VISANDO O CONTROLE DA FERRUGEM ASIÁTICA (*Phakopsora pachyrhizi*) DA SOJA, ESTADO SÃO PAULO, SAFRA 2010/11

FURLAN, S.H.¹; FONTES, J.C.²; FRANCO, D.A.S.¹

¹ Instituto Biológico, C. P. 70, CEP 13.012-970, silvania@biologico.sp.gov.br; ² Plantec, C.P. 39, Itacemápolis, SP.

A ferrugem asiática (*P. pachyrhizi*) constitui-se na doença que mais prejuízos tem ocasionado na cultura da soja desde a safra 2001/02. A principal medida para o seu controle tem sido o uso de fungicidas aplicados em parte aérea. Através de ensaios cooperativos em diversas regiões produtoras do país tem-se avaliado por várias safras a eficiência de misturas de triazóis + estrobilurinas e triazóis isolados. O objetivo desse trabalho foi verificar o comportamento de novos fungicidas e aqueles já recomendados no controle desta doença e no rendimento da cultura.

O ensaio foi conduzido com a cultivar BMX Potência RR, município de Itacemápolis, SP, com semeadura realizada tardiamente, em 28/01/11, visando obter maior pressão de uredosporos durante o cultivo, visto que a ocorrência da doença foi tardia nesta safra. Os produtos e doses utilizadas constam na Tabela 1, sendo aplicados com pulverizador costal a base de CO₂, volume de 200 L/ha, em dois estádios de desenvolvimento da cultura, R1 (pré-florescimento) e R4 (crescimento de vagens).

A doença foi avaliada pela severidade das plantas (% de área foliar atacada) na área útil da parcela (duas linhas centrais), nos estádios R5.2; R5.3 e R5.4; AACPD e porcentagem de desfolha em R6. Realizou-se a pesagem das parcelas colhidas em 5 m² para a determinação da produtividade e do peso de 1.000 grãos. Ainda, atribuíram-se notas de 1 a 4, de acordo com o grau de fitotoxicidade, onde 1= sem sintomas, 2= sintoma leve, 3= sintoma moderado e 4= sintoma severo.

Foi utilizado o delineamento experimental de blocos ao acaso, com 18 tratamentos e quatro repetições. Os dados foram interpretados estatisticamente por

meio de análise de variância e as médias comparadas pelo teste de Scott-Knott, a 5% de probabilidade.

As condições climáticas foram favoráveis ao desenvolvimento da ferrugem asiática, com irrigações complementares no período crítico da cultura. A severidade da doença alcançou 86,0% nas plantas testemunhas em R5.4, enquanto os tratamentos com fungicidas apresentaram valores entre 15,3% a 60,8%. Os maiores valores foram apresentados pelos tratamentos com os triazóis isolados, Alto 100 e Folicur. Entre as misturas, as mais eficientes foram Horos + Nimbus, Fox + Áureo, Aproach Prima + Nimbus e Opera + Assist com as maiores porcentagens de controle relativo (72,4 % a 83,7 %), notadamente as duas primeiras, conforme confirma a AACPD (Tabela 2).

Quanto à desfolha, Folicur e Alto 100 apresentaram os maiores valores. Os tratamentos com as menores porcentagens foram Horos + Nimbus, Aproach Prima + Nimbus, Opera + Assist, NTX 3200 + Assist e ISB021 F + Nimbus (Tabela 3).

Foram proporcionados acréscimos significativos (45,1 % a 92,7 %) de produtividade pela aplicação dos fungicidas. Os maiores valores foram obtidos por Aproach Prima + Nimbus, Piori Xtra + Nimbus e Azimut + Nimbus (Tabela 3).

Os pesos de 1000 grãos dos tratamentos com fungicidas foram superiores à testemunha. Com exceção da mistura de Nativo + Áureo, as demais foram superiores aos triazóis isolados (Tabela 3).

Houve fitotoxicidade de alguns fungicidas nas plantas de soja da cv. BMX Potência RR, principalmente pelos tratamentos de números 14, 17 e 18, com notas de 3 a 4, seguidos dos tratamentos 11 e 16, ambos com nota 2,5 (Figura 4).

Tabela 1. Produtos e doses utilizados na cultura da soja, cv. BMX Potência RR, para o controle da ferrugem asiática, em duas aplicações, em R1 e R4. Iracemápolis, SP. 2011.

Tratamento	Ingrediente ativo	Dose <i>L p.c. ha⁻¹</i>
1 Testemunha	-----	---
2 Folicur	tebuconazol	0,50
3 Alto 100	ciproconazol	0,30
4 Priori Xtra + Nimbus (0,5% v/v)	azoxistrobina & ciproconazol	0,30
5 Opera + Assist (0,5 L/ha)	piraclostrobina & epoxiconazol	0,50
6 Nativo + Aureo (0,5 L/ha)	trifloxistrobina & tebuconazol	0,50
7 Aproach Prima + Nimbus (0,45 L/ha)	picoxistrobina & ciproconazol	0,30
8 SphereMax + Aureo (0,5 L/ha)	trifloxistrobina & ciproconazol	0,15
9 ISB021F (Domark XL) + Nimbus (0,5 L/ha)	azoxistrobina & tetraconazol	0,50
10 Fox + Aureo (0,4 L/ha)	trifloxistrobina & prothioconazol	0,40
11 BAS 556 01F+ Assist (0,5 L/ha)	piraclostrobina & metconazol	0,50
12 ENVOY + Lanzar (0,3% v/v)	piraclostrobina & epoxiconazol	0,60
13 NTX 3200 + Assist (0,5% v/v)	azoxistrobina	0,35
14 NTX 3900 + Assist (0,5% v/v)	azoxistrobina & tebuconazol	0,50
15 Plantvax 750 WP + Silwett (0,05%)	oxicarboxim	1,20
16 Azimut + Nimbus (0,5% v/v)	azoxistrobina & tebuconazol	0,50
17 Horos + Nimbus (0,5% v/v)	picoxistrobina & tebuconazol	0,50
18 MILFF 0453 + Nimbus (0,5% v/v)	azoxistrobina & epoxiconazol	0,60

Tabela 2. Porcentagem de severidade da ferrugem asiática da soja, AACPD e porcentagem de eficácia relativa. Iracemápolis, SP. 2011.

Tratamentos	Dose <i>L p.c. ha⁻¹</i>	Severidade Ferrugem Asiática			AACPD	Eficácia relativa %
		R5.2	R5.3	R5.4		
1. Testemunha	-	52,5 a	75,0 a	86,0 a	1310,0 a	-
2. Folicur	0,50	28,8 b	41,3 b	57,3 b	791,0 b	41,6
3. Alto 100	0,30	32,5 b	45,0 b	60,8 b	854,4 b	36,5
4. Priori Xtra + Nimbus (0,5% v/v)	0,30	18,5 c	26,8 c	36,5 c	507,9 c	62,4
5. Opera + Assist (0,5 L/ha)	0,50	15,0 d	19,0 d	26,5 d	371,0 d	72,4
6. Nativo + Aureo (0,5 L/ha)	0,50	21,3 c	32,0 c	42,8 c	598,2 c	55,6
7. Aproach Prima + Nimbus (0,45 L/ha)	0,30	11,0 d	15,0 d	20,3 d	285,6 d	78,8
8. SphereMax + Aureo (0,5 L/ha)	0,15	19,3 c	27,5 c	36,5 c	515,6 c	61,6
9. ISB021F (Domark XL) + Nimbus (0,5 L/ha)	0,50	17,5 c	25,3 c	33,0 c	469,4 c	65,0
10. Fox + Aureo (0,4 L/ha)	0,40	13,8 d	12,3 d	18,5 d	263,9 d	80,3
11. BAS 556 01F+ Assist (0,5 L/ha)	0,50	12,3 d	16,3 d	22,0 d	311,2 d	76,9
12. ENVOY + Lanzar (0,3% v/v)	0,60	20,8 c	29,0 c	38,0 c	541,8 c	59,5
13. NTX 3200 + Assist (0,5% v/v)	0,35	20,0 c	30,0 c	40,3 c	562,1 c	58,3
14. NTX 3900 + Assist (0,5% v/v)	0,50	14,8 d	22,5 c	29,5 c	415,8 c	69,1
15. Plantvax 750 WP + Silwett (0,05%)	1,20	30,8 b	43,8 b	61,3 b	843,5 b	37,8
16. Azimut + Nimbus (0,5% v/v)	0,50	19,5 c	26,8 c	32,3 c	482,0 c	63,5
17. Horos + Nimbus (0,5% v/v)	0,50	9,3 d	11,3 d	15,3 d	218,8 d	83,7
18. MILFF 0453 + Nimbus (0,5% v/v)	0,60	19,0 c	28,5 c	34,5 c	507,5 c	61,7
CV (%)	-	13,24	10,99	9,04	10,6	-

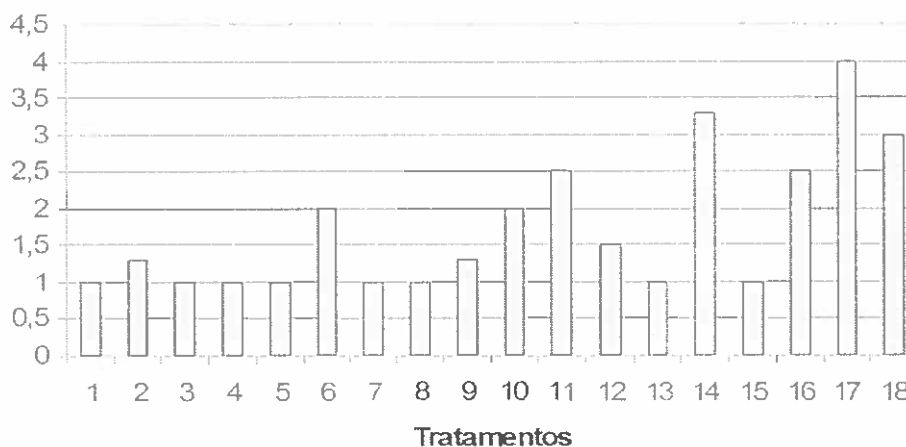
Médias seguidas pela mesma letra não diferem entre si pelo teste Scott-Knott.

Tabela 3. Efeito de fungicidas na porcentagem de desfolha, na produtividade (kg/ha) e no peso de 1000 grãos de soja (g).

Tratamentos	Dose <i>L p.c. ha⁻¹</i>	Desfolha	Produtividade <i>kg ha⁻¹</i>	Peso 1000 grãos <i>g</i>
		--- R6 --- %		
1. Testemunha	-	85,0 a	858 e	155,0 c
2. Folicur	0,50	53,8 b	1252 c	169,0 b
3. Alto 100	0,30	64,5 b	1245 c	169,7 b
4. Priori Xtra + Nimbus (0,5% v/v)	0,30	26,3 c	1579 a	171,1 b
5. Opera + Assist (0,5 L/ha)	0,50	20,0 d	1448 b	177,1 a
6. Nativo + Aureo (0,5 L/ha)	0,50	32,0 c	1502 b	168,2 b
7. Aproach Prima + Nimbus (0,45 L/ha)	0,30	16,3 d	1653 a	176,3 a
8. SphereMax + Aureo (0,5 L/ha)	0,15	30,0 c	1335 c	182,1 a
9. ISB021F (Domark XL) + Nimbus (0,5 L/ha)	0,50	21,3 d	1253 c	177,5 a
10. Fox + Aureo (0,4 L/ha)	0,40	27,5 c	1522 b	178,9 a
11. BAS 556 01F+ Assist (0,5 L/ha)	0,50	23,8 c	1323 c	187,1 a
12. ENVOY + Lanzar (0,3% v/v)	0,60	27,0 c	1505 b	175,5 a
13. NTX 3200 + Assist (0,5% v/v)	0,35	22,0 d	1245 c	176,5 a
14. NTX 3900 + Assist (0,5% v/v)	0,50	26,3 c	1367 b	179,2 a
15. Plantvax 750 WP + Silwett (0,05%)	1,20	62,0 b	1049 d	165,5 b
16. Azimut + Nimbus (0,5% v/v)	0,50	25,5 c	1557 a	177,1 a
17. Horos + Nimbus (0,5% v/v)	0,50	16,3 d	1452 b	180,0 a
18. MILFF 0453 + Nimbus (0,5% v/v)	0,60	25,0 c	1494 b	178,9 a
CV (%)	-	11,09	9,71	4,47

A.R. (Aumento relativo): Médias seguidas pela mesma letra não diferem entre si pelo teste Scott-Knott.

Notas de Fitotoxicidade (1 a 4)

**Figura 1.** Fitotoxicidade (notas de 1 a 4, onde 1= sem sintoma; 2= sintoma leve; 3= sintoma moderado e 4= sintoma severo) atribuída ao efeito dos fungicidas nas plantas de soja, cultivar BMX Potência RR, para cada tratamento (1 a 18).

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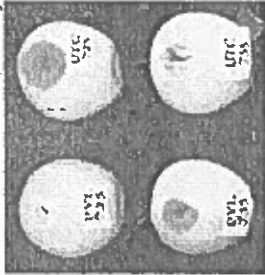
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Monitoring Methods

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To establish a baseline using bioassay procedures, a reliable technique must be developed. The technique must also be able to be used in subsequent monitoring operations investigating possible development of resistance. If and when resistance develops, the same method may be used to identify resistant isolates, but it is possible that more specific methods will have been developed at that time.

In a joint effort to create standardized methods, members of different FRAC Working Groups have reviewed and collected detailed, ready-to-use bioassay techniques to monitor fungal pathogens of economic importance.



The following requirements were considered while establishing these techniques:

- The method must be robust, reliable and repeatable
- It must be as simple as possible to operate in terms of technology and user skills
- It should be as cheap to operate as possible and capable of a high throughput in a short time

- The data obtained must be able to be related to sensitivity responses in the field

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NOTAS CIENTÍFICAS

Efeito de óleo essencial de laranja associados a fungicidas no controle de doenças foliares do trigo

Effect of orange essential oil associated with fungicides to control wheat leaf diseases

Cezar Coradini¹ *

Fernando Piccinini¹

Geovane Boschmann Reimche¹

Ivan Francisco Dressler da Costa¹

Sérgio Luiz de Oliveira Machado¹

¹Departamento de Defesa Fitossanitária, Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, Rio Grande do Sul (RS), Brasil.

RESUMO

Foi conduzido um experimento em campo na safra de 2012 com o objetivo de avaliar a contribuição do óleo essencial de laranja na performance dos fungicidas visando o controle de doenças foliares do trigo. O delineamento experimental foi o de blocos ao acaso em arranjo fatorial $(3 \times 5) + 1$, com quatro repetições. O fator A foi composto pelos fungicidas (dose do produto comercial) epoxiconazol $(0,75 \text{ L ha}^{-1})$, piraclostrobina $(0,80 \text{ L ha}^{-1})$ e epoxiconazol+piraclostrobina $(0,75 \text{ L ha}^{-1})$ e o fator B pelos adjuvantes (doses) óleo mineral (500 mL ha^{-1}) e óleo essencial de laranja $(0, 50, 100 \text{ e } 150 \text{ mL ha}^{-1})$. O tratamento adicional compreendeu-se pela testemunha (água). O controle de manchas foliares na folha bandeira foi mais eficaz com a aplicação de epoxiconazol e de epoxiconazol+piraclostrobina independentemente do adjuvante usado ser óleo mineral ou óleo essencial de laranja, enquanto que os adjuvantes testados não interferiram no controle da ferrugem. O peso do hectolítro, massa de mil grãos e a produtividade do trigo não foram afetados negativamente pelas diferentes concentrações do óleo essencial de laranja.

Palavras-chave Triticum aestivum L.; manchas foliare; ferrugem da folha; adjuvantes

ABSTRACT

A field experiment was conducted during the 2012 growing season with the aim of evaluating the contribution of orange essential oil to the performance of fungicides on the control of wheat leaf diseases. The experimental design was in randomized blocks, as factorial arrangement $(3 \times 5) + 1$, and four replicates. Factor A was composed of the fungicides (dose of the commercial product) epoxiconazole (0.75 L ha^{-1}) , pyraclostrobin (0.80 L ha^{-1}) and epoxiconazole+pyraclostrobin (0.75 L ha^{-1}) and factor B was composed of the adjuvants (doses) mineral oil (500 mL ha^{-1}) and orange essential oil $(0, 50, 100 \text{ and } 150 \text{ mL ha}^{-1})$. The additional treatment was represented by a control (distilled water). Flag leaf spot control was more efficient with the use of epoxiconazole and epoxiconazole+pyraclostrobin whether the used adjuvant was mineral oil or orange essential oil, while the tested adjuvants did not affect rust control. The hectoliter weight, mass of one thousand grains and wheat yield were not negatively affected by the different concentrations of orange essential oil.

Keywords Triticum aestivum L.; leaf spots; leaf rust; adjuvants

The most common foliar diseases in wheat crop are brown spot (*Cochliobolus sativus* (Ito; Kurib.), Drechs eg Dastur. (Anamorph: *Bipolaris sorokiniana* Sacc. In. Sorok.), Speckled spot (*Septoria tritici* Roberge in Desmaz (teleomorph: *Mycosphaerella graminicola* (Fuckel) J. Schöt. In Chon) and leaf rust (*Puccinia recondita* Rob. Ex.

Desm. F.sp. *tritici* Heriks. & Henn). up to 80% in productivity. ⁽⁴⁾ Adjuvants are used to modify the physical and biological properties of spray mixtures to enhance the performance of pesticides. ⁽³⁾ Recently, natural adjuvants based on essential oils extracted from the peel of oranges (*Citrus* spp. L.) have been developed. However, there is a lack of information on their efficiency when associated with fungicides for disease control.

In this context, this study aimed to evaluate the effect of orange essential oil associated with epoxiconazole, piraclostrobin and epoxiconazole + piraclostrobin fungicides on leaf rust and leaf spot complex control and on wheat yield. The experiment was conducted in the 2012 agricultural harvest in the city of Itaara, (29 ° 32' S, 53 ° 45' W;), RS, Brazil using a randomized complete block design in factorial arrangement (3x5 + 1) with four repetitions. Each experimental unit consisted of 12 rows of wheat (cv. Quartz ⁽⁸⁾) 0.17m apart and 5.0m long. Factor A was composed of the fungicides (doses) epoxiconazole (Opus ⁽⁹⁾ SC) (0.75L ha⁻¹), piraclostrobin (Comet ⁽⁸⁾ CE) (0.80L ha⁻¹) and epoxiconazole + piraclostrobin (Opera ⁽⁸⁾ SE) (0.75L ha⁻¹) and factor B by adjuvants (doses): mineral oil (Assist ⁽⁸⁾) (500ml ha⁻¹) and orange essential oil doses (Orobor ⁽⁸⁾) (0, 50, 100 and 150ml ha⁻¹), with additional treatment represented by the control (water). Two treatments were applied with the aid of a CO₂ pressure sprayer, with an application bar with four XR 11002 tips spaced 0.5 m apart.

Wheat Flowering ⁽⁵⁾. Disease severity was obtained through the percentage of affected area in 25 flag leaves and flag -1, randomly evaluating 25 flag leaves and 25 flag leaves -1 in the useful area of the plots. Evaluations were performed at 15, 22, 29, 36 and 43 days after the first application (DAA). Grain mass (13% humidity), hectoliter weight (PH) and thousand grain mass (MMG) were determined. Data were subjected to tests of the assumptions of the mathematical model and submitted to analysis of variance (ANOVA) (p≤0.05). Means were compared by t-test (p≤0.05) and control (water) was compared to means using Dunnett test (p≤0.05).

The analysis of variance showed that there was a significant interaction (p≤0.05) between the studied factors for area below the leaf spot progress curve (AACPMFb) (Table 1), epoxiconazole + piraclostrobin fungicides differed significantly from non-adjuvant treatment regardless of the adjuvant in use (mineral oil and orange essential oil). In the concentrations (50, 100 and 150mL ha⁻¹) of orange essential oil in the spray syrup there was control of leaf stems, not differing (p≤0,05) from mineral oil treatment. The difference in efficacy of epoxiconazole + piraclostrobin with different adjuvant treatments compared to treatment without adjuvant may be due to the effect of the adjuvant that second ⁽²⁾, serve to provide greater foliage coverage, promoting better protection. For rust area below the curve progress flag leaf (AACPFb), area under the curve rust progress flag leaf ⁽¹⁾ (AACPFb⁻¹) and the area under the flag leaf spot progress curve⁻¹ (AACPMFb⁻¹), the interaction was not significant (data not shown). AACPFb, AACPFb⁻¹ and AACPMFb did not differ statistically between the fungicides and adjuvants applied, but showed better performance when compared to the control (water). The

adjuvants tested did not differ ($p \leq 0.05$) for PH, MMG and grain yield (data not shown), however, differed from control (water). Mineral adjuvants may have a phytotoxic effect on plants and may reduce growth and interfere with plant development⁽¹⁾. Thus, these results show that the replacement of mineral oil with orange essential oil (50 to 150mL ha⁻¹) in the syrup. spraying does not change the control of the set of diseases analyzed, as well as the PH, MMG and the grain yield of wheat.

Table 1 Area below the progress curve of leaf blemishes on the wheat flag leaf in response to fungicides and different concentrations of orange essential oil in the spray solution.

Treatments	Adjuvants					
	SA 4	OM 5 ₅₀₀	OEL 6 ₅₀	OEL 6 ₁₀₀	OEL 6 ₁₅₀	Average
Epoxiconazole 1	B 15 bA	B 12 bA	B 15 bA	B 17 tab	B 13 aa	15 b
Piraclostrobin 2	B 22 tab	B 24 aA	B 25 aA	B 24 aA	B 17 aa	23 to
Epoxiconazole + Piraclostrobin 3	B 27 aA	B 12 bB	B 12 bB	B 12 bB	B 13 ab	15 b
Control (water)	A 373					
Average	22 to	16 to	17 ab	17 ab	14 b	17.6
CV (%)	14.7					

* means followed by the same uppercase letter in the rows and lowercase in the columns do not differ from each other by the t test ($p \leq 0.05$). Means not preceded by the same capital letter differ from the control (water) by Dunnett's test ($p \leq 0.05$);

- 1 Opus® SC;
- 2 Comet® EC;
- 3 Opera® SE;
- 4 without adjuvant;
- 5 mineral oil (Assist®);
- 6 orange essential oil (Orobor®).

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Autor para correspondência: Cezar Coradini (cezarcoradini@yahoo.com.br)



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FCA/UNESP - Depto. De Produção Vegetal
Caixa Postal 237
18603-970 - Botucatu, SP Brasil
Tel.: (55 14) 3811 7262
Fax: (55 14) 3811 7206



summa.phyto@gmail.com

Winter wheat (*Triticum aestivum* L.) tolerance to mixtures of herbicides and fungicides applied at different timings

Melody A. Robinson¹, Michael J. Cowbrough², Peter H. Sikkema³, and François J. Tardif^{1,4}

¹Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada N1G 2W1;

²Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, Ontario, Canada N1G 4Y2; and

³University of Guelph Ridgetown Campus, Ridgetown, Ontario, Canada N0P 2C0.

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Robinson, M. A., Cowbrough, M. J., Sikkema, P. H. and Tardif, F. J. 2013. Winter wheat (*Triticum aestivum* L.) tolerance to mixtures of herbicides and fungicides applied at different timings. *Can. J. Plant Sci.* 93: 491–501. Farmers commonly tank-mix herbicides and fungicides to reduce application costs. In the spring of 2008, there were reports of winter wheat injury with the application of herbicide–fungicide tank-mixes early in the growing season. This study was established to determine the tolerance of winter wheat to herbicide–fungicide mixtures as influenced by time of application. Field studies were conducted at four Ontario locations in 2009 and 2010 with three herbicides and four fungicides. Herbicide–fungicide tank-mixes were applied early, under cold conditions, and late at growth stage Zadoks 37–39. Dichlorprop/2,4-D mixed with tebuconazole caused up to 15% injury when applied early and up to 29% injury when applied late. Bromoxynil/MPCA mixed with tebuconazole injured wheat up to 15% when applied early but only 10% when applied late. Other herbicide and fungicide mixes caused a lower level of injury. Visible injury was transient and did not reduce winter wheat yields. The likelihood of tank-mixes causing injury was greater when they were applied late. The fungicide tebuconazole caused the highest level of injury when mixed with herbicides and injury was particularly high with dichlorprop/2,4-D.

Key words: Dichlorprop/2,4-D, bromoxynil/MCPA, pyrasulfotole/bromoxynil, fungicide, herbicide injury

Robinson, M. A., Cowbrough, M. J., Sikkema, P. H. et Tardif, F. J. 2013. Tolérance du blé d'hiver (*Triticum aestivum* L.) aux mélanges d'herbicides et de fongicides appliqués à divers moments. *Can. J. Plant Sci.* 93: 491–501. Les agriculteurs mélangent couramment les herbicides et les fongicides en citerne pour économiser sur le coût des applications. Au printemps 2008, certains rapports indiquaient que le blé d'hiver avait subi des dommages consécutivement à l'application d'un mélange d'herbicides et de fongicides au début de la saison végétative. La présente étude devait préciser la tolérance du blé d'hiver à de tels mélanges selon le moment de leur application. Les auteurs ont procédé à des études au champ sur trois herbicides et quatre fongicides, à quatre endroits de l'Ontario, en 2009 et 2010. Les mélanges ont été appliqués hâtivement, quand il faisait encore froid, et tardivement, au stade de croissance Zadoks 37–39. Le dichlorprop/2,4-D mélangé à du tebuconazole engendre jusqu'à 15 % de dommages quand on l'applique hâtivement et jusqu'à 29 %, quand l'application est tardive. Une application hâtive de bromoxynil/MPCA mélangé à du tebuconazole suscite jusqu'à 15 % de dommages, contre 10 % pour une application tardive. Les autres mélanges d'herbicides et de fongicides sont moins dommageables. Les dommages visibles sont passagers et ne réduisent pas le rendement du blé d'hiver. Les risques de dommages sont plus grands quand le mélange est appliqué tardivement. Le fongicide tebuconazole entraîne les dommages les plus importants quand on le mélange à des herbicides, particulièrement du dichlorprop/2,4-D.

Mots clés: Dichlorprop/2,4-D, bromoxynil/MCPA, pyrasulfotole/bromoxynil, fongicide, dommages causés par les herbicides

Herbicides are often combined with other agro-chemicals such as adjuvants, fertilizers, fungicides, insecticides, or other herbicides as part of modern pest management practices. Combinations of agrochemicals are utilized to increase the spectrum of pests controlled, or to reduce the application costs associated with pesticide applications. Unpredicted interactions can occur when two or more biologically active chemicals are applied in combination (Hatzios and Penner 1985). In 2008, winter wheat growers in Ontario observed injury after herbicide–fungicide tank-mixes were applied early in the growing season when temperatures were near 0°C (Peter Johnson,

Ontario Ministry of Agriculture, Food and Rural Affairs, personal communication). There are no Ontario data to suggest whether herbicide–fungicides mixes result in crop injury, or if the injury is influenced by weather conditions at the time of application.

Biotic and abiotic stresses may occur simultaneously during winter wheat development. As a result, the application of agrochemicals used to address some of the biotic stresses may occur concurrently with the presence of abiotic stresses. The interactions of herbicide–fungicide mixtures in higher plants have not been studied as extensively as the interactions of

⁴Corresponding author (e-mail: ftardif@uoguelph.ca).

Abbreviations: DAT, days after treatment; TKW, 1000-kernel weight

herbicides with other agrochemicals such as adjuvants, fertilizer additives or insecticides (Hatzios and Penner 1985). Substantial winter wheat yield loss can result from weed competition or from damage caused by diseases if pest control strategies are not implemented in a timely manner (Welsh et al. 1999; Wegulo et al. 2011).

There is no information in the literature on winter wheat tolerance to herbicide–fungicide tank-mixes but there is documentation for some other crops. For example, the triazine herbicides and the fungicide fenaminosulf are antagonistic in oats, soybean and cucumber resulting in reduced efficacy of both pesticides (Corbin and Sheets 1968; Hatzios and Penner 1985). Conversely, the fungicide pyraclostrobin and the herbicide thifensulfuron-methyl were synergistic, causing tomato injury when applied as a mixture (Robinson and Nurse 2009). Azoxystrobin is a fungicide in the same chemical family as pyraclostrobin, which caused injury to sugarbeet when combined with the herbicides desmedipham-phenmedipham plus ethofumesate (Stump et al. 2002). Pyraclostrobin and azoxystrobin are two fungicides commonly used in winter wheat in Ontario and they are often tank-mixed with herbicides (Peter Johnson, personal communication).

Weather factors such as temperature and moisture may influence the interactions of herbicide plus fungicide tank-mixes, such that the activity may be enhanced or reduced. However, these effects have not been well documented (Green 1989; Kudsk 1999). An understanding of the interactions between herbicides and fungicides would be helpful in optimising pest management in winter wheat.

The objectives of this research were to evaluate the effects of herbicide–fungicide tank-mixes on winter wheat injury and yield, and to determine the impact of application timing on winter wheat tolerance.

MATERIALS AND METHODS

Field experiments were conducted in 2009 and 2010 at the Elora, Exeter, Ridgetown and Woodstock Research Stations located in southern Ontario. At Elora and Woodstock, seedbed preparation consisted of either a chisel plow or an offset disk followed by a cultipacker, while at Exeter and Ridgetown, the winter wheat was established under a no-till regime with direct seeding into previous crop residue (soybeans). Soft red winter wheat (cv. Pioneer 25R47) was planted in rows 17.5 cm apart at seeding densities ranging from 3.5 to 4.5 million seeds per hectare. Sites were fertilized in the early spring (March) with 120 kg ha⁻¹ of nitrogen once conditions allowed as per standard practices at these stations. Potassium and phosphorus were applied according to soil test results. Plots were organized in a randomized complete block design with four replicates at each location. Plot size was 1.5 m × 5.75 m at Elora and Woodstock, 2 m × 10 m at Exeter and 2 m × 8 m at Ridgetown.

All herbicide and fungicide treatments were applied at recommended label rates (Table 1). Treatments were applied with a wheel-mounted small plot sprayer fitted with TeeJet flat fan XR 8001 nozzles at Elora and Woodstock and with a backpack sprayer fitted with Hypro FC-ULD-120-01 nozzles at Exeter and Hypro GRD-120-01 nozzles at Ridgetown. The herbicides tested were dichlorprop/2,4-D, bromoxynil/MCPA, and pyrasulfotole/bromoxynil. Ammonium sulfate (BASF Canada Inc., Mississauga, ON) was added to the pyrasulfotole/bromoxynil treatment, at a rate of 1 L ha⁻¹. The fungicides tested were the strobilurin pyraclostrobin, the triazole tebuconazole, and two fungicides combining a triazole and a strobilurin, propiconazole/trifloxystrobin and azoxystrobin/propiconazole. Treatments were organized so that each herbicide and fungicide was applied separately and in combination. Dates of seeding, herbicide–fungicide application, and final grain harvest varied according to sites (Table 2). Each treatment was applied at two timings: the early application occurred after Apr. 15 in 2009 and 2010, when a temperature of approximately 0°C was forecasted, while the late application was applied at Zadoks stage 37–39 (Zadoks et al. 1974). While the early application was timed based on weather pattern, crop stage at this timing ranged between Zadoks 14 and 21. Daily maximum and minimum temperatures on the day of treatment application at each location are listed in Table 3. Winter wheat injury was assessed visually, approximately 7, 14, 28 and 42 d after treatment (DAT) on a scale of 0 to 100% where a value of 0% was defined as no visible plant injury, and a value of 100% was defined as plant death. Injury included any visible “departure” from normal leaf appearance including chlorosis and necrosis. Before final grain harvest, winter wheat plant height (cm) was determined in each plot by taking a random sample of 10 plants. The plots were harvested with a small plot combine. After harvest, 1000-kernel weight (g) (TKW) was assessed and test weight (kg hL⁻¹) was determined using the Cox funnel procedure according to federal guidelines (Canadian Grain Commission 2011).

Table 1. Active ingredient and dose for herbicides and fungicides

Active ingredient	Dose (g a.i. or a.e. ha ⁻¹)
<i>Herbicides</i>	
Dichlorprop/2,4-D	524 + 493
Bromoxynil/MCPA	280 + 280
Pyrasulfotole/bromoxynil	31 + 175
<i>Fungicides</i>	
Pyraclostrobin	150
Tebuconazole	126
Propiconazole/trifloxystrobin	625 + 62.5
Azoxystrobin/propiconazole	75 + 125

Table 2. Dates of winter wheat seeding, herbicide application at each treatment timing, and final grain harvest in each year at each location

Location	Year	Seeding date ^a	Early	Late	Harvest
Elora	2009	2008 Oct. 10	May 03	May 30	Aug. 06
	2010	2009 Sep. 17	Apr. 22	May 19	Jul. 27
Exeter	2009	2008 Sep. 26	Apr. 23	May 29	Jul. 27
	2010	2009 Sep. 18	Apr. 19	May 12	Jul. 07
Ridgetown	2009	2008 Oct. 24	Apr. 23	May 23	Jul. 29
	2010	2009 Oct. 15	Apr. 19	May 19	Jul. 08
Woodstock	2009	2008 Oct. 25	May 02	May 31	Aug. 07
	2010	2009 Oct. 10	Apr. 23	May 28	Aug. 01

^aSeeding was done in the fall prior to the year of testing.

Statistical Analysis

For yield data, variances were partitioned into the random effects of location, year, location by year, treatment by year, location by treatment and block by location within year, and the fixed effects of herbicide, fungicide and herbicide $\times$ fungicide, using the PROC MIXED procedure of SAS (SAS/STAT 9.2, 2009, SAS Institute, Inc. Cary, NC). Data for location and year were pooled unless the location by treatment or year by location by treatment effect was significant, in which case locations and/or years were analyzed separately. Data were subjected to ANOVA, and means were separated using Fisher's protected LSD test at the 0.05% level of significance. Error assumptions of the variance analysis including a random, homogeneous and normal distribution of error were confirmed. For the data to meet the assumption of normality, outlying data points were removed based on their non-alignment shown in a plot of residuals against ranked residuals and the box plot of the PROC UNIVARIATE procedure of SAS. Plant height, test weight, TKW, and injury were analyzed similarly. Visible injury data, however,

did not meet the error assumption of a normal distribution and so data were subjected to an arcsin square root transformation; transformations did not improve the normality of residuals so untransformed data was used. It has been shown that F-tests have minimal sensitivity to small deviations from normality (Box 1953; Box and Watson 1962). A histogram of residuals indicated a near-normal pattern for visible injury data (data not shown).

RESULTS

Visible Injury

Different types of visible injury were observed with specific herbicide–fungicide tank-mixes. Dichlorprop/2,4-D plus tebuconazole was consistently the most injurious tank-mix (Tables 4 and 5). This mixture caused dark brown–black foliar fleckings localized where the spray droplet made contact with the leaf surface (Fig. 1). Bromoxynil/MCPA + tebuconazole injury appeared as white foliar flecking, with some small dark brown spots (Fig. 2).

Pyrasulfotole/bromoxynil + tebuconazole injury appeared as patches on the leaf surface that were lightly bleached and lighter in colour (Fig. 3).

Bromoxynil/MCPA + azoxystrobin/propiconazole caused distinct small to medium white (bleached) linear splotches, well defined by a necrotic contour (Fig. 4).

Application of fungicides alone to winter wheat under cold conditions did not result in any injury at 7 DAT, regardless of sites or years (Table 4). In contrast, dichlorprop/2,4-D alone applied early under cold conditions caused injury at three of four sites in each year at 7 DAT. Some herbicide–fungicide combinations caused injury that was higher than would have been expected from each product applied alone. The fungicide tebuconazole applied in a tank-mix with a herbicide caused the highest level of visible injury compared with the other fungicide–herbicide combinations. This fungicide applied as a tank-mix with pyrasulfotole/bromoxynil caused visible injury at five of the eight site/years with injury ranging from 2.3 to 5.5%. Similarly, greater injury was found with this fungicide when tank-mixed with dichlorprop/2,4-D or bromoxynil/MCPA at four out of seven site/years. The addition of azoxystrobin/

Table 3. Daily high and low temperatures at each location for early/late and late application timings

Year	Location ^a	Early		Late	
		Low	High	Low	High
(°C)					
2009	Elora	1.7	17.3	4.6	18.7
	Exeter	1.0	13.0	11.5	24.0
	Ridgetown	1.3	11.1	9.2	24.0
	Woodstock	2.5	14.0	3.0	13.5
2010	Elora	0.2	11.2	6.1	22.9
	Exeter	−2.0	13.0	5.3	12.2
	Ridgetown	−2.2	14.5	5.6	22.2
	Woodstock	0.5	16.0	17.0	28.0

^aDaily temperatures retrieved from Environment Canada weather stations closest to field locations. Stations used were Elora Reference Climate Station (RCS), Nairn, Ridgetown RCS and Woodstock/Foldens for Elora, Exeter, Ridgetown and Woodstock locations, respectively. Weather data can be retrieved via www.climate.weatheroffice.gc.ca.

Table 4. Average percent winter wheat visible injury for each herbicide, fungicide or herbicide/fungicide combination treatment following an early application at all locations* 7 d after treatment in 2009 and 2010

Treatment	Injury											
	2009						2010					
	Elora		Exeter		Woodstock		Exeter		Ridgetown		Woodstock	
	----- (%) -----											
<i>No herbicide</i>												
No fungicide	0	C	0	D	0	D	0	G	0	E	0	E
Pyraclostrobin	0	C	0	D	0	D	1	FG	0	E	0	E
Tebuconazole	0	C	1	D	0	D	1	FG	0	E	0	E
Propiconazole/trifloxystrobin	0	C	0	D	0	D	0	G	0	E	0	E
Azoxystrobin/propiconazole	0	C	0	D	0	D	0	G	0	E	0	E
<i>Dichlorprop/2,4-D</i>												
No fungicide	2	B	5	AB	3.5	BC	3.5	BCDE	1	D	5	C
Pyraclostrobin	2	B	5.8	A	5	B	4	BCD	1	D	8	B
Tebuconazole	2.5	A	6.5	A	11.3	A	5	ABC	3	B	14.5	A
Propiconazole/trifloxystrobin	2	B	4.3	ABC	4.5	BC	4	BCD	1	D	5.3	C
Azoxystrobin/propiconazole	2	B	5	AB	4	BC	2.8	CDEF	1	D	8.5	B
<i>Bromoxynil/MCPA</i>												
No fungicide	0	C	0	D	0	D	0	G	1	D	0	E
Pyraclostrobin	0	C	0	D	0	D	1.5	EFG	0	E	0	E
Tebuconazole	0	C	1.8	CD	2.8	BC	6.5	A	6.3	A	13.5	A
Propiconazole/trifloxystrobin	0	C	0	D	0	D	4	BCD	1	D	0	E
Azoxystrobin/propiconazole	0	C	0	D	2.3	CD	3.3	BCDEF	2	C	5.5	C
<i>Pyrasulfotole/bromoxynil</i>												
No fungicide	0	C	0	D	0	D	2	DEFG	1	D	0	E
Pyraclostrobin	0	C	0	D	0	D	2.3	DEFG	1	D	0	E
Tebuconazole	0	C	2.3	BCD	4.5	BC	5.5	AB	2.5	BC	3	D
Propiconazole/trifloxystrobin	0	C	0	D	0	D	1.5	EFG	1	D	0	E
Azoxystrobin/propiconazole	0	C	0	D	0	D	2	DEFG	0	E	0	E

*No injury was recorded at Ridgetown in 2009 and Elora in 2010 so the data are not presented in the table.

A–G Within each column, means followed by the same letter are not different according to the LSD test ($P = 0.05$).

propiconazole to bromoxynil/MPCA enhanced wheat injury at Exeter and Woodstock in 2010, and also enhanced injury when tank-mixed with dichlorprop/2,4-D at Woodstock in 2010. The highest level of injury was 14.5% in 2010 at Woodstock with the application of tebuconazole with dichlorprop/2,4-D. The fungicides pyraclostrobin and azoxystrobin/propiconazole did not increase injury caused by any of the herbicides. Injury on winter wheat was transient with most herbicide–fungicide combinations. Tebuconazole mixed with dichlorprop/2,4-D and with bromoxynil/MPCA were the only combinations that caused visible injury at 14 DAT (data not shown). By 28 DAT injury level was reduced, except for tebuconazole with dichlorprop/2,4-D at Elora which was increased to 10% (data not shown).

Late application of fungicides did not cause any injury when evaluated at 7 DAT (Table 5). The injury caused by herbicides varied depending on the active ingredient.

Pyrasulfotole/bromoxynil and bromoxynil/MPCA were safe and did not cause any injury when applied late. In contrast, injury with dichlorprop/2,4-D ranged from 0 to 11.5%. Injury was apparent only at the Ridgetown site and was greatest in 2009. The addition of

fungicides to herbicides increased visible injury; however, the extent of injury varied depending on the particular herbicide–fungicide combination as well as sites and years. Of the four fungicides, tebuconazole increased injury the most with each of the three herbicides evaluated. The combination of tebuconazole with dichlorprop/2,4-D was the most injurious with injury of up to 28.5% at Ridgetown in 2009; however, there was no injury with this combination at Exeter in 2010. Visible injury occurred when tebuconazole was tank-mixed with bromoxynil/MPCA and with pyrasulfotole/bromoxynil at all sites and years. The addition of azoxystrobin/propiconazole to bromoxynil/MPCA was injurious at 7 of 8 site years with injury level as high as 7.3% at Elora in 2009. This fungicide can also cause injury when tank-mixed with dichlorprop/2,4-D, but enhanced injury was only observed at Elora and Ridgetown in 2009.

Propiconazole/trifloxystrobin enhanced bromoxynil/MPCA injury to a lesser extent than other combinations, but injury was observed at Ridgetown in 2009 and Exeter, Ridgetown, and Woodstock in 2010. Propiconazole/trifloxystrobin enhanced dichlorprop/2,4-D injury at Ridgetown in 2009, but injury level was not

Table 5. Average percent visible injury for each herbicide, fungicide or herbicide/fungicide combination treatment following a late application at all locations seven days after treatment in 2009 and 2010

Treatment	Injury																
	2009			2010													
	Elora	Exeter	Ridgetown	Woodstock	Elora	Exeter	Woodstock	Ridgetown									
	(%)																
No herbicide																	
No fungicide	0.0	D	0.0	G	0.0	D	0.0	F	0.0	C							
Pyraclostrobin	0.0	D	0.0	G	0.0	D	0.0	C	0.0	C							
Tebuconazole	0.0	D	0.0	G	0.0	D	0.0	C	0.0	F	0.0	C					
Propiconazole/trifloxystrobin	0.0	D	0.0	G	0.0	D	0.0	C	0.0	F	0.0	C					
Azoxystrobin/propiconazole	0.0	D	0.0	G	0.0	D	0.0	C	0.0	F	0.0	C					
Dichlorprop 2,4-D																	
No fungicide	3.1	CD	0.0	B	11.5	D	0.0	E	2.3	BCD	0.0	C	0.0	F	2.3	B	
Pyraclostrobin	4.3	C	0.0	B	15.0	C	0.0	E	2.0	BCD	0.0	C	2.0	D	3.5	B	
Tebuconazole	12.3	A	6.5	A	28.5	A	3.3	C	5.0	A	1.0	BC	4.3	C	7.0	A	
Propiconazole/trifloxystrobin	6.0	BC	0.0	B	17.3	B	0.0	E	2.5	BC	0.5	C	0.0	F	2.5	B	
Azoxystrobin/propiconazole	7.3	B	0.0	B	15.0	C	0.0	E	2.0	BCD	1.0	BC	0.0	F	2.3	B	
Bromoxynil MCPA																	
No fungicide	0.0	D	0.0	B	0.0	G	0.0	E	0.0	D	0.0	C	0.0	F	0.0	C	
Pyraclostrobin	0.0	D	0.0	B	0.0	G	0.0	E	0.8	CD	0.0	C	0.0	F	0.0	C	
Tebuconazole	7.8	B	6.5	A	10.0	D	8.0	A	3.3	AB	2.8	AB	8.5	A	6.0	A	
Propiconazole/trifloxystrobin	0.0	D	0.0	B	3.5	F	1.0	D	0.0	D	3.0	A	1.3	E	3.0	B	
Azoxystrobin/propiconazole	7.3	B	0.0	B	5.5	E	2.5	C	3.5	AB	1.3	ABC	1.5	DE	2.5	B	
Pyrasulfotole bromoxynil																	
No fungicide	0.0	D	0.0	B	0.0	G	0.0	E	0.0	D	0.0	C	0.0	F	0.0	C	
Pyraclostrobin	0.0	D	0.0	B	0.0	G	0.0	E	0.0	D	0.0	C	0.0	F	0.0	C	
Tebuconazole	7.8	B	5.0	A	5.3	E	4.3	E	3.0	ABC	1.5	ABC	5.3	B	2.3	B	
Propiconazole/trifloxystrobin	0.0	D	0.0	B	0.1	G	0.0	E	0.0	D	0.0	C	1.0	E	0.5	C	
Azoxystrobin/propiconazole	0.0	D	0.0	B	0.0	G	0.0	E	0.0	D	0.0	C	0.0	F	0.0	C	

A–G Within each column, means followed by the same letter are not different according to the LSD test ($P=0.05$).

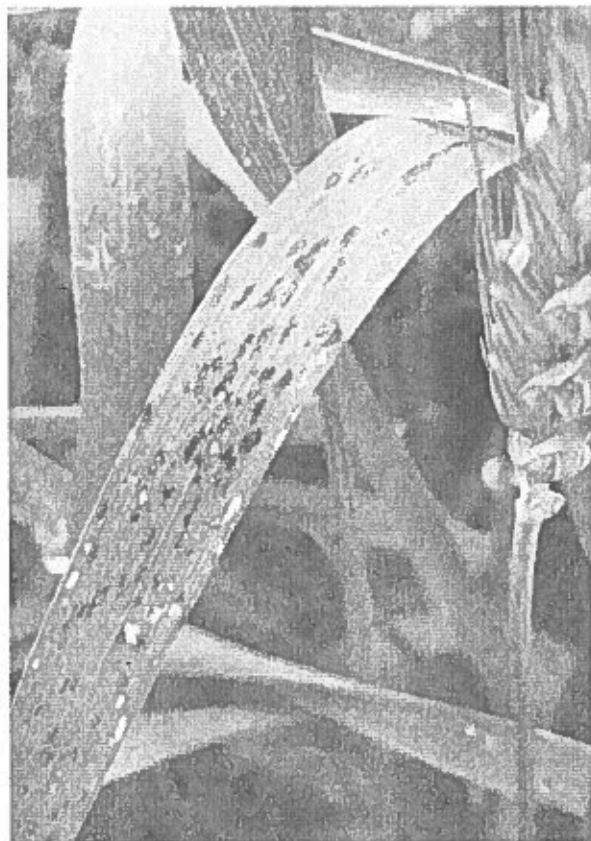


Fig. 1. Dark foliar flecking injury caused by the mixture of dichlorprop/2,4-D and tebuconazole.

increased at the other site/years. There was no increase in injury when pyraclostrobin was applied in a tank-mix with bromoxynil/MCPA or pyrasulfotole/bromoxynil and there was only a slight increase in injury when applied in combination with dichlorprop/2,4-D at Ridgetown in 2009 and Woodstock in 2010. The injury observed was transient with less injury observed at 14 and 28 DAT (data not shown).

Yield

Following early treatment applications, when averaged over 2 yr. yields were 7.4 t ha^{-1} at Exeter and 7.7 t ha^{-1} at Ridgetown, and did not differ among treatments (Table 6). At Woodstock, there was an important difference between years with yields averaging 5.4 t ha^{-1} in 2009 and 3.9 t ha^{-1} in 2010, but there was no difference among treatments in either year. The only treatment difference was between azoxystrobin/propiconazole alone and dichlorprop/2,4-D mixed with tebuconazole at Elora (average of 2009 and 2010). All other treatments had similar yields and in no instance did mixing a fungicide with a herbicide decrease yield compared with each product alone (Table 6).

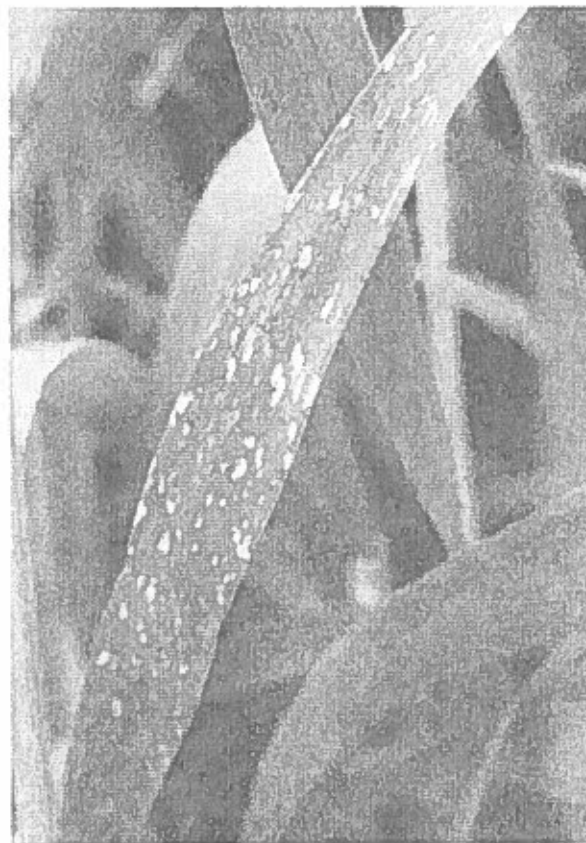


Fig. 2. Light foliar flecking with some dark spots caused by the mixture of bromoxynil/MCPA and tebuconazole.

Yields of treatments applied late varied among locations. At Elora, the highest yield was observed following treatment with the tank-mix of pyrasulfotole/bromoxynil + pyraclostrobin, which was higher than the value observed in the untreated check, pyrasulfotole/bromoxynil or bromoxynil/MCPA treatments (Table 7). At Ridgetown, pyraclostrobin alone resulted in greater yields than bromoxynil/MPCA, bromoxynil/MPCA + tebuconazole, bromoxynil/MPCA + propiconazole/trifloxystrobin, pyrasulfotole/bromoxynil + tebuconazole and pyrasulfotole/bromoxynil + propiconazole/trifloxystrobin; however, none of these treatments differed from the untreated control (Table 7). At Woodstock, the bromoxynil/MPCA + pyraclostrobin tank-mix resulted in yields higher than dichlorprop/2,4-D alone; however, neither was different from the untreated control. There were no yield differences observed at the Exeter location (data not shown).

Plant Height

Height was measured as this variable has often been associated with herbicide phytotoxicity in winter wheat (Soltani et al. 2006; Sikkema et al. 2007). However, regardless of application timing, none of the



Fig. 3. Injury caused by the mixture of pyrasulfotole/bromoxynil plus tebuconazole. Foliage appears lighter and bleached, with no well-defined contour. This symptomology was unique to this tank-mix.

herbicide-fungicide combinations affected the height of winter wheat (data not shown).

Thousand-kernel Weight

The herbicide-fungicide combinations applied early during cold temperatures did not affect the TKW of winter wheat (data not shown) but differences were observed after late applications at Elora and Woodstock (Table 8). At Elora, plants treated with pyraclostrobin alone had a mean TKW of 38.2 g, 15% higher than that of untreated plants, which had a TKW of 33.2 g. Thousand kernel weight was reduced with tebuconazole, dichlorprop/2,4-D, bromoxynil/MCPA and pyrasulfotole/bromoxynil applied alone. At Exeter, bromoxynil/MPCA + pyraclostrobin, bromoxynil/MPCA + tebuconazole and bromoxynil/MPCA + azoxystrobin/propiconazole resulted in higher TKW than the untreated control, as well as higher TKW than the fungicides pyraclostrobin, tebuconazole or azoxystrobin/propiconazole applied alone (Table 8). Thousand kernel weight



Fig. 4. Injury caused by the mixture of bromoxynil/MCPA plus azoxystrobin/propiconazole. Symptoms included large bleached area with well defined necrotic contour and generally linear in shape.

is an indication of kernel mass and a higher TKW can be indicative of a greater potential flour extraction.

Test Weight

The herbicide-fungicide combinations applied early generally did not affect the test weight of winter wheat except at Woodstock in 2010. At this location, bromoxynil/MPCA alone, bromoxynil/MPCA + azoxystrobin/propiconazole, pyrasulfotole/bromoxynil + pyraclostrobin, pyrasulfotole/bromoxynil + tebuconazole and pyrasulfotole/bromoxynil + propiconazole/trifloxystrobin applied early resulted in test weights that were greater than the untreated control (data not shown). Treatments did not affect test weight at any other sites or at Woodstock in 2009.

Treatments applied late did not impact test weight at any locations except Elora. At Elora, bromoxynil/MPCA + pyraclostrobin had a greater test weight than pyrasulfotole/bromoxynil applied alone, however both did not differ from all other treatments, including the control (data not shown).

Table 6. Average winter wheat grain yield for each herbicide, fungicide or herbicide/fungicide combination treatment following an early application at all locations 7 d after treatment in 2009 and 2010

Treatment	Injury									
						Woodstock				
	Elora		Exeter		Ridgetown		2009		2010	
	Yield (t ha ⁻¹)									
<i>No herbicide</i>										
No fungicide	4.49	AB	7.43	A	7.47	A	5.22	A	3.56	A
Pyraclostrobin	4.81	AB	7.74	A	7.51	A	4.85	A	3.79	A
Tebuconazole	4.68	AB	7.68	A	7.56	A	5.69	A	3.68	A
Propiconazole/trifloxystrobin	4.69	AB	7.65	A	8.00	A	6.16	A	3.80	A
Azoxystrobin/propiconazole	4.92	A	7.70	A	7.73	A	5.78	A	3.68	A
<i>Dichlorprop/2,4-D</i>										
No fungicide	4.44	AB	7.26	A	7.66	A	5.54	A	3.30	A
Pyraclostrobin	4.49	AB	7.48	A	7.96	A	4.92	A	3.82	A
Tebuconazole	4.16	B	7.41	A	7.55	A	5.29	A	4.21	A
Propiconazole/trifloxystrobin	4.46	AB	7.26	A	7.87	A	5.37	A	3.63	A
Azoxystrobin/propiconazole	4.44	AB	7.43	A	7.76	A	5.71	A	3.82	A
<i>Bromoxynil/MCPA</i>										
No fungicide	4.37	AB	7.27	A	7.62	A	5.81	A	4.22	A
Pyraclostrobin	4.79	AB	7.42	A	7.70	A	5.63	A	3.79	A
Tebuconazole	4.78	AB	7.14	A	7.47	A	5.55	A	4.02	A
Propiconazole/trifloxystrobin	4.66	AB	7.33	A	7.74	A	5.12	A	3.90	A
Azoxystrobin/propiconazole	4.73	AB	7.32	A	7.58	A	5.29	A	4.64	A
<i>Pyrasulfotole/bromoxynil</i>										
No fungicide	4.41	AB	7.28	A	7.71	A	5.87	A	3.97	A
Pyraclostrobin	4.86	AB	7.49	A	7.76	A	5.04	A	4.49	A
Tebuconazole	4.72	AB	7.49	A	7.61	A	5.60	A	4.18	A
Propiconazole/trifloxystrobin	4.74	AB	7.47	A	7.66	A	5.27	A	3.89	A
Azoxystrobin/propiconazole	4.75	AB	7.39	A	7.67	A	5.94	A	3.96	A

A, B Within each column, means followed by the same letter are not different according to the LSD test ($P=0.05$).

DISCUSSION

In both 2009 and 2010, all tank-mixes that included the fungicide tebuconazole consistently caused visible injury greater than either of the single components of the mixture applied alone. Tebuconazole caused injury when mixed with dichlorprop/2,4-D, bromoxynil/MPCA and pyrasulfotole/bromoxynil. In previous studies, tebuconazole was reported to reduce the efficacy of certain herbicides when applied as a tank-mix; however, no injury or yield reductions were observed (Lancaster et al. 2005a, b). In contrast, tebuconazole phytotoxicity has been reported in wheat and in other crops such as cocoa and rockmelon (Holderness 1990; Vawdrey 1994; Hunt et al. 2008). This fungicide is known to be inherently toxic to plants at rates required for adequate fungicidal action (Pedersen 2007; Hunt et al. 2008). It is conceivable that the phytotoxicity observed when herbicides were mixed with tebuconazole was due to a reciprocal action. The fungicide or components of its commercial formulation may increase uptake of the herbicides, while components of the herbicide formulations could also enhance tebuconazole leaf penetration, accelerating its phytotoxic action. This double action would account for the specificity of injury we observed with each tebuconazole/herbicide tank-mix (Figs. 1 to 3). Interestingly, of

all the combinations that were tested in this research, bromoxynil/MPCA+tebuconazole was the only registered tank-mix found on both product labels (Bayer CropScience Inc. Suite 100, 3131 114th Avenue S.E., Calgary, AB), and was one of the most injurious tank-mixes in this study.

Another injurious tank-mix was bromoxynil/MPCA+azoxystrobin/propiconazole. Azoxystrobin/propiconazole is a pre-mixed fungicide, made up of two active ingredients from the strobilurin and triazole fungicide families. Strobilurin fungicides have been documented to cause crop injury when tank-mixed with certain herbicides (Stump et al. 2002; Robinson and Nurse 2009). The phytotoxic effect of strobilurin fungicides is enhanced when tank-mixes include products that solubilize the plant cuticle (such as surfactants and some pesticides) and facilitate increased passage of strobilurin into the living plant tissue instead of remaining on the leaf surface, resulting in injury (Stump et al. 2002; Vincelli 2002; Agrios 2005). In fact, the product label for azoxystrobin/propiconazole issues a warning that phytotoxic effects may be encountered when azoxystrobin+propiconazole is tank-mixed with products formulated as emulsifiable concentrates (Syngenta Crop Protection Canada, Inc., 140 Research Lane, Guelph, ON).

Table 7. Average winter wheat grain yield for each herbicide, fungicide or herbicide/fungicide combination treatment following a late application at all locations 7 d after treatment averaged over 2009 and 2010

Treatment	Injury							
	Elora		Exeter		Ridgetown		Woodstock	
	Yield (t ha ⁻¹)							
<i>No herbicide</i>								
No fungicide	4.90	BC	6.98	A	7.54	AB	4.42	AB
Pyraclostrobin	5.58	AB	7.50	A	8.18	A	4.98	AB
Tebuconazole	4.97	ABC	7.33	A	7.70	AB	4.96	AB
Propiconazole/trifloxystrobin	5.30	ABC	7.45	A	7.47	AB	4.76	AB
Azoxystrobin/propiconazole	5.66	AB	7.45	A	7.30	AB	5.30	AB
<i>Dichlorprop/2,4-D</i>								
No fungicide	4.97	ABC	7.21	A	7.33	AB	4.02	B
Pyraclostrobin	5.70	AB	7.33	A	7.47	AB	5.06	AB
Tebuconazole	5.13	ABC	7.21	A	7.48	AB	4.63	AB
Propiconazole/trifloxystrobin	5.23	ABC	7.40	A	7.58	AB	4.81	AB
Azoxystrobin/propiconazole	5.31	ABC	7.45	A	7.68	AB	5.00	AB
<i>Bromoxynil/MCPA</i>								
No fungicide	4.64	C	7.04	A	7.13	B	5.03	AB
Pyraclostrobin	5.42	ABC	7.20	A	7.54	AB	5.65	A
Tebuconazole	4.97	ABC	7.02	A	7.12	B	5.27	AB
Propiconazole/trifloxystrobin	4.99	ABC	7.18	A	6.99	B	5.22	AB
Azoxystrobin/propiconazole	5.31	ABC	7.16	A	7.39	AB	5.30	AB
<i>Pyrasulfotole/bromoxynil</i>								
No fungicide	4.61	C	7.10	A	7.39	AB	4.86	AB
Pyraclostrobin	5.75	A	7.34	A	7.40	AB	5.20	AB
Tebuconazole	4.97	ABC	7.00	A	7.02	B	4.90	AB
Propiconazole/trifloxystrobin	5.27	ABC	7.37	A	7.20	B	5.27	AB
Azoxystrobin/propiconazole	5.41	ABC	7.44	A	7.62	AB	4.70	AB

A–C Within each column, means followed by the same letter are not different according to the LSD test ($P=0.05$).

Although dichlorprop/2,4-D alone as well as all mixtures including dichlorprop/2,4-D consistently caused visible injury, the injury that was observed with the dichlorprop/2,4-D + tebuconazole tank-mix was unique. Symptoms of the dichlorprop/2,4-D + tebuconazole tank-mix were a dark, localized, brownish-black flecking, often resembling a distinct spray pattern on the uppermost leaves of the canopy at the time of application (Fig. 1). The injury symptoms associated with dichlorprop/2,4-D applied alone or in a tank-mix with the other fungicides were growth distortion and a stretching and subsequent flattening of the canopy (change of leaf angle such that they were more parallel to the ground) with no visible chlorosis or flecking. Wheat grew out of this injury and no plant height reduction was observed (data not shown). Damage to winter wheat caused by dichlorprop/2,4-D has been documented in other studies, causing up to 3.5% injury when applied in the fall and reducing plant height in the following spring (Soltani et al. 2006). 2,4-D applied alone or in combination with other herbicides often reduces plant height (similar to the pre-senescence canopy “flattening” injury mentioned above) and yield (Martin et al. 1989; Soltani et al. 2006; Leaden et al. 2007).

When treatments were applied late, injury most often occurred on the flag leaf of the crop. Flag leaf health is

correlated with cereal crop yield (James et al. 1968; Briggs and Aytensu 1980). Many fungicide applications are timed to prevent pathogen invasion and subsequent death of the flag leaf (Agrios 2005; Wegulo et al. 2009, 2011; Ali et al. 2010). Since some tank-mixes such as dichlorprop/2,4-D + tebuconazole caused visible injury to the flag leaf, we expected that yield reductions due to this injury would occur; however, they did not occur. This indicates that winter wheat plants are sufficiently plastic and capable of recovering from injury less than 15% (in most cases) that occurs on the flag leaf due to the tank-mixes tested in this experiment.

In summary, the incidence of winter wheat injury due to herbicide–fungicide tank-mixes is increased when applied late. Herbicide–fungicide tank-mixes applied early, under cold temperature conditions do not cause crop injury greater than when they are applied late. Cold temperatures during application do not accentuate injury of winter wheat from herbicide–fungicide tank-mixes.

Dichlorprop/2,4-D + tebuconazole, bromoxynil/MPCA + tebuconazole, pyrasulfotole/bromoxynil + tebuconazole and bromoxynil/MPCA + azoxystrobin/propiconazole were the herbicide–fungicide tank-mixes that consistently caused the greatest injury. Dichlorprop/2,4-D + tebuconazole caused injury at most locations and at both timings. Herbicide–fungicide tank-mixes including the fungicide tebuconazole are likely to cause injury as

Table 8. Average 1000-kernel weight (TKW) following the late application at all locations in 2009 and 2010

Treatment	TKW							
	Elora		Exeter		Ridgetown		Woodstock	
	(g)							
<i>No herbicide</i>								
Untreated Control	33.2	BC	38.0	B	37.9	A	36.3	A
Pyraclostrobin	38.2	A	38.0	B	38.7	A	37.6	A
Tebuconazole	32.4	C	37.3	B	39.1	A	37.5	A
Propiconazole/trifloxystrobin	34.9	ABC	37.8	B	38.5	A	36.9	A
Azoxystrobin/propiconazole	35.2	ABC	37.4	B	39.2	A	37.6	A
<i>Dichlorprop[2,4-D]</i>								
Untreated Control	32.2	C	37.3	B	39.1	A	35.4	A
Pyraclostrobin	37.1	AB	39.0	AB	39.7	A	39.4	A
Tebuconazole	35.4	ABC	38.4	AB	39.7	A	38.6	A
Propiconazole/trifloxystrobin	36.1	ABC	38.7	AB	39.7	A	38.9	A
Azoxystrobin/propiconazole	36.4	ABC	38.3	AB	39.6	A	39.1	A
<i>Bromoxynil/MCPA</i>								
Untreated Control	33.1	BC	38.8	AB	39.6	A	37.3	A
Pyraclostrobin	36.6	ABC	40.5	A	39.7	A	38.9	A
Tebuconazole	34.8	ABC	40.4	A	40.2	A	39.4	A
Propiconazole/trifloxystrobin	36.2	ABC	39.6	AB	39.4	A	39.4	A
Azoxystrobin/propiconazole	35.0	ABC	40.1	A	40.0	A	39.7	A
<i>Pyrasulfotole/bromoxynil</i>								
Untreated Control	33.0	BC	37.5	B	38.4	A	36.2	A
Pyraclostrobin	35.4	ABC	38.7	AB	38.7	A	38.5	A
Tebuconazole	34.0	ABC	38.7	AB	39.6	A	38.1	A
Propiconazole/trifloxystrobin	34.0	ABC	39.0	AB	39.0	A	37.6	A
Azoxystrobin/propiconazole	34.1	ABC	39.1	AB	39.2	A	37.6	A

A–C Within each column, means followed by the same letter are not different according to the LSD test ($P=0.05$).

this was observed when tebuconazole was mixed with all of the herbicides tested. However, it is important to note that the injury from herbicide–fungicide tank-mixes observed in this study was transient. Tank-mixes of herbicides and fungicides did not reduce winter wheat yield, height, TKW and test weight.

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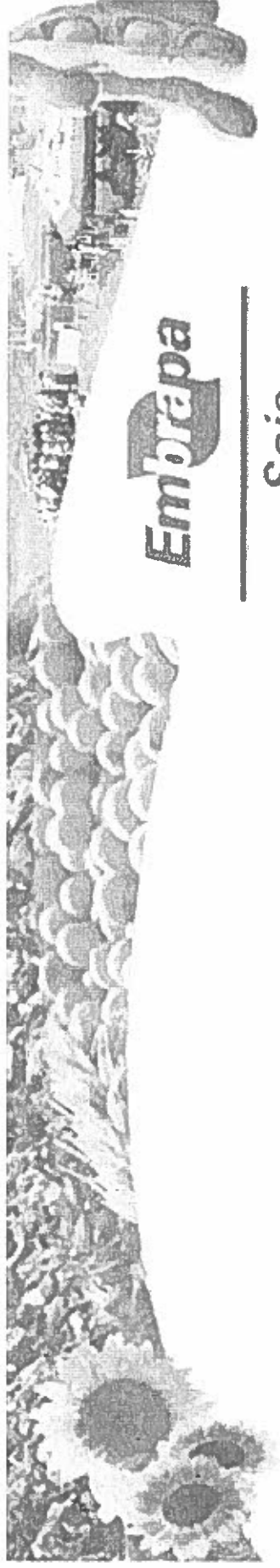
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Changes in performance of SBR fungicides over the years and new management strategies adopted in Brazil

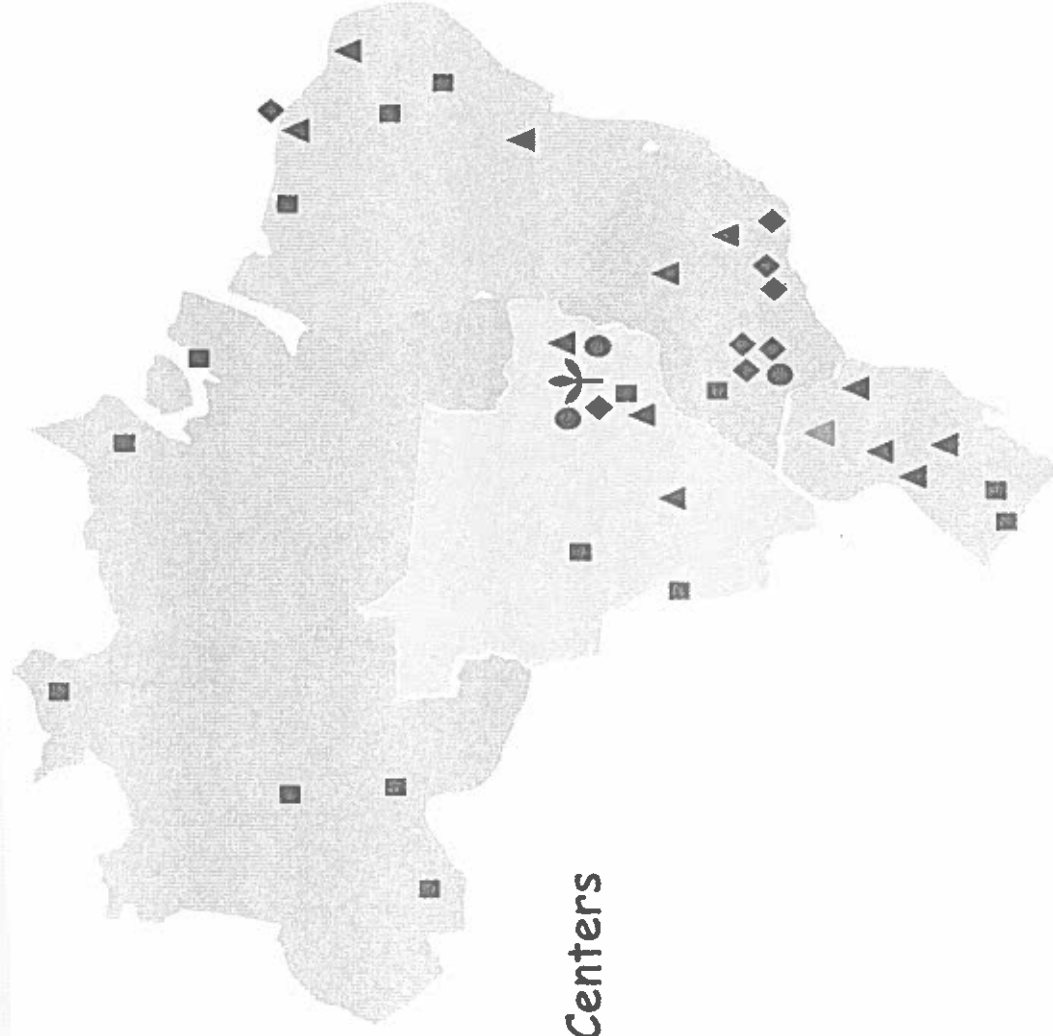
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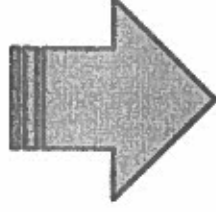
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Anti Rust Consortium

First report of ASR in Brazil - May 2001

Anti Rust Consortium (ARC) was created in 2004



Annual meetings to discuss and define the recommendations
 Warning system - 100 labs in the main soybean producing regions
 Update the website's warning system in real time



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www.consorcioantiferrugem.net



Consórcio Antiferrugem
União de Estados e Municípios para a Defesa da Soja

Menu

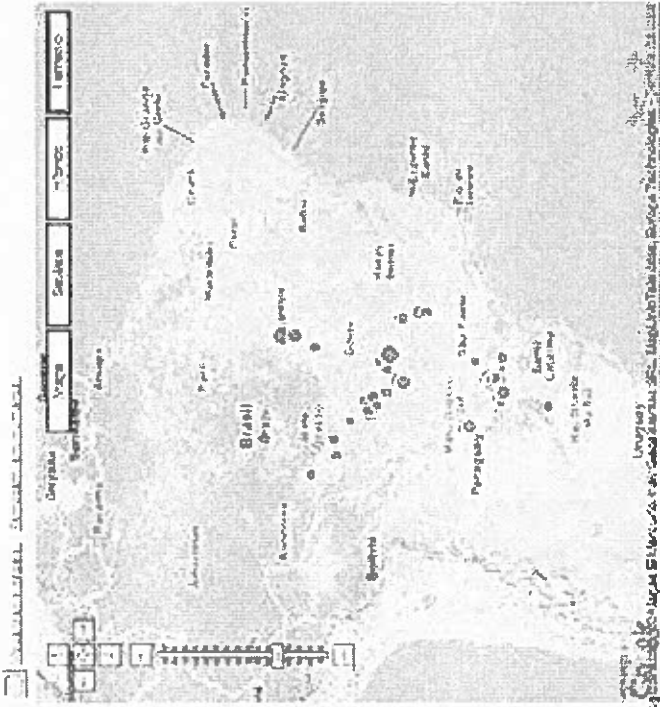
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O que é o Consórcio

O Consórcio Antiferrugem é uma entidade sem fins lucrativos, criada em 2004, com o objetivo de promover a defesa da soja no Brasil, através da troca de informações e recursos entre produtores e pesquisadores.

O Consórcio é formado por produtores de soja de todo o Brasil, que se uniram para enfrentar os problemas de ferrugem que afetam a produção.

O Consórcio atua em várias frentes, incluindo a realização de pesquisas, a produção de materiais educativos e a organização de eventos para a troca de experiências entre produtores.



O mapa mostra a distribuição da produção de soja no Brasil, com destaque para as regiões de maior concentração, como o Centro-Oeste e o Sul. Também são indicadas as áreas afetadas pela ferrugem, que se estendem por grande parte do território nacional.

Objetivos do Consórcio

- Defender a produção de soja no Brasil.
- Promover a troca de informações entre produtores e pesquisadores.
- Realizar pesquisas e desenvolver tecnologias para a defesa da soja.
- Organizar eventos para a troca de experiências entre produtores.

Parceiros do Consórcio

O Consórcio Antiferrugem é apoiado por diversos parceiros, incluindo o Ministério da Agricultura, o Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e a Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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Telefone: (11) 1234-5678

E-mail: contato@consorcioantiferrugem.net

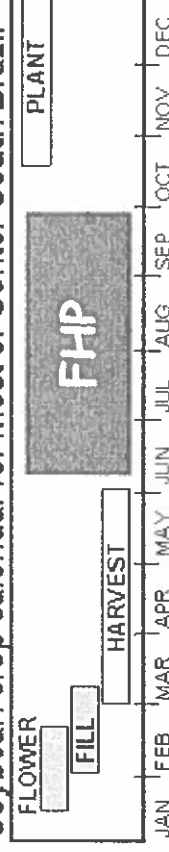
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- Ministério da Agricultura
- CNPq
- FAPESP
- Associação Brasileira de Produtores de Soja

Strategies adopted by ARC

- **Crop management in the winter season**
 - ✓ Eliminate volunteer plants;
 - ✓ Avoid second summer crop;
 - ✓ "Free host period" (FHP) – no soybeans in the winter season

Soybean crop calendar for most of Center-South Brazil



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Free host period

State	Jun 15	July 1	August 15	September 15	October 15	November 1	November 30
TO							
PA							
MA							
BA							
GO							
MT							
MS							
DF							
SP							
MG							
PR							
SC							



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Strategies adopted by ARC

- **Crop Management in the winter season**
 - ✓ Eliminate volunteer plants;
 - ✓ Avoid Second summer crop;
 - ✓ "Free host period" (FHP) - no soybeans in the winter season (July - Sep)
- **Early sowing with early maturity cultivar**
- **Monitor disease presence in the field and region**
- **Fungicides at first symptoms or preventive**
- **Resistant cultivars**



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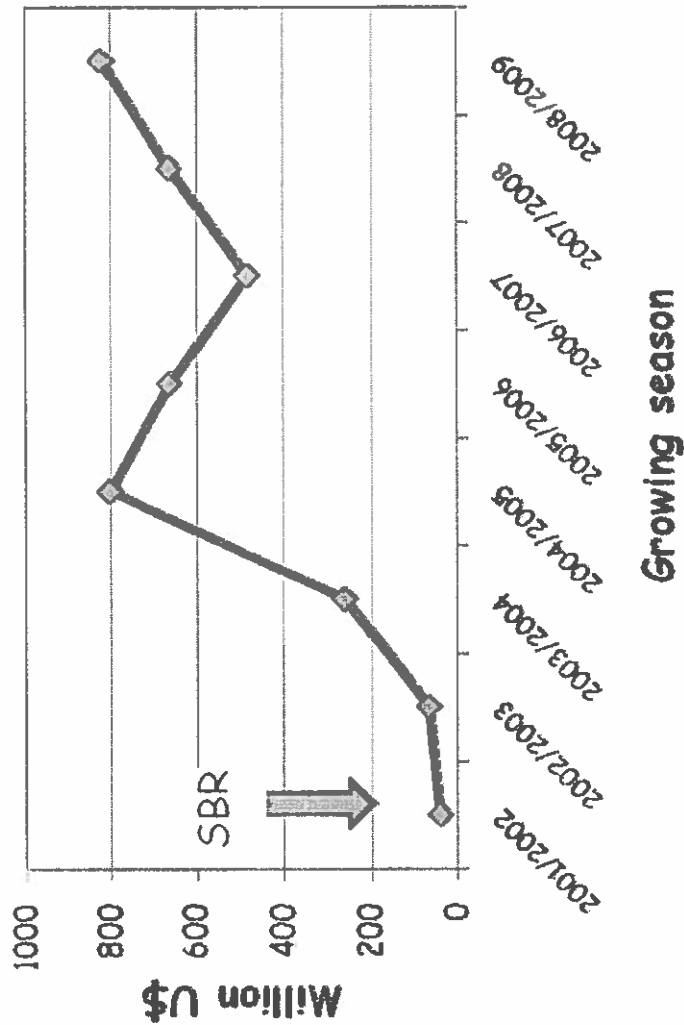


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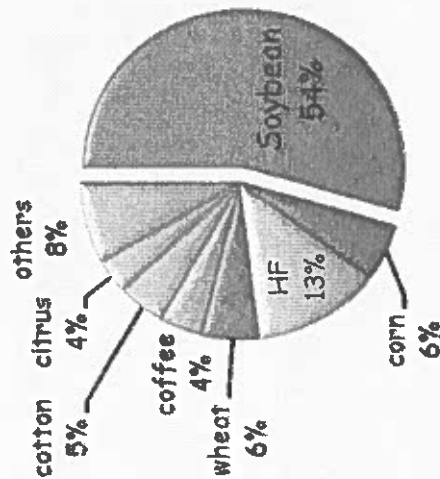
Fungicide soybean market

Fungicide soybean market evolution in Brazil

Million U\$

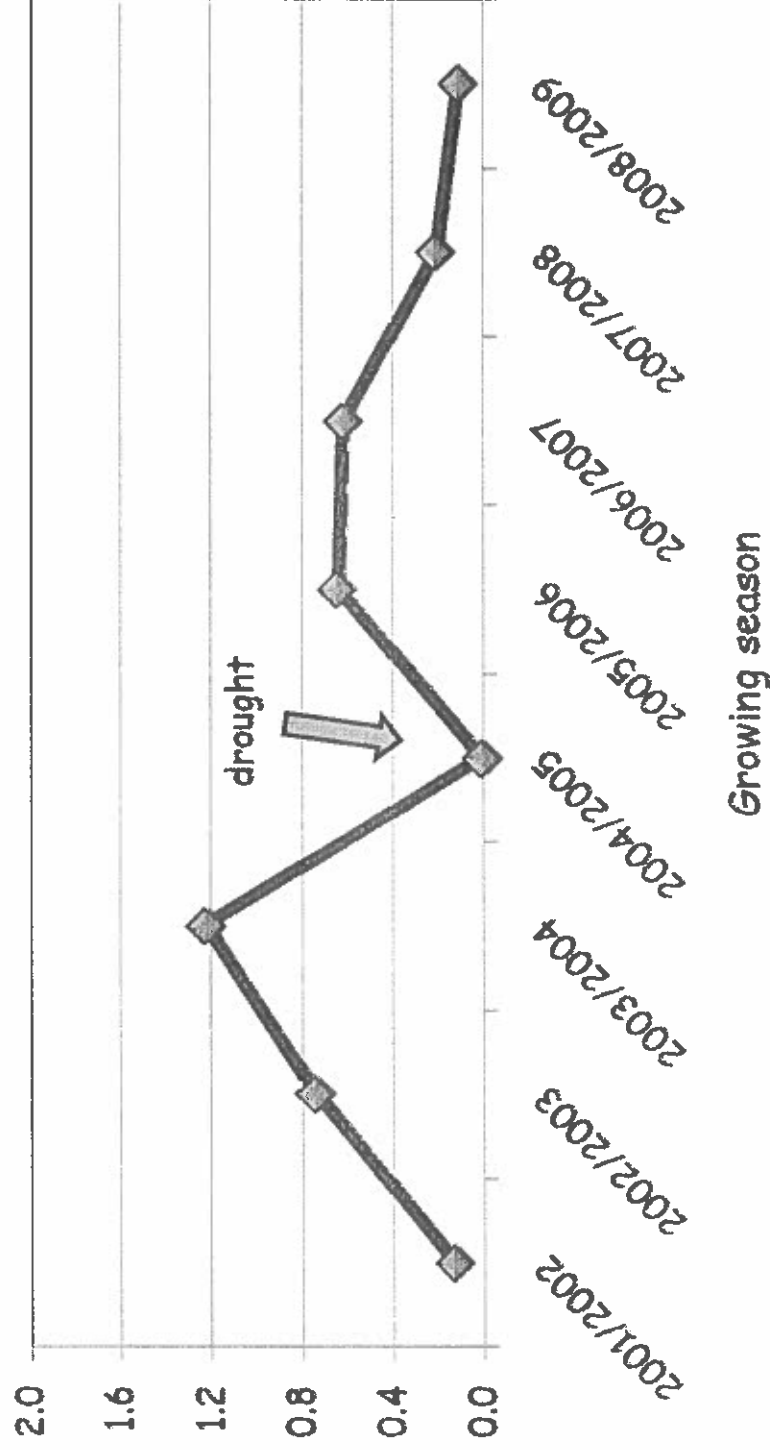


Fungicide market in Brazil
U\$1,5 B (2008)



Grain losses due to SBR

Grain losses (Million U\$)



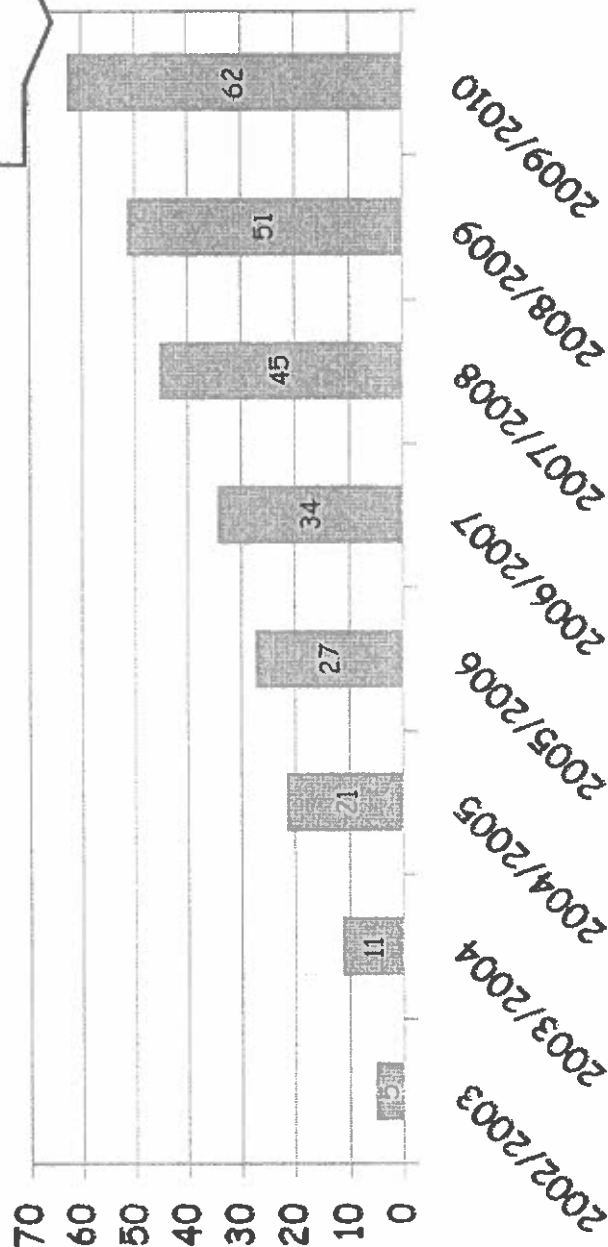
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Labeled fungicides

Number of labeled fungicides

6 triazoles
2 strobilurin
5 triazoles + strobilurin
1 triazole + benzimidazole



Growing season

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4. Resistance culture

5. Perspectives for 2009/10



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Trial network for chemical control

Objective:

compare fungicides in the same situation (later in the season)

Material and methods (standard protocols):

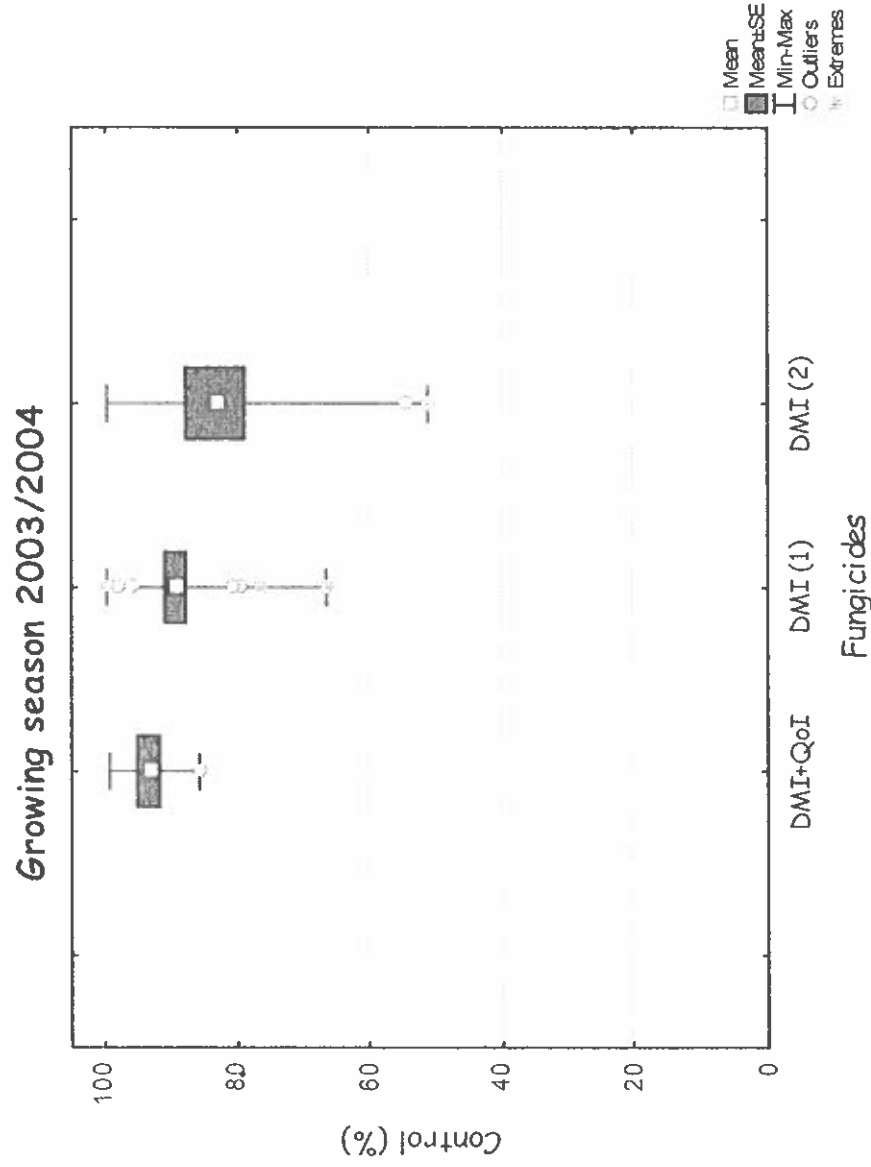
- ✓ randomized complete block with four replications (6 rows wide and 6 m long);
- ✓ two or three sequential application (maximum interval 21 days), starting at R2 or at first symptoms (vegetative);
- ✓ CO₂ pressurized back pack sprayer;
- ✓ 150 - 200 l ha⁻¹;
- ✓ severity assessment in the application time and at R5-R6;
- ✓ yield
- ✓ all soybean region (Universities, Embrapa, foundations, and private extension companies)



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DMI + QoI x DMI - 2003/2004

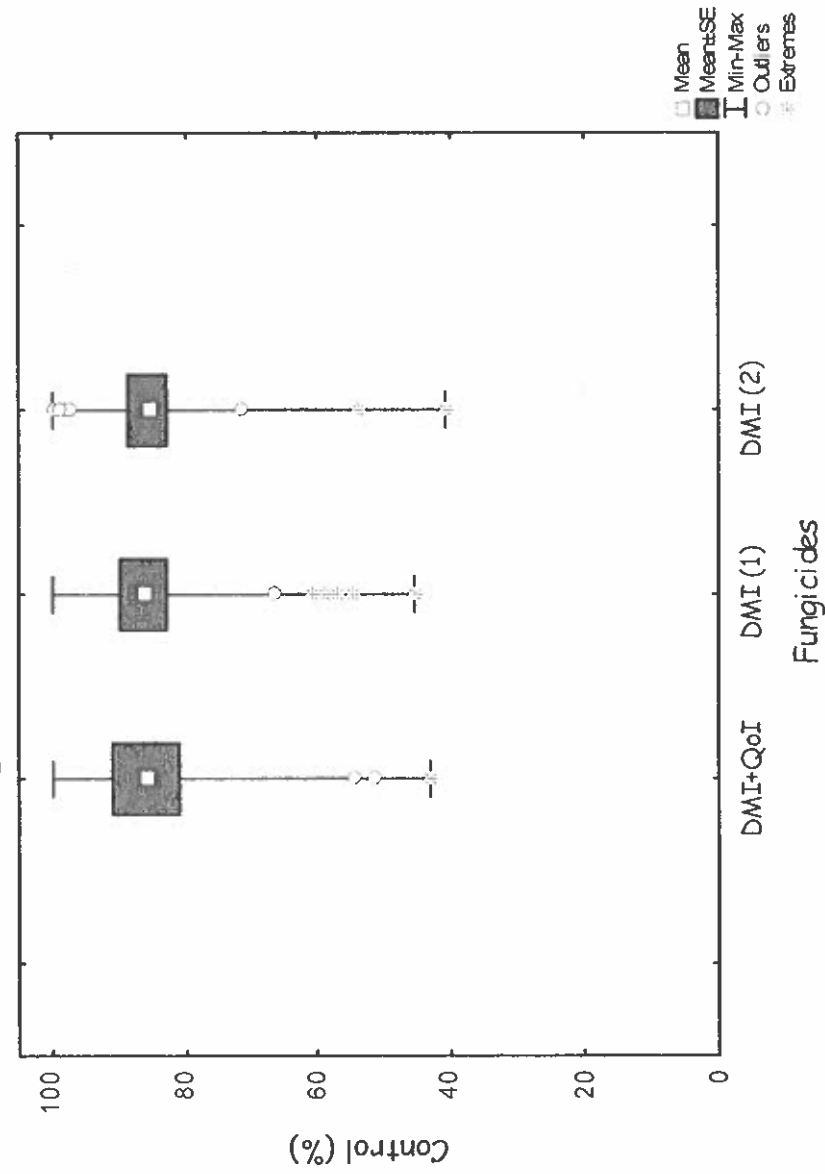


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DMI + QoI x DMI - 2004/2005

Growing season 2004/2005

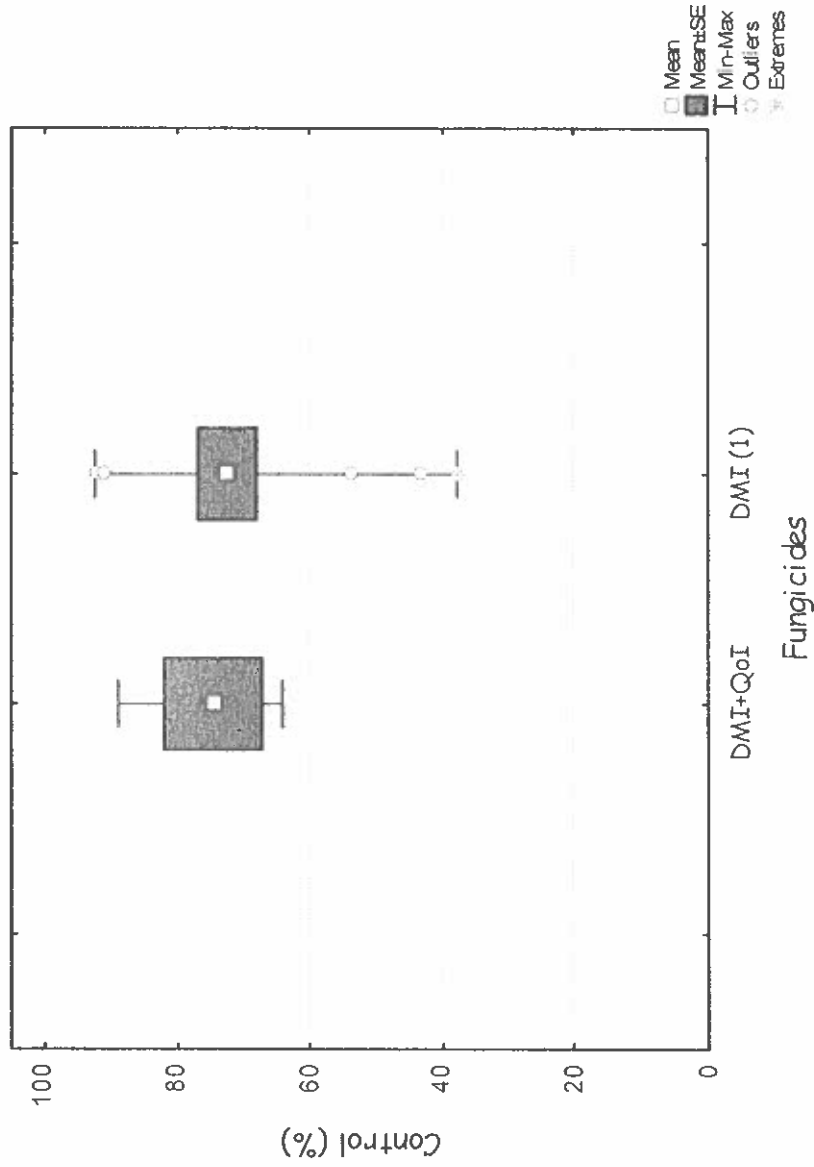


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DMI + QoI x DMI - 2005/2006

Growing season 2005/2006



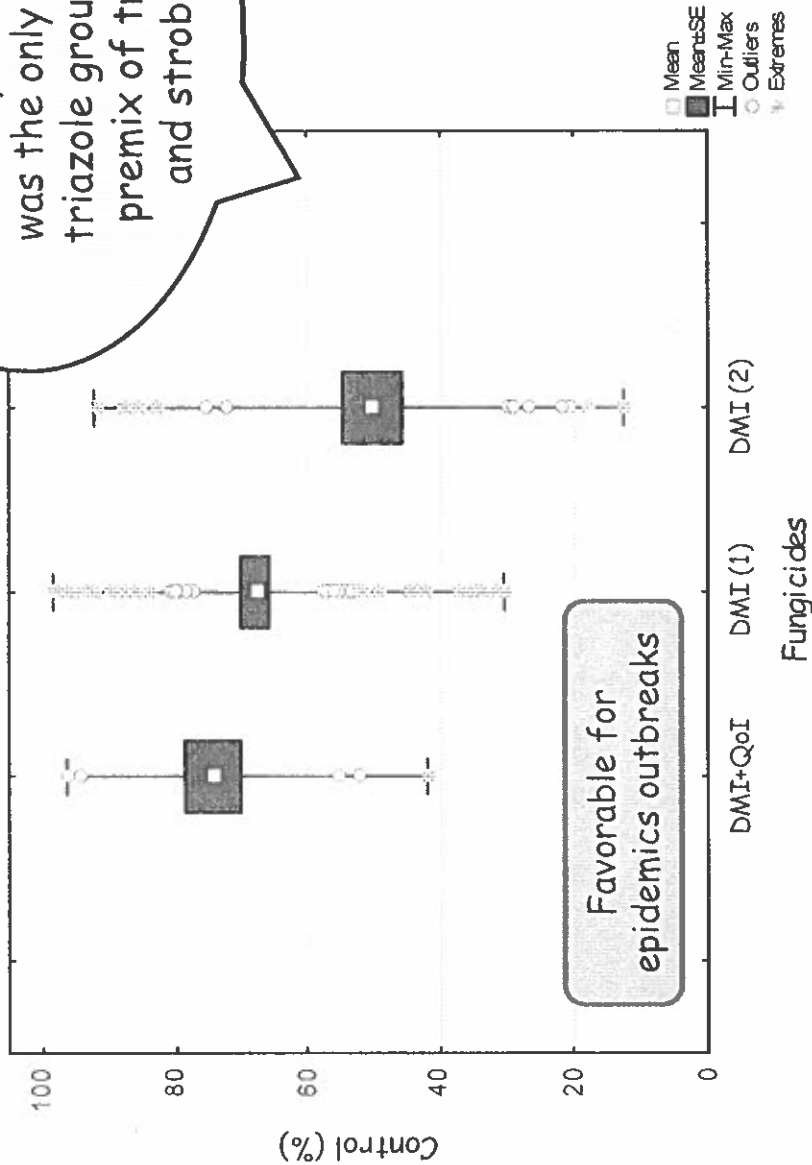
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DMI + QoI x DMI - 2006/2007

Fungicides were grouped according to efficacy and DMI (1) was the only straight triazole grouped with premix of triazoles and strobilurin.

Growing season 2006/2007



Crop protection 28:774-782 (2009)

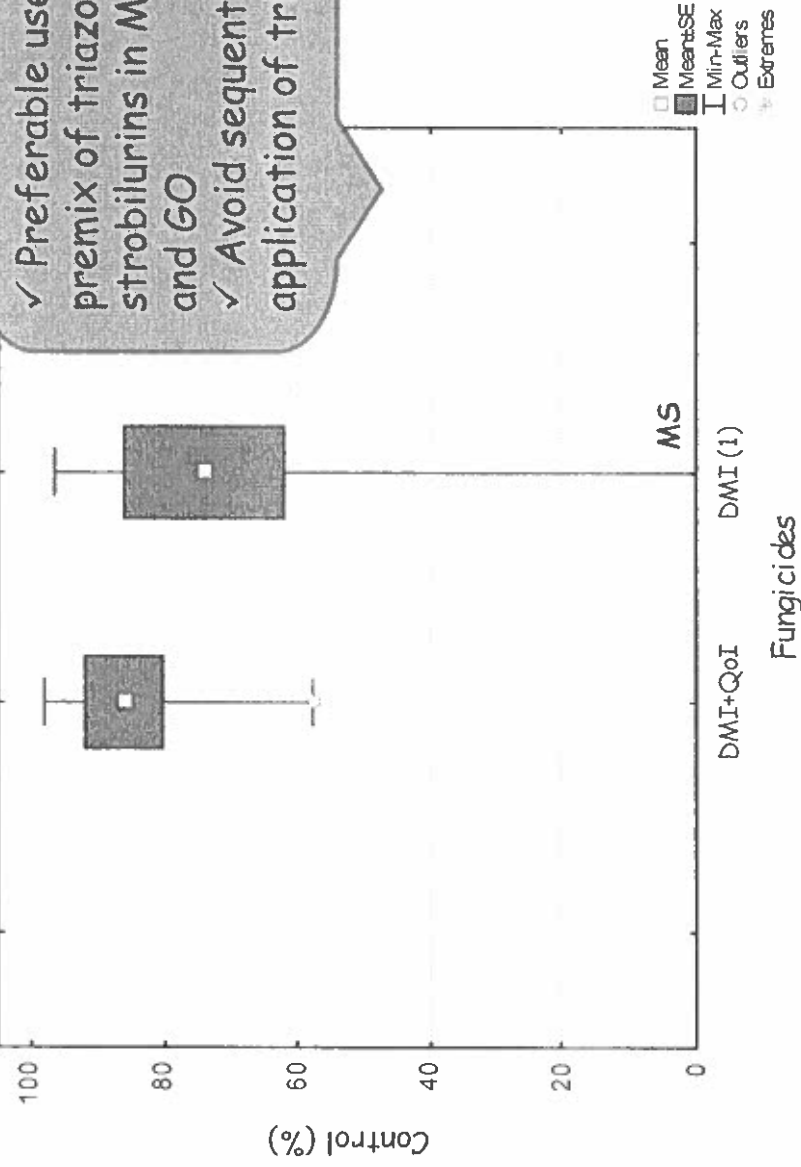


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DMI + QoI x DMI - 2007/2008

Growing season 2007/2008

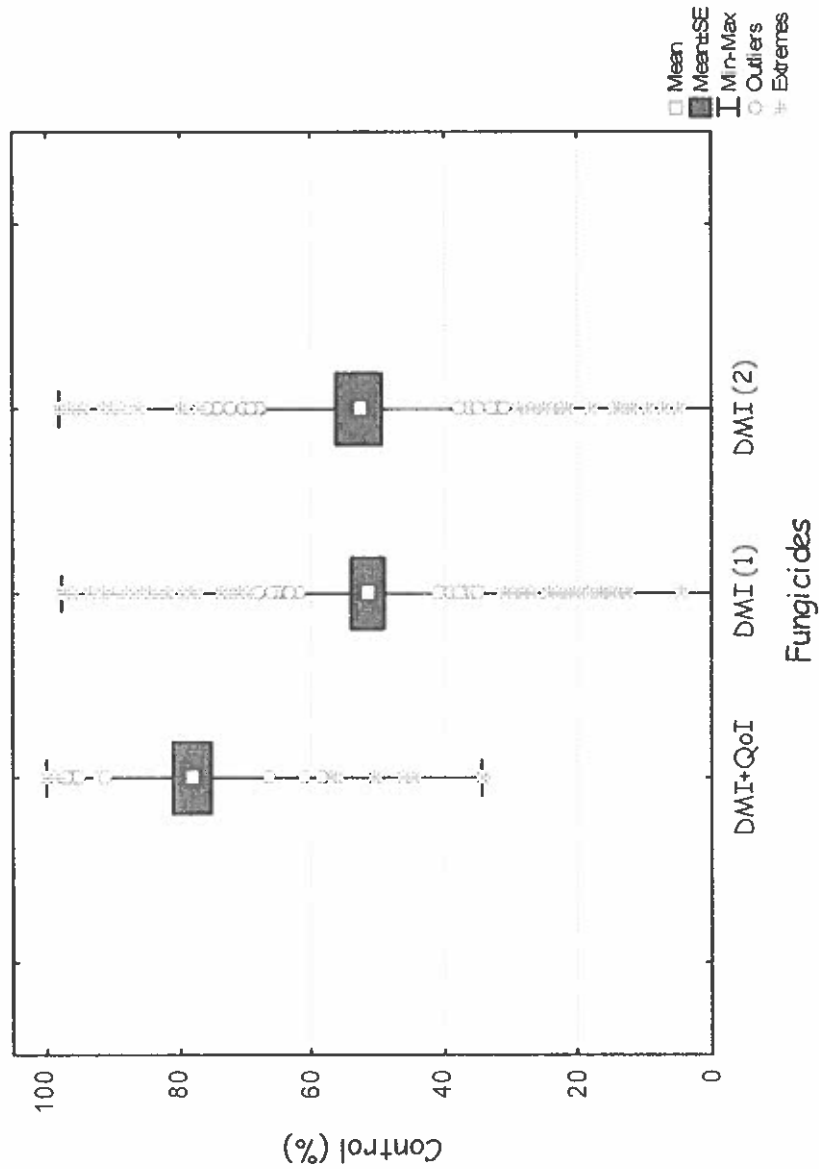


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DMI + QoI x DMI - 2008/2009

Growing season 2008/2009





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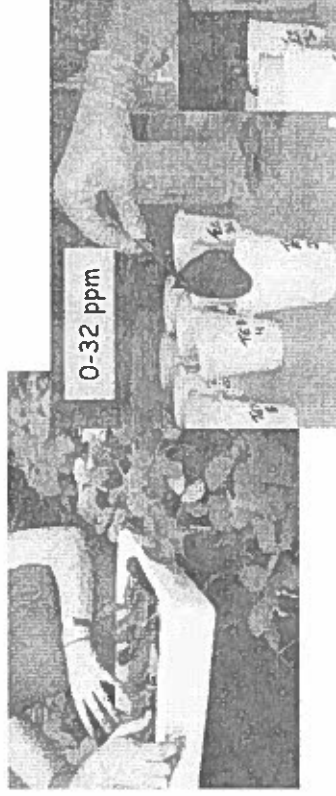
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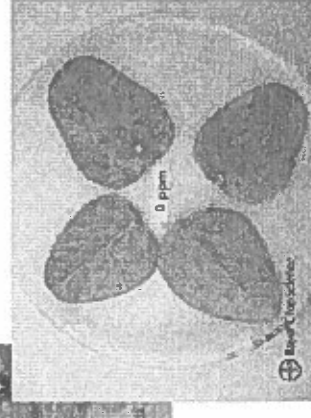
Sensitivity monitoring tests

Bayer CropScience monitoring data are supporting FRAC recommendation (<http://www.frac.info/>)

2005 until 2009 - detached leaf method

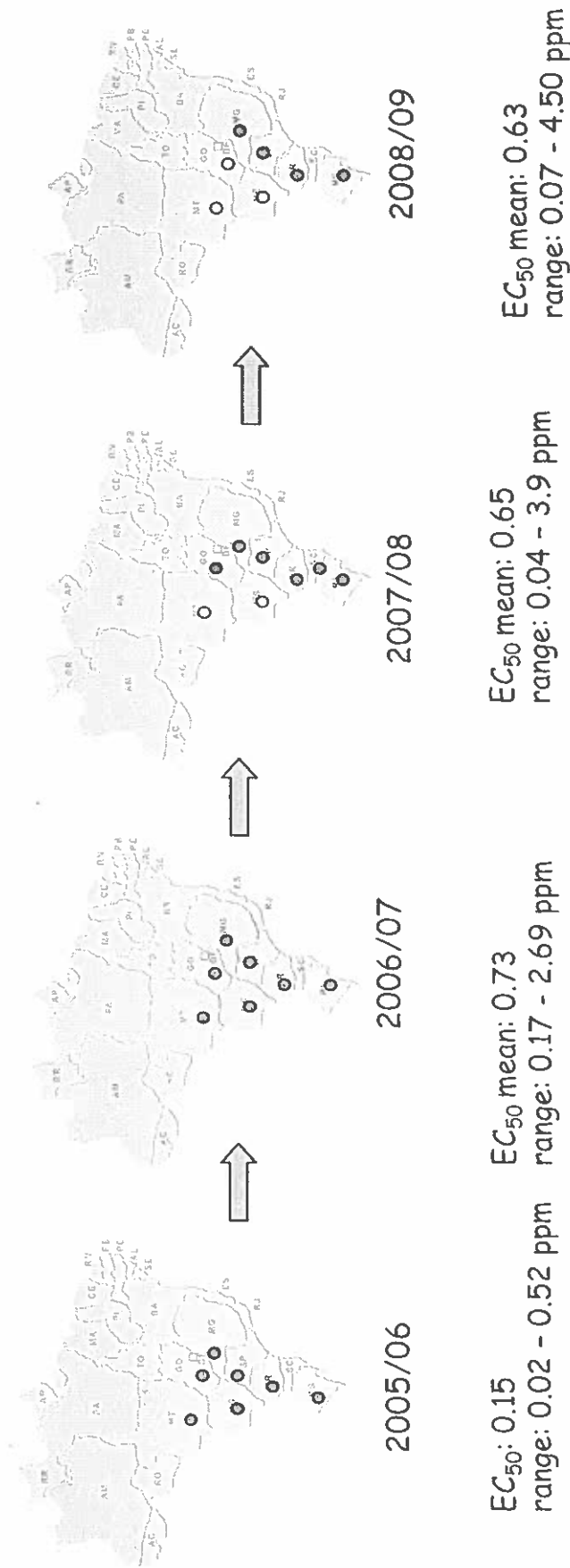


Within the first 6 hours of germination no significant sterol biosynthesis can be detected



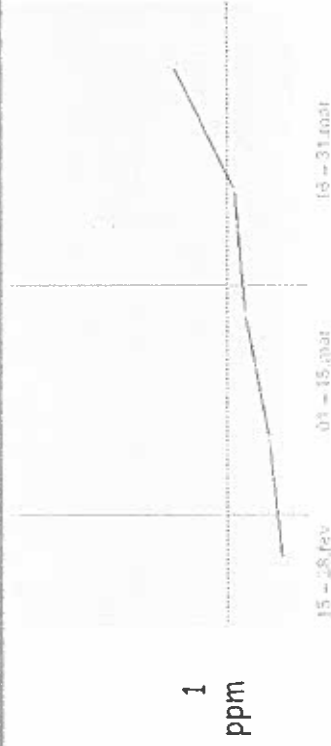
The EC_{50} (half maximal effective concentration) value is used to compare among the regions and during season time

Sensitivity monitoring tests



Sensitivity monitoring tests

EC50 values within the growing season



Londrina - Paraná

The less sensitive parts of the *P. pachyrhizi* population disappeared in the winter season period
back-shift / sensitivity recovery observable between seasons



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Why does this selection happened?

↓ Fungicide Classes *	↓ Fungicide Risk	Combined Risk	
benzimidazoles dicarboximides phenylamides QoI fungicides **	high = 3	3	9
carboxamides SBI fungicides aminopyrimidines phenylpyrroles phosphorothiolates	medium = 2	2	6
multi site fungicides (e.g. dithiocarbamates Copper, Sulphur) MBI-R inhibitors SAR inducers	low = 1	1	3
Pathogen groups *	Pathogen risk ↑	low = 1	medium = 2
	Pathogen groups * ↑	high = 3	
seed borne pathogens (e.g. <i>Pyrenophthora</i> spp., <i>Ustilago</i> spp.)		<i>Rhynchosporium secalis</i> <i>Septoria tritici</i>	<i>Erysiphe graminis</i> <i>Botrytis cinerea</i> <i>Penicillium</i> spp. <i>Magnaporthe grisea</i> <i>Venturia inaequalis</i> <i>Mycosphaerella fijiensis</i> <i>Phytophthora infestans</i>
soil-borne pathogens (e.g. <i>Phytophthora</i> spp.)		<i>rust fungi</i> <i>Kunzeomyces</i> spp. <i>Tapscottia</i> spp.	

Source: FRAC



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Why does this selection happened?

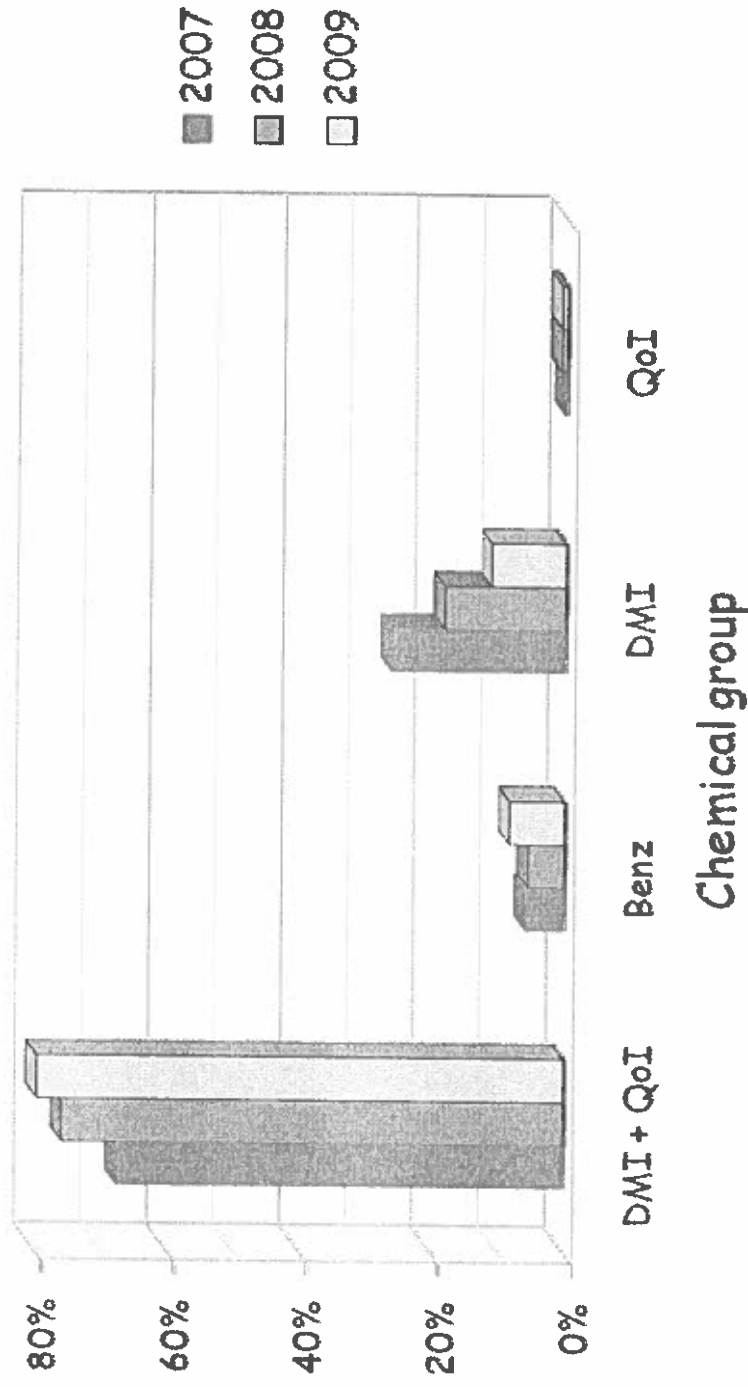
- ✓ Large number of applications, curative applications;
- ✓ Use of straight azole in sequential applications;
- ✓ Underrate;
- ✓ Large areas treated with the same product



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Market share of foliar fungicides (%)



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Resistant cultivars

BRS GO 7560 – maturity group 7.5

Source: [(Tracy x Abura) x (D67 x R48)] x Embrapa 59
Single recessive major gene (PI 203398)

Genetic and molecular biology 31:505-511 (2008)



Recommendation: same than susceptible cultivars
fungicides at first symptoms or preventive

Advantage: stability in cases of delayed control



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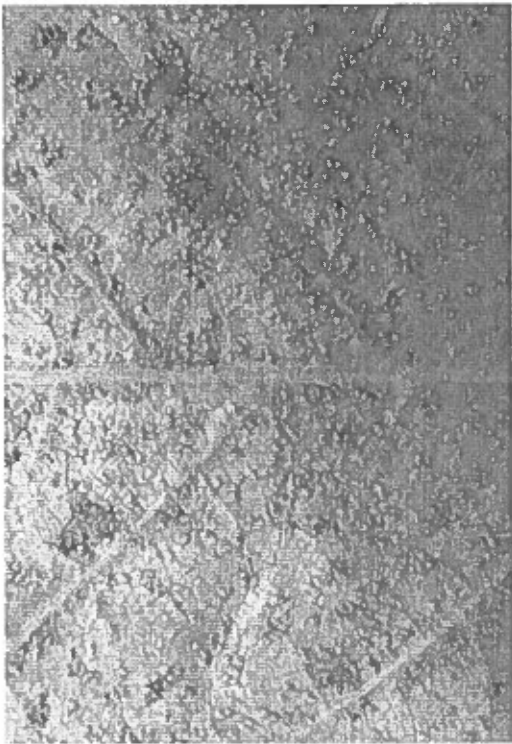


Resistant cultivars

BRSGO 7560



Susceptible



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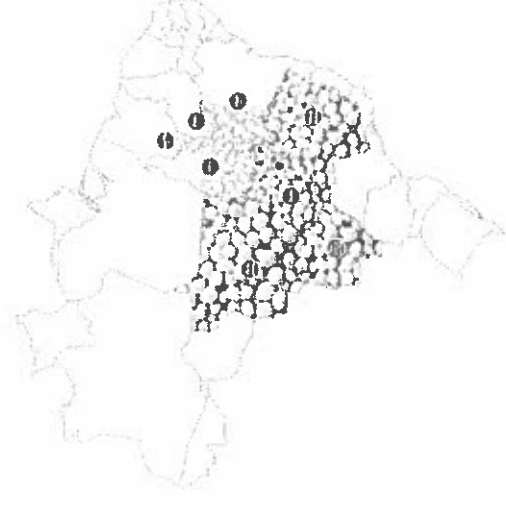
Resistant cultivars

TMG 801 - maturity group 8.2
TMG 803 - maturity group 8.7

Source: different major genes
(Rpp2, Rpp4 or Rpp5)

Recommendation:
scouting until R3;
only apply fungicide before if you have symptoms otherwise apply at R3;
scouting for second and third reapplications

Advantage:
reduce the number of fungicide application
stability in cases of delayed control



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Resistant cultivars

TMG 803

Susceptible

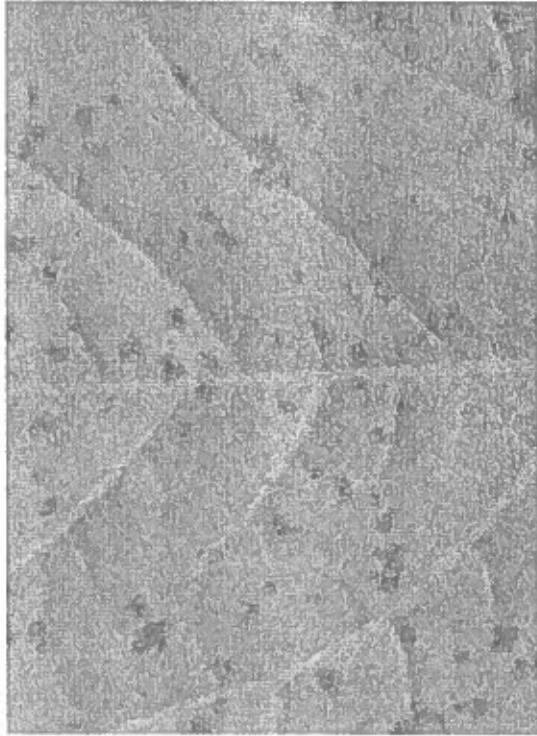


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Resistant cultivars

TMG 801



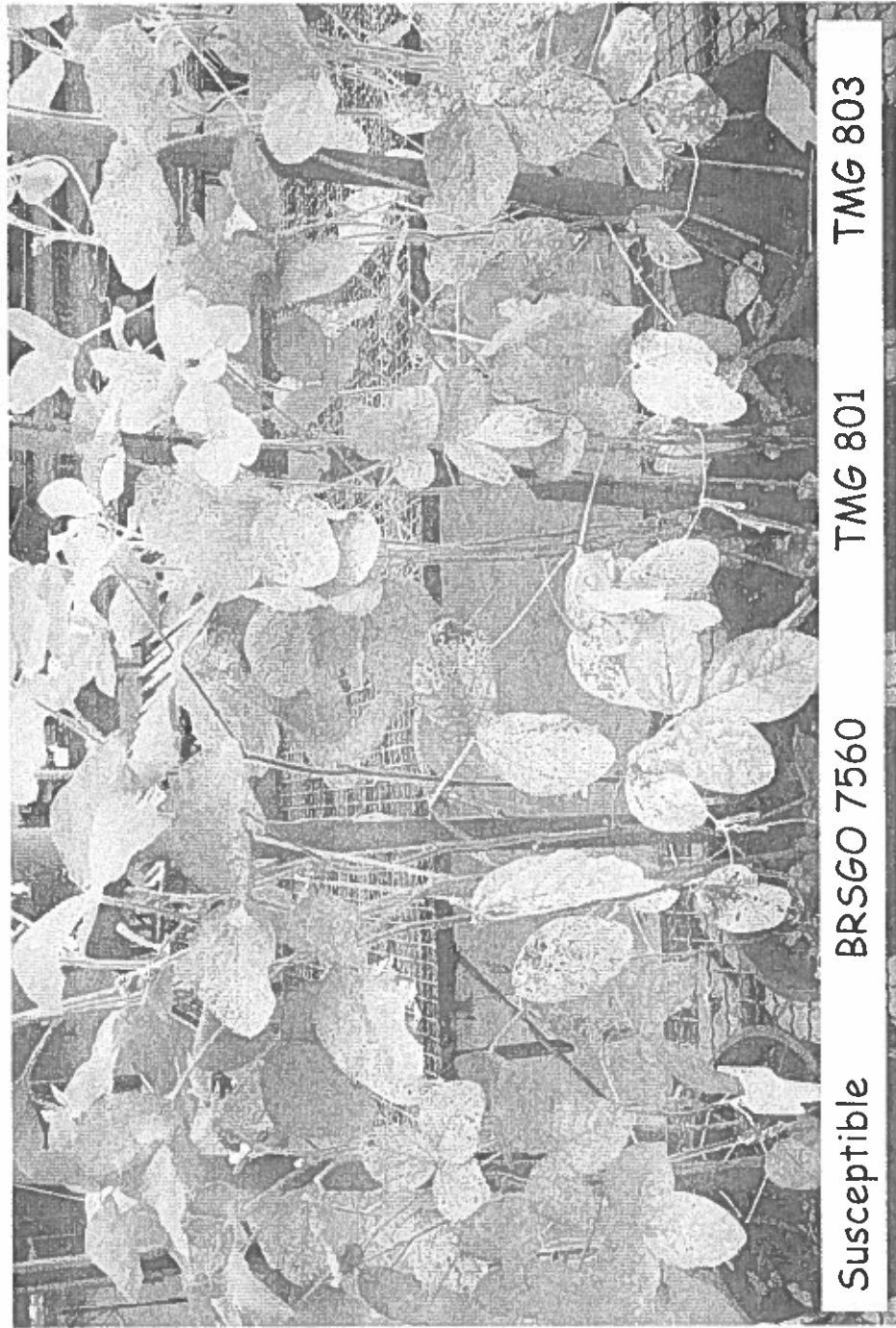
Susceptible



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Resistant cultivars



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2009/10

✓ Unusually mild and wet winter;

✓ *El Nino* Southern Oscillation (ENSO) phenomenon forecast with well distributed rain until the end of December for most soybean region in

Brazil;

✓ ASR started in commercial crops one month before 2008/09 but most of the cases after flowering stage;

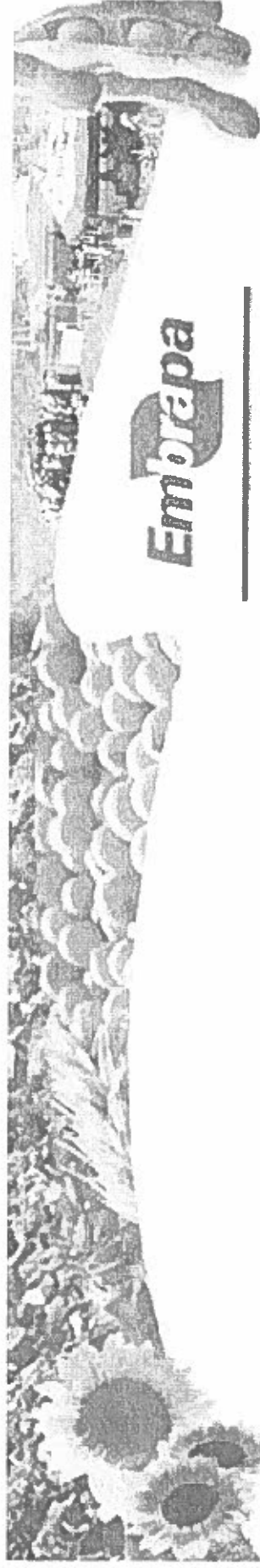
✓ Resistant cultivars are in test in the field

**SBR has shown that no crop season is alike;
new challenges appear every year**



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Embrapa

Soja

Acknowledgements

Organizing committee

Fabiano Siqueri - Mato Grosso Foundation

Pedro Singer - Bayer CropScience

Pierri Spolti and Emerson Del Ponte - UFRGS

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Protective, curative and eradivative effects of fungicides to control soybean rust caused by *Phakopsora pachyrhizi*, i....

Article in *Fitopatologia Brasileira* · November 2003

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SYNGENTA EXHIBIT 1038
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Efeitos Protetor, Curativo e Erradicante de Fungicidas no Controle da Ferrugem da Soja Causada por *Phakopsora pachyrhizi*, em Casa de Vegetação

Cláudia V. Godoy¹ & Marcelo G. Canteri²

¹Embrapa Soja, Cx. Postal 231, CEP 86001-970, Londrina, PR, fax: (43) 3371-6100, e-mail: godoy@cnpsa.embrapa.br;

²Universidade Estadual de Londrina, CEP 86051-990, Londrina, PR, fax: (43) 3371-4697, e-mail: canteri@uel.br

(Aceito para publicação em 04/11/2003)

Autor para correspondência: Cláudia V. Godoy

GODOY, C.V. & CANTERI, M.G. Efeitos protetor, curativo e erradicante de fungicidas no controle da ferrugem da soja causada por *Phakopsora pachyrhizi*, em casa de vegetação. Fitopatologia Brasileira 29:097-101. 2004.

RESUMO

Os efeitos protetor, curativo e erradicante de fungicidas sistêmicos (azoxystrobin 50 g i.a./ha + nimbus 0,5%, carbendazim 250 g i.a./ha, tebuconazole 100 g i.a./ha, difenoconazole 50 g i.a./ha e epoxiconazole 25 g i.a./ha + pyraclostrobin 66,5 g i.a./ha) foram avaliados em plantas de soja (*Glycine max*) inoculadas com suspensão de uredósporos de *Phakopsora pachyrhizi*, em casa de vegetação. Para avaliar o efeito protetor, as plantas foram tratadas com os fungicidas e inoculadas quatro, oito e 14 dias após o tratamento. Os efeitos curativo e erradicante foram avaliados em plantas previamente inoculadas com uma suspensão de uredósporos e tratadas com os fungicidas após dois, quatro e oito dias. A severidade foi quantificada 16 dias após as inoculações. Com exceção do fungicida carbendazim, os demais apresentaram efeito protetor com controle acima de 90%,

até oito dias após o tratamento. Plantas inoculadas 14 dias após o tratamento com carbendazim apresentaram severidade estatisticamente semelhante à testemunha sem controle, enquanto as plantas tratadas com os fungicidas dos grupos dos inibidores da biossíntese de ergosterol e com as estrobilurinas apresentaram controle acima de 60%. Nenhum produto mostrou efeito erradicante, quando aplicado durante o período de incubação da doença, no entanto, todos os fungicidas reduziram a severidade da doença e a viabilidade dos uredósporos. Com exceção do carbendazim, todos os fungicidas inibiram acima de 60% a germinação de uredósporos, quando aplicados até oito dias após a inoculação, no período de incubação da doença.

Palavras-chave adicionais: controle químico, ferrugem asiática, *Glycine max*.

ABSTRACT

Protective, curative and eradivative effects of fungicides to control soybean rust caused by *Phakopsora pachyrhizi*, in greenhouse

Protective, curative and eradivative effects of systemic fungicides (azoxystrobin 50 g a.i./ha + nimbus 0.5%, carbendazim 250 g a.i./ha, tebuconazole 100 g a.i./ha, difenoconazole 50 g a.i./ha e epoxiconazole 25 g a.i./ha + pyraclostrobin 66.5 g a.i./ha) were evaluated in soybean (*Glycine max*) plants inoculated with a urediniospore suspension of *Phakopsora pachyrhizi* in greenhouse. To evaluate the protective effect plants were treated with fungicides and inoculated four, eight and 14 days after the treatment. To evaluate the curative and eradivative effects, the plants were inoculated with a urediniospore suspension of *P. pachyrhizi* and treated with fungicides after two, four and eight days. Disease severity was

assessed 16 days after each inoculation. With the exception of the fungicide carbendazim, all of the fungicides inhibited over 90% of urediniospore germination until eight days after the treatment. Plants inoculated 16 days after the treatment with carbendazim presented severity statistically similar to unsprayed plants, while plants treated with fungicides of ergosterol biosynthesis inhibitors and strobilurin groups showed over 60% germination control. None of the tested fungicides provided an eradivative effect when applied before symptoms developed. However, all treatments reduced disease severity and urediniospore viability. With the exception of carbendazim, all fungicides inhibited over 60% of urediniospore germination until eight days after inoculation, in the incubation period of the disease.

A ferrugem da soja [*Glycine max* (L.) Merrill] causada por *Phakopsora pachyrhizi* H. Sydow & Sydow foi relatada pela primeira vez no Brasil no final da safra de 2000/01 (Yorinori *et al.*, 2002a), representando uma grande ameaça para os países do continente americano. Duas espécies do gênero *Phakopsora* incidem na cultura da soja (Ono *et al.*, 1992): *P. meibomiae* Arthur (Arthur) e *P. pachyrhizi*. *Phakopsora meibomiae* foi relatada pela primeira vez no Brasil em 1979, em Minas Gerais, por Deslandes (1979), sendo inicialmente classificada em função do hospedeiro como *P. pachyrhizi*.

Carvalho & Figueiredo (2000) constataram que até essa data a única espécie presente no Brasil era *P. meibomiae*, considerada espécie menos agressiva e de ocorrência endêmica, em regiões com temperaturas mais amenas. *Phakopsora pachyrhizi* ocorre no Hemisfério Leste, desde 1902, sendo considerada altamente agressiva, causando danos de 10 a 40% na Tailândia, 10-90% na Índia, 10-50% no sul da China, 23-90% em Taiwan e 40% no Japão (Sinclair & Hartman, 1999). Plantas severamente infetadas apresentam desfolha precoce, comprometendo a formação e o enchimento

de vagens e o peso final dos grãos (Yang *et al.* 1991).

Existem relatos de genes dominantes para resistência, denominados Rpp1 a Rpp4, identificados em introduções de plantas e cultivares. No entanto, a estabilidade dessa resistência é duvidosa, devido à grande variabilidade do patógeno (Hartman *et al.*, 1994). Estudos realizados pela Embrapa Soja identificaram 11 cultivares com resistência a ferrugem. No entanto, a maioria das cultivares comerciais atualmente utilizadas foi classificada como suscetível (Yorinori *et al.*, 2002b). A disponibilidade limitada de cultivares resistentes faz com que o manejo da cultura, por meio de épocas de semeadura e aplicação de defensivos, seja uma alternativa, devendo ser feito de forma racional para não inviabilizar a cultura e agredir o meio ambiente de forma indiscriminada. Os trabalhos disponíveis sobre controle químico da ferrugem, na literatura, são limitados e ausentes nas nossas condições devido à recente introdução da doença. Estudos mostram que, em condições severas de epidemia, são necessárias de três a cinco aplicações em intervalos de dez dias (Sinclair & Hartman, 1996). Na África, onde a doença apareceu em 1990, são utilizados os fungicidas pertencentes ao grupo dos inibidores da biossíntese de ergosterol (triazóis) (Caldwell *et al.*, 2002).

No Brasil, a utilização de fungicidas para o controle de oídio (*Microspheera diffusa* Cooke & Peck) e do complexo de doenças de final de ciclo (*Cercospora kikuchii* Matsu. & Tomoyasu e *Septoria glycines* Hemmi) é prática recomendada onde as doenças ocorrem, sendo a maioria dos fungicidas registrados para a cultura da soja pertencentes aos grupos dos triazóis, estrobilurinas e benzimidazóis.

O objetivo deste trabalho foi avaliar os efeitos protetor, curativo e erradicante de fungicidas registrados para a cultura da soja no controle da ferrugem causada por *P. pachyrhizi*, em casa de vegetação. O efeito protetor ou residual refere-se à proteção da planta conferida pela aplicação do produto antes da deposição do patógeno. O efeito curativo é aquele onde ocorre atenuação dos sintomas ou reparação dos danos provocados pelo patógeno, sendo uma ação dirigida contra o patógeno, após o estabelecimento de seu contato efetivo com o hospedeiro. O efeito erradicante é a atuação direta sobre o patógeno, na sua fonte de inóculo (Kimati, 1995).

Sementes da cultivar MG/BR 46 (Conquista) foram semeadas em vasos de plástico com 4 kg de mistura de terra argilosa, arcia e esterco (2:1:2), deixando-se quatro plantas por vaso. As plantas foram mantidas em casa de vegetação, durante toda condução do ensaio.

O fungo *P. pachyrhizi* foi mantido em plantas da cultivar MG/BR 46 (Conquista) e os uredósporos foram coletados lavando-se folhas infetadas com ajuda de pincel e suspensos em água destilada contendo tween 20 (0,1 ml/l) no momento do preparo da suspensão, para as inoculações.

Para avaliar o efeito protetor ou residual, os fungicidas foram previamente aplicados às plantas no estágio V4 (Fehr & Caviness, 1977), utilizando um pulverizador manual, com volume aproximado de 10 ml por vaso. Os tratamentos utilizados foram: 1 - testemunha sem aplicação; 2 - azoxystrobin 50 g i.a./ha + nimbus 0,5% v/v; 3 - carbendazin 250 g i.a./ha; 4 -

tebuconazole 100 g i.a./ha; 5 - difenoconazole 50 g i.a./ha e 6 - epoxiconazole 25 g i.a./ha + pyraclostrobin 66,5 g i.a./ha. A suspensão (3×10^4 uredósporos por ml) foi pulverizada sobre a terceira e quarta folha das plantas aos quatro, oito e 14 dias após o tratamento com fungicidas. As avaliações foram realizadas 16 dias após cada inoculação e a porcentagem de controle para cada tratamento foi calculada em relação à testemunha não tratada.

Para avaliação dos efeitos curativo e erradicante, as plantas foram previamente inoculadas com a suspensão (3×10^4 uredósporos por ml) no estágio V4, inoculando todas as folhas, e posteriormente tratadas com os fungicidas após dois, quatro e oito dias.

O delineamento experimental foi inteiramente casualizado, em arranjo fatorial, com três repetições, cada unidade experimental sendo constituída por um vaso com quatro plantas. Os tratamentos com fungicidas foram realizados pela manhã e as inoculações ao final da tarde. Após às 18:00 h, o sistema de nebulização da casa de vegetação foi acionado, em intervalos de 3 h, durante 10 min, até às 6:00 h do dia seguinte, visando garantir a presença de água livre na folha, favorecendo o processo de infecção. A temperatura da casa de vegetação durante toda condução do ensaio oscilou entre 19-28 °C.

As avaliações foram realizadas 16 dias após cada inoculação, determinando-se o número de lesões por cm² e o tamanho médio das lesões com auxílio de uma escala (Bassanezi, 1995). A severidade foi avaliada no terceiro e quarto trifólios principais de cada planta.

A viabilidade dos uredósporos de *P. pachyrhizi* foi avaliada no ensaio para controle curativo. Uredósporos coletados lavando-se aproximadamente dez folhas infetadas de cada tratamento, com ajuda de pincel, e suspensos em água destilada contendo tween 20 (0,1 ml/l) foram ajustados por meio de diluição para concentração de 10^4 esporos por ml e transferidos para placas de Petri (0,1 ml por placa) contendo meio ágar-água. As placas foram incubadas a 24 °C, no escuro por 24 h. Após a incubação, foi avaliada a porcentagem de germinação com auxílio de microscópio óptico. Uredósporos foram considerados germinados quando o comprimento do tubo germinativo ultrapassou o diâmetro do esporo. O delineamento experimental foi inteiramente casualizado, em arranjo fatorial, com três repetições, sendo cada repetição constituída de uma placa de Petri.

As análises estatísticas foram realizadas por meio do teste de Tukey a 5% de significância, para comparação de médias, utilizando o programa STATISTICA (StatSoft, Inc). Os valores de severidade e germinação em porcentagem foram transformados para análise utilizando $\arcsen \sqrt{x/100}$.

Os primeiros sintomas de *P. pachyrhizi* foram observados nove a 11 dias após as inoculações, sendo próximo ao período de incubação relatado por Melching *et al.* (1989). A severidade das plantas sem aplicação de fungicida, no ensaio para avaliação do efeito protetor foi 10,2%, na média dos três períodos de inoculação (Tabela 1). Todos os fungicidas apresentaram efeito protetor, aos quatro dias após o tratamento, mas as plantas tratadas com triazóis (tebuconazole e

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difenoconazole) e com a mistura de triazól e estrobilurina (epoxiconazole + pyraclostrobin) não manifestaram sintomas. Aos oito dias após a aplicação de fungicidas, foram observados sintomas nas plantas de todos tratamentos. No entanto, os tratamentos com triazóis, estrobilurina e mistura dos dois princípios ativos apresentaram controle superior a 90%, em relação à testemunha. O fungicida que mostrou menor residual foi o carbendazim, apresentando 75, 25 e 21% de controle, em relação à testemunha, aos quatro, oito e 14 dias após o tratamento com fungicida, respectivamente. Plantas inoculadas oito dias após o tratamento com carbendazim apresentaram severidade estatisticamente semelhante à testemunha sem tratamento. As plantas inoculadas 14 dias após os tratamentos com tebuconazole e epoxiconazole + pyraclostrobin apresentaram 88 e 90% de controle, respectivamente e os tratamentos com azoxystrobin e difenoconazole apresentaram 69 e 61% de controle, respectivamente. Na média dos três períodos de inoculação, os tratamentos que proporcionaram a menor severidade foram epoxiconazole + pyraclostrobin, tebuconazole, azoxystrobin e difenoconazole, não diferindo entre si pelo teste de Tukey ao nível de 5% de significância.

Sintomas de fitotoxicidade do tipo engrossamento e escurecimento das folhas foram observados para os tratamentos com triazóis e mistura de triazól + estrobilurina, no entanto, as plantas tratadas com difenoconazole recuperaram seu desenvolvimento, dez dias após o tratamento. O tratamento com tebuconazole resultou em plantas menores com folhas mais escuras até o final do ensaio. Esses sintomas são relatados para triazóis em outras culturas (Dickens, 1990). As plantas tratadas com epoxiconazole + pyraclostrobin apresentaram redução de crescimento, folhas encarquilhadas e encurtamento de entrenós dificultando a avaliação dos resultados. Esses sintomas severos de fitotoxicidade podem ter sido causados pelo alto volume de calda aplicada em plantas novas (estádio V4) e pelo ambiente da casa de vegetação. No campo, essa condição não deve se repetir, pois esse produto é registrado para a cultura na dose testada e não são observados esses sintomas pelos agricultores.

Todos os fungicidas reduziram a infecção quando aplicados na ausência de sintomas, durante o período de

incubação (Tabela 2). No entanto, nenhum impediu o desenvolvimento da doença, atuando de forma erradicante, quando aplicados dois dias após a inoculação. Não foi observada interação fatorial significativa nesse ensaio. A aplicação de azoxystrobin e da mistura epoxiconazole + pyraclostrobin inibiram 93% do desenvolvimento dos sintomas quando aplicados dois dias após a inoculação. Aos quatro dias após a inoculação, azoxystrobin e epoxiconazole + pyraclostrobin inibiram 93 e 85% do desenvolvimento dos sintomas, respectivamente. Aos oito dias após a inoculação, a eficiência dos fungicidas variou entre 56% (azoxystrobin) e 10% (difenoconazole). Na média, todos fungicidas foram eficientes na redução dos sintomas, quando aplicados no período de incubação da doença, sendo estatisticamente diferentes da testemunha não tratada com fungicida pelo teste de Tukey ao nível de 5% de significância. O efeito curativo dos fungicidas foi reduzido à medida que se atrasou a aplicação do fungicida, sendo a severidade média das plantas aos oito dias após a inoculação (5,8%) estatisticamente superior a severidade média observada nas plantas aos dois e quatro dias após a inoculação (1,8 e 2,4%, respectivamente).

Embora nenhum tratamento tenha tido efeito erradicante quando aplicado até dois dias após a inoculação, a diminuição da viabilidade dos uredósporos, comprovada pelo teste de germinação em ágar-água (Tabela 3) mostrou o efeito curativo dos fungicidas. A porcentagem média de germinação dos uredósporos das plantas sem tratamento foi de 55,1%. Não foi observada interação fatorial significativa nesse ensaio. Na média, todos tratamentos com fungicidas foram eficientes na redução da germinação dos uredósporos, sendo a germinação estatisticamente inferior à testemunha sem tratamento pelo teste de Tukey ao nível de 5% de significância. Com exceção do carbendazim, todos os fungicidas inibiram acima de 60% a germinação de uredósporos, quando aplicados até oito dias após a inoculação, no período de incubação da doença.

O padrão de esporulação de *P. pachyrhizi* caracteriza-se por apresentar curva de produção diária de esporos com vários picos de máxima esporulação distribuídos durante todo período infeccioso (Melching *et al.*, 1979). A redução nos sintomas, associada à menor viabilidade dos uredósporos após

TABELA 1 - Efeito protetor e residual. Severidade $\pm$ desvio padrão e controle (%) das plantas de soja (*Glycine max*) tratadas com fungicidas e inoculadas com suspensão de uredósporos de *Phakopsora pachyrhizi* aos quatro, oito e 14 dias após os tratamentos (DAT)

TRATAMENTO	4 DAT		8 DAT		14 DAT		Severidade média (%)
	Severidade (%)	Controle	Severidade (%)	Controle	Severidade (%)	Controle	
1. Testemunha sem aplicação	9,1 $\pm$ 2,5 a A	0	10,3 $\pm$ 2,1 a A	0	11,3 $\pm$ 4,2 a A	0	10,2 a
2. Azoxystrobin 50 g i.a./ha	0,6 $\pm$ 0,6 bc A	93	0,8 $\pm$ 0,7 b A	92	3,5 $\pm$ 1,3 cd A	69	1,6 c
3. Carbendazim 250 g i.a./ha	2,3 $\pm$ 0,3 b B	75	7,7 $\pm$ 1,4 a A	25	8,9 $\pm$ 0,6 ab A	21	6,3 b
4. Tebuconazole 100 g i.a./ha	0,0 c B	100	0,1 $\pm$ 0,2 b B	99	1,3 $\pm$ 0,3 cd A	88	0,5 c
5. Difenoconazole 50 g i.a./ha	0,0 c B	100	0,8 $\pm$ 1 b B	92	4,4 $\pm$ 1 bc A	61	1,7 c
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	0,0 c B	100	0,1 $\pm$ 0,1 b B	99	1,1 $\pm$ 0,6 d A	90	0,4 c
Severidade média (%)	2,0 C		3,3 B		5,1 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

TABELA 2 - Efeito curativo dos tratamentos. Severidade $\pm$ desvio padrão e controle (%) das plantas de soja (*Glycine max*) tratadas com fungicidas dois, quatro e oito dias após a inoculação (DAI) com suspensão de uredósporos de *Phakopsora pachyrhizi*

TRATAMENTO	2 DAI		4 DAI		8 DAI		Severidade média (%)
	Severidade (%)	Controle	Severidade (%)	Controle	Severidade (%)	Controle	
1. Testemunha sem aplicação	5,7 $\pm$ 1,5	0	6,7 $\pm$ 1,7	0	7,7 $\pm$ 2,2	0	6,7 a
2. Azoxystrobin 50 g i.a./ha	0,4 $\pm$ 0,7	93	0,5 $\pm$ 0,5	93	3,4 $\pm$ 1,7	56	1,4 c
3. Carbenfazin 250 g i.a./ha	1,4 $\pm$ 0,5	75	1,5 $\pm$ 1,3	78	5,8 $\pm$ 0,4	25	2,9 bc
4. Tebuconazole 100 g i.a./ha	1,9 $\pm$ 0,4	67	2,4 $\pm$ 0,8	64	5,2 $\pm$ 1,2	32	3,2 b
5. Difenoconazole 50 g i.a./ha	0,9 $\pm$ 0,5	84	2,1 $\pm$ 0,7	69	6,9 $\pm$ 1,2	10	3,3 b
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	0,4 $\pm$ 0,1	93	1,0 $\pm$ 0,5	85	5,9 $\pm$ 1,6	23	2,4 bc
Severidade média (%)	1,8 B		2,4 B		5,8 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

TABELA 3 - Porcentagem de germinação e germinação relativa de uredósporos coletados nas plantas de soja (*Glycine max*) tratadas com fungicidas dois, quatro e oito dias após a inoculação (DAI) com suspensão de uredósporos *Phakopsora pachyrhizi*

TRATAMENTO	2 DAI		4 DAI		8 DAI		Germinação média (%)
	Germinação (%)	Germinação relativa (%)	Germinação (%)	Germinação relativa (%)	Germinação (%)	Germinação relativa (%)	
1. Testemunha sem aplicação	49,4 $\pm$ 6,2	100	60,3 $\pm$ 8,3	100	55,5 $\pm$ 5,2	100	55,1 a
2. Azoxystrobin 50 g i.a./ha	7,8 $\pm$ 4,2	16	11,2 $\pm$ 6,3	19	8,7 $\pm$ 1,2	16	9,2 b
3. Carbenfazin 250 g i.a./ha	8,1 $\pm$ 5,8	16	12,7 $\pm$ 5,6	21	36,6 $\pm$ 0,5	66	19,1 b
4. Tebuconazole 100 g i.a./ha	5,5 $\pm$ 3,2	11	5,6 $\pm$ 4,7	9	11,2 $\pm$ 0,8	20	7,4 b
5. Difenoconazole 50 g i.a./ha	5,2 $\pm$ 7,4	11	8,0 $\pm$ 1,3	13	20,8 $\pm$ 5,9	37	11,3 b
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	3,8 $\pm$ 1,6	8	13,7 $\pm$ 8,9	23	13,2 $\pm$ 4,9	24	10,2 b
Germinação média (%)	13,3 B		18,6 AB		24,3 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

a aplicação de fungicidas, pode retardar o progresso da doença no campo para patógenos que apresentam esse padrão de esporulação.

O fungicida carbenfazin, pertencente ao grupo dos benzimidazóis, apresentou a menor eficiência de controle, tanto na aplicação preventiva como na curativa. Os resultados disponíveis na literatura a respeito desse grupo de fungicidas para controle da ferrugem da soja são contraditórios, sendo eficientes em alguns trabalhos e ineficientes em outros (Sinclair & Hartman, 1996).

Os resultados mostraram que os fungicidas testados, já registrados para a cultura da soja, pertencentes aos grupos estrobilurinas e triazóis, sozinhos e em misturas, são eficientes no controle de *P. pachyrhizi*. A presença de mais de um grupo com eficiência comprovada é importante no que diz respeito ao manejo de resistência de fungos a fungicidas. Fungicidas com modo de ação específico possuem um maior risco de seleção de populações resistentes do patógeno, devendo-se, dessa forma, alternar produtos com diferentes modos de ação ou utilizar misturas prontas dos dois grupos (Kimati, 1995).

Em condições de campo, como as re-inoculações naturais sucedem-se durante todo período infeccioso, devem-se realizar estudos sobre número e épocas de aplicação dos fungicidas para evitar danos com a ferrugem da soja. Estudos sobre a eficiência de diferentes fungicidas no controle curativo,

após a observação da doença no campo, devem ser realizados, para verificar a real necessidade de aplicações preventivas.

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Efeitos Protetor, Curativo e Erradicante de Fungicidas no Controle da Ferrugem da Soja Causada por *Phakopsora pachyrhizi*, em Casa de Vegetação

Cláudia V. Godoy¹ & Marcelo G. Canteri²

¹Embrapa Soja, Cx. Postal 231, CEP 86001-970, Londrina, PR, fax: (43) 3371-6100, e-mail: godoy@cnpso.embrapa.br;

²Universidade Estadual de Londrina, CEP 86051-990, Londrina, PR, fax: (43) 3371-4697, e-mail: canteri@ucl.br

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Autor para correspondência: Cláudia V. Godoy

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RESUMO

Os efeitos protetor, curativo e erradicante de fungicidas sistêmicos (azoxystrobin 50 g i.a./ha + nimbus 0,5%, carbendazim 250 g i.a./ha, tebuconazole 100 g i.a./ha, difenoconazole 50 g i.a./ha e epoxiconazole 25 g i.a./ha + pyraclostrobin 66,5 g i.a./ha) foram avaliados em plantas de soja (*Glycine max*) inoculadas com suspensão de uredósporos de *Phakopsora pachyrhizi*, em casa de vegetação. Para avaliar o efeito protetor, as plantas foram tratadas com os fungicidas e inoculadas quatro, oito e 14 dias após o tratamento. Os efeitos curativo e erradicante foram avaliados em plantas previamente inoculadas com uma suspensão de uredósporos e tratadas com os fungicidas após dois, quatro e oito dias. A severidade foi quantificada 16 dias após as inoculações. Com exceção do fungicida carbendazim, os demais apresentaram efeito protetor com controle acima de 90%,

até oito dias após o tratamento. Plantas inoculadas 14 dias após o tratamento com carbendazim apresentaram severidade estatisticamente semelhante à testemunha sem controle, enquanto as plantas tratadas com os fungicidas dos grupos dos inibidores da biossíntese de ergosterol e com as estrobilurinas apresentaram controle acima de 60%. Nenhum produto mostrou efeito erradicante, quando aplicado durante o período de incubação da doença, no entanto, todos os fungicidas reduziram a severidade da doença e a viabilidade dos uredósporos. Com exceção do carbendazim, todos os fungicidas inibiram acima de 60% a germinação de uredósporos, quando aplicados até oito dias após a inoculação, no período de incubação da doença.

Palavras-chave adicionais: controle químico, ferrugem asiática, *Glycine max*.

ABSTRACT

Protective, curative and eradivative effects of fungicides to control soybean rust caused by *Phakopsora pachyrhizi*, in greenhouse

Protective, curative and eradivative effects of systemic fungicides (azoxystrobin 50 g a.i./ha + nimbus 0.5%, carbendazim 250 g a.i./ha, tebuconazole 100 g a.i./ha, difenoconazole 50 g a.i./ha e epoxiconazole 25 g a.i./ha + pyraclostrobin 66.5 g a.i./ha) were evaluated in soybean (*Glycine max*) plants inoculated with a urediniospore suspension of *Phakopsora pachyrhizi*, in greenhouse. To evaluate the protective effect plants were treated with fungicides and inoculated four, eight and 14 days after the treatment. To evaluate the curative and eradivative effects, the plants were inoculated with a urediniospore suspension of *P. pachyrhizi* and treated with fungicides after two, four and eight days. Disease severity was

assessed 16 days after each inoculation. With the exception of the fungicide carbendazim, all of the fungicides inhibited over 90% of urediniospore germination until eight days after the treatment. Plants inoculated 16 days after the treatment with carbendazim presented severity statistically similar to unsprayed plants, while plants treated with fungicides of ergosterol biosynthesis inhibitors and strobilurin groups showed over 60% germination control. None of the tested fungicides provided an eradivative effect when applied before symptoms developed. However, all treatments reduced disease severity and urediniospore viability. With the exception of carbendazim, all fungicides inhibited over 60% of urediniospore germination until eight days after inoculation, in the incubation period of the disease.

A ferrugem da soja [*Glycine max* (L.) Merrill] causada por *Phakopsora pachyrhizi* H. Sydow & Sydow foi relatada pela primeira vez no Brasil no final da safra de 2000/01 (Yorinori *et al.*, 2002a), representando uma grande ameaça para os países do continente americano. Duas espécies do gênero *Phakopsora* incidem na cultura da soja (Ono *et al.*, 1992): *P. meibomiae* Arthur (Arthur) e *P. pachyrhizi*. *Phakopsora meibomiae* foi relatada pela primeira vez no Brasil em 1979, em Minas Gerais, por Deslandes (1979), sendo inicialmente classificada em função do hospedeiro como *P. pachyrhizi*.

Carvalho & Figueiredo (2000) constataram que até essa data a única espécie presente no Brasil era *P. meibomiae*, considerada espécie menos agressiva e de ocorrência endêmica, em regiões com temperaturas mais amenas. *Phakopsora pachyrhizi* ocorre no Hemisfério Leste, desde 1902, sendo considerada altamente agressiva, causando danos de 10 a 40% na Tailândia, 10-90% na Índia, 10-50% no sul da China, 23-90% em Taiwan e 40% no Japão (Sinclair & Hartman, 1999). Plantas severamente infetadas apresentam desfolha precoce, comprometendo a formação e o enchimento

de vagens e o peso final dos grãos (Yang *et al.* 1991).

Existem relatos de genes dominantes para resistência, denominados Rpp1 a Rpp4, identificados em introduções de plantas e cultivares. No entanto, a estabilidade dessa resistência é duvidosa, devido à grande variabilidade do patógeno (Hartman *et al.*, 1994). Estudos realizados pela Embrapa Soja identificaram 11 cultivares com resistência a ferrugem. No entanto, a maioria das cultivares comerciais atualmente utilizadas foi classificada como suscetível (Yorinori *et al.*, 2002b). A disponibilidade limitada de cultivares resistentes faz com que o manejo da cultura, por meio de épocas de semeadura e aplicação de defensivos, seja uma alternativa, devendo ser feito de forma racional para não inviabilizar a cultura e agredir o meio ambiente de forma indiscriminada. Os trabalhos disponíveis sobre controle químico da ferrugem, na literatura, são limitados e ausentes nas nossas condições devido à recente introdução da doença. Estudos mostram que, em condições severas de epidemia, são necessárias de três a cinco aplicações em intervalos de dez dias (Sinclair & Hartman, 1996). Na África, onde a doença apareceu em 1990, são utilizados os fungicidas pertencentes ao grupo dos inibidores da biossíntese de ergosterol (triazóis) (Caldwell *et al.*, 2002).

No Brasil, a utilização de fungicidas para o controle de oídio (*Microspheera diffusa* Cooke & Peck) e do complexo de doenças de final de ciclo (*Cercospora kikuchii* Matsu. & Tomoyasu e *Septoria glycines* Hemmi) é prática recomendada onde as doenças ocorrem, sendo a maioria dos fungicidas registrados para a cultura da soja pertencentes aos grupos dos triazóis, estrobilurinas e benzimidazóis.

O objetivo deste trabalho foi avaliar os efeitos protetor, curativo e erradicante de fungicidas registrados para a cultura da soja no controle da ferrugem causada por *P. pachyrhizi*, em casa de vegetação. O efeito protetor ou residual refere-se à proteção da planta conferida pela aplicação do produto antes da deposição do patógeno. O efeito curativo é aquele onde ocorre atenuação dos sintomas ou reparação dos danos provocados pelo patógeno, sendo uma ação dirigida contra o patógeno, após o estabelecimento de seu contato efetivo com o hospedeiro. O efeito erradicante é a atuação direta sobre o patógeno, na sua fonte de inóculo (Kimat, 1995).

Sementes da cultivar MG/BR 46 (Conquista) foram semeadas em vasos de plástico com 4 kg de mistura de terra argilosa, areia e esterco (2:1:2), deixando-se quatro plantas por vaso. As plantas foram mantidas em casa de vegetação, durante toda condução do ensaio.

O fungo *P. pachyrhizi* foi mantido em plantas da cultivar MG/BR 46 (Conquista) e os uredósporos foram coletados lavando-se folhas infetadas com ajuda de pincel e suspensos em água destilada contendo tween 20 (0,1 ml/l) no momento do preparo da suspensão, para as inoculações.

Para avaliar o efeito protetor ou residual, os fungicidas foram previamente aplicados às plantas no estádio V4 (Fehr & Caviness, 1977), utilizando um pulverizador manual, com volume aproximado de 10 ml por vaso. Os tratamentos utilizados foram: 1 - testemunha sem aplicação; 2 - azoxystrobin 50 g i.a./ha + nimbus 0,5% v/v; 3 - carbendazin 250 g i.a./ha; 4 -

tebuconazole 100 g i.a./ha; 5 - difenoconazole 50 g i.a./ha e 6 - epoxiconazole 25 g i.a./ha + pyraclostrobin 66,5 g i.a./ha. A suspensão (3×10^4 uredósporos por ml) foi pulverizada sobre a terceira e quarta folha das plantas aos quatro, oito e 14 dias após o tratamento com fungicidas. As avaliações foram realizadas 16 dias após cada inoculação e a porcentagem de controle para cada tratamento foi calculada em relação à testemunha não tratada.

Para avaliação dos efeitos curativo e erradicante, as plantas foram previamente inoculadas com a suspensão (3×10^4 uredósporos por ml) no estádio V4, inoculando todas as folhas, e posteriormente tratadas com os fungicidas após dois, quatro e oito dias.

O delineamento experimental foi inteiramente casualizado, em arranjo fatorial, com três repetições, cada unidade experimental sendo constituída por um vaso com quatro plantas. Os tratamentos com fungicidas foram realizados pela manhã e as inoculações ao final da tarde. Após às 18:00 h, o sistema de nebulização da casa de vegetação foi acionado, em intervalos de 3 h, durante 10 min, até às 6.00 h do dia seguinte, visando garantir a presença de água livre na folha, favorecendo o processo de infecção. A temperatura da casa de vegetação durante toda condução do ensaio oscilou entre 19-28 °C.

As avaliações foram realizadas 16 dias após cada inoculação, determinando-se o número de lesões por cm² e o tamanho médio das lesões com auxílio de uma escala (Bassanezi, 1995). A severidade foi avaliada no terceiro e quarto trifólios principais de cada planta.

A viabilidade dos uredósporos de *P. pachyrhizi* foi avaliada no ensaio para controle curativo. Uredósporos coletados lavando-se aproximadamente dez folhas infetadas de cada tratamento, com ajuda de pincel, e suspensos em água destilada contendo tween 20 (0,1 ml/l) foram ajustados por meio de diluição para concentração de 10^4 esporos por ml e transferidos para placas de Petri (0,1 ml por placa) contendo meio ágar-água. As placas foram incubadas a 24 °C, no escuro por 24 h. Após a incubação, foi avaliada a porcentagem de germinação com auxílio de microscópio óptico. Uredósporos foram considerados germinados quando o comprimento do tubo germinativo ultrapassou o diâmetro do esporo. O delineamento experimental foi inteiramente casualizado, em arranjo fatorial, com três repetições, sendo cada repetição constituída de uma placa de Petri.

As análises estatísticas foram realizadas por meio do teste de Tukey a 5% de significância, para comparação de médias, utilizando o programa STATISTICA (StatSoft, Inc). Os valores de severidade e germinação em porcentagem foram transformados para análise utilizando $\arcsin \sqrt{x/100}$.

Os primeiros sintomas de *P. pachyrhizi* foram observados nove a 11 dias após as inoculações, sendo próximo ao período de incubação relatado por Melching *et al.* (1989). A severidade das plantas sem aplicação de fungicida, no ensaio para avaliação do efeito protetor foi 10,2%, na média dos três períodos de inoculação (Tabela 1). Todos os fungicidas apresentaram efeito protetor, aos quatro dias após o tratamento, mas as plantas tratadas com triazóis (tebuconazole e

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difenoconazole) e com a mistura de triazol e estrobilurina (epoxiconazole + pyraclostrobin) não manifestaram sintomas. Aos oito dias após a aplicação de fungicidas, foram observados sintomas nas plantas de todos tratamentos. No entanto, os tratamentos com triazóis, estrobilurina e mistura dos dois princípios ativos apresentaram controle superior a 90%, em relação à testemunha. O fungicida que mostrou menor residual foi o carbendazim, apresentando 75, 25 e 21% de controle, em relação à testemunha, aos quatro, oito e 14 dias após o tratamento com fungicida, respectivamente. Plantas inoculadas oito dias após o tratamento com carbendazim apresentaram severidade estatisticamente semelhante à testemunha sem tratamento. As plantas inoculadas 14 dias após os tratamentos com tebuconazole e epoxiconazole + pyraclostrobin apresentaram 88 e 90% de controle, respectivamente e os tratamentos com azoxystrobin e difenoconazole apresentaram 69 e 61% de controle, respectivamente. Na média dos três períodos de inoculação, os tratamentos que proporcionaram a menor severidade foram epoxiconazole + pyraclostrobin, tebuconazole, azoxystrobin e difenoconazole, não diferindo entre si pelo teste de Tukey ao nível de 5% de significância.

Sintomas de fitotoxicidade do tipo engrossamento e escurecimento das folhas foram observados para os tratamentos com triazóis e mistura de triazol + estrobilurina, no entanto, as plantas tratadas com difenoconazole recuperaram seu desenvolvimento, dez dias após o tratamento. O tratamento com tebuconazole resultou em plantas menores com folhas mais escuras até o final do ensaio. Esses sintomas são relatados para triazóis em outras culturas (Dickens, 1990). As plantas tratadas com epoxiconazole + pyraclostrobin apresentaram redução de crescimento, folhas encarquilhadas e encurtamento de entrenós dificultando a avaliação dos resultados. Esses sintomas severos de fitotoxicidade podem ter sido causados pelo alto volume de calda aplicada em plantas novas (estádio V4) e pelo ambiente da casa de vegetação. No campo, essa condição não deve se repetir, pois esse produto é registrado para a cultura na dose testada e não são observados esses sintomas pelos agricultores.

Todos os fungicidas reduziram a infecção quando aplicados na ausência de sintomas, durante o período de

incubação (Tabela 2). No entanto, nenhum impediu o desenvolvimento da doença, atuando de forma erradicante, quando aplicados dois dias após a inoculação. Não foi observada interação fatorial significativa nesse ensaio. A aplicação de azoxystrobin e da mistura epoxiconazole + pyraclostrobin inibiram 93% do desenvolvimento dos sintomas quando aplicados dois dias após a inoculação. Aos quatro dias após a inoculação, azoxystrobin e epoxiconazole + pyraclostrobin inibiram 93 e 85% do desenvolvimento dos sintomas, respectivamente. Aos oito dias após a inoculação, a eficiência dos fungicidas variou entre 56% (azoxystrobin) e 10% (difenoconazole). Na média, todos fungicidas foram eficientes na redução dos sintomas, quando aplicados no período de incubação da doença, sendo estatisticamente diferentes da testemunha não tratada com fungicida pelo teste de Tukey ao nível de 5% de significância. O efeito curativo dos fungicidas foi reduzido à medida que se atrasou a aplicação do fungicida, sendo a severidade média das plantas aos oito dias após a inoculação (5,8%) estatisticamente superior a severidade média observada nas plantas aos dois e quatro dias após a inoculação (1,8 e 2,4%, respectivamente).

Embora nenhum tratamento tenha tido efeito erradicante quando aplicado até dois dias após a inoculação, a diminuição da viabilidade dos uredósporos, comprovada pelo teste de germinação em ágar-água (Tabela 3) mostrou o efeito curativo dos fungicidas. A porcentagem média de germinação dos uredósporos das plantas sem tratamento foi de 55,1%. Não foi observada interação fatorial significativa nesse ensaio. Na média, todos tratamentos com fungicidas foram eficientes na redução da germinação dos uredósporos, sendo a germinação estatisticamente inferior à testemunha sem tratamento pelo teste de Tukey ao nível de 5% de significância. Com exceção do carbendazim, todos os fungicidas inibiram acima de 60% a germinação de uredósporos, quando aplicados até oito dias após a inoculação, no período de incubação da doença.

O padrão de esporulação de *P. pachyrhizi* caracteriza-se por apresentar curva de produção diária de esporos com vários picos de máxima esporulação distribuídos durante todo período infeccioso (Melching *et al.*, 1979). A redução nos sintomas, associada à menor viabilidade dos uredósporos após

TABELA 1 - Efeito protetor e residual. Severidade $\pm$ desvio padrão e controle (%) das plantas de soja (*Glycine max*) tratadas com fungicidas e inoculadas com suspensão de uredósporos de *Phakopsora pachyrhizi* aos quatro, oito e 14 dias após os tratamentos (DAT)

TRATAMENTO	4 DAT		8 DAT		14 DAT		Severidade média (%)
	Severidade (%)	Controle	Severidade (%)	Controle	Severidade (%)	Controle	
1. Testemunha sem aplicação	9,1 $\pm$ 2,5 a A	0	10,3 $\pm$ 2,1 a A	0	11,3 $\pm$ 4,2 a A	0	10,2 a
2. Azoxystrobin 50 g i.a./ha	0,6 $\pm$ 0,6 bc A	93	0,8 $\pm$ 0,7 b A	92	3,5 $\pm$ 1,3 cd A	69	1,6 c
3. Carbendazim 250 g i.a./ha	2,3 $\pm$ 0,3 b B	75	7,7 $\pm$ 1,4 a A	25	8,9 $\pm$ 0,6 ab A	21	6,3 b
4. Tebuconazole 100 g i.a./ha	0,0 c B	100	0,1 $\pm$ 0,2 b B	99	1,3 $\pm$ 0,3 cd A	88	0,5 c
5. Difenoconazole 50 g i.a./ha	0,0 c B	100	0,8 $\pm$ 1 b B	92	4,4 $\pm$ 1 bc A	61	1,7 c
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	0,0 c B	100	0,1 $\pm$ 0,1 b B	99	1,1 $\pm$ 0,6 d A	90	0,4 c
Severidade média (%)	2,0 C		3,3 B		5,1 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

TABELA 2 - Efeito curativo dos tratamentos. Severidade $\pm$ desvio padrão e controle (%) das plantas de soja (*Glycine max*) tratadas com fungicidas dois, quatro e oito dias após a inoculação (DAI) com suspensão de uredósporos de *Phakopsora pachyrhizi*

TRATAMENTO	2 DAI		4 DAI		8 DAI		Severidade média (%)
	Severidade (%)	Controle	Severidade (%)	Controle	Severidade (%)	Controle	
1. Testemunha sem aplicação	5,7 $\pm$ 1,5	0	6,7 $\pm$ 1,7	0	7,7 $\pm$ 2,2	0	6,7 a
2. Azoxystrobin 50 g i.a./ha	0,4 $\pm$ 0,7	93	0,5 $\pm$ 0,5	93	3,4 $\pm$ 1,7	56	1,4 c
3. Carbendazin 250 g i.a./ha	1,4 $\pm$ 0,5	75	1,5 $\pm$ 1,3	78	5,8 $\pm$ 0,4	25	2,9 bc
4. Tebuconazole 100 g i.a./ha	1,9 $\pm$ 0,4	67	2,4 $\pm$ 0,8	64	5,2 $\pm$ 1,2	32	3,2 b
5. Difenoconazole 50 g i.a./ha	0,9 $\pm$ 0,5	84	2,1 $\pm$ 0,7	69	6,9 $\pm$ 1,2	10	3,3 b
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	0,4 $\pm$ 0,1	93	1,0 $\pm$ 0,5	85	5,9 $\pm$ 1,6	23	2,4 bc
Severidade média (%)	1,8 B		2,4 B		5,8 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

TABELA 3 - Porcentagem de germinação e germinação relativa de uredósporos coletados nas plantas de soja (*Glycine max*) tratadas com fungicidas dois, quatro e oito dias após a inoculação (DAI) com suspensão de uredósporos *Phakopsora pachyrhizi*

TRATAMENTO	2 DAI		4 DAI		8 DAI		Germinação média (%)
	Germinação (%)	Germinação relativa (%)	Germinação (%)	Germinação relativa (%)	Germinação (%)	Germinação relativa (%)	
1. Testemunha sem aplicação	49,4 $\pm$ 6,2	100	60,3 $\pm$ 8,3	100	55,5 $\pm$ 5,2	100	55,1 a
2. Azoxystrobin 50 g i.a./ha	7,8 $\pm$ 4,2	16	11,2 $\pm$ 6,3	19	8,7 $\pm$ 1,2	16	9,2 b
3. Carbendazin 250 g i.a./ha	8,1 $\pm$ 5,8	16	12,7 $\pm$ 5,6	21	36,6 $\pm$ 0,5	66	19,1 b
4. Tebuconazole 100 g i.a./ha	5,5 $\pm$ 3,2	11	5,6 $\pm$ 4,7	9	11,2 $\pm$ 0,8	20	7,4 b
5. Difenoconazole 50 g i.a./ha	5,2 $\pm$ 7,4	11	8,0 $\pm$ 1,3	13	20,8 $\pm$ 5,9	37	11,3 b
6. Epoxiconazole 25 + pyraclostrobin 66,5 g i.a./ha	3,8 $\pm$ 1,6	8	13,7 $\pm$ 8,9	23	13,2 $\pm$ 4,9	24	10,2 b
Germinação média (%)	13,3 B		18,6 AB		24,3 A		

Médias seguidas por letras distintas, minúsculas na vertical e maiúsculas na horizontal, diferem entre si, ao nível de 5% de probabilidade, pelo teste de Tukey.

a aplicação de fungicidas, pode retardar o progresso da doença no campo para patógenos que apresentam esse padrão de esporulação.

O fungicida carbendazin, pertencente ao grupo dos benzimidazóis, apresentou a menor eficiência de controle, tanto na aplicação preventiva como na curativa. Os resultados disponíveis na literatura a respeito desse grupo de fungicidas para controle da ferrugem da soja são contraditórios, sendo eficientes em alguns trabalhos e ineficientes em outros (Sinclair & Hartman, 1996).

Os resultados mostraram que os fungicidas testados, já registrados para a cultura da soja, pertencentes aos grupos estrobilurinas e triazóis, sozinhos e em misturas, são eficientes no controle de *P. pachyrhizi*. A presença de mais de um grupo com eficiência comprovada é importante no que diz respeito ao manejo de resistência de fungos a fungicidas. Fungicidas com modo de ação específico possuem um maior risco de seleção de populações resistentes do patógeno, devendo-se, dessa forma, alternar produtos com diferentes modos de ação ou utilizar misturas prontas dos dois grupos (Kimati, 1995).

Em condições de campo, como as re-inoculações naturais sucedem-se durante todo período infeccioso, devem-se realizar estudos sobre número e épocas de aplicação dos fungicidas para evitar danos com a ferrugem da soja. Estudos sobre a eficiência de diferentes fungicidas no controle curativo,

após a observação da doença no campo, devem ser realizados, para verificar a real necessidade de aplicações preventivas.

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Epidemics of Soybean Rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003

J. T. Yorinori, Embrapa Soja, 86001-970, Londrina, PR, Brazil; W. M. Paiva, CRIA, Capitán Miranda, Paraguay; R. D. Frederick, USDA/ARS, Foreign Disease-Weed Science Research Unit, 1301 Ditto Ave., Fort Detrick, MD 21702 USA; L. M. Costamilan and P. F. Bertagnolli, Embrapa Trigo, 99001-970, Passo Fundo, Brazil; G. E. Hartman, USDA/ARS, Department of Crop Sciences, University of Illinois, Urbana-Champaign, IL, USA; C. V. Godoy, Embrapa Soja, 86001-970, Londrina, PR, Brazil; and J. Nunes, Jr., CTPA, Goiania, GO, Brazil

ABSTRACT

Yorinori, J. T., Paiva, W. M., Frederick, R. D., Costamilan, L. M., Bertagnolli, P. F., Hartman, G. E., Godoy, C. V., and Nunes, J., Jr. 2005. Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Dis. 89:675-677.

In 5 March 2001, a severe rust outbreak was recorded at Pitapó, Paraguay, and the causal organism was determined to be *Phakopsora pachyrhizi* using polymerase chain reaction (PCR) and DNA sequence analysis. In May, rust surveys showed spread throughout most of Paraguay and into western and northern Paraná, Brazil. In the 2001–02 season, rust was widespread in Paraguay, but losses were reduced due to severe drought; however, in Brazil it spread to more than 60% of the soybean acreage, causing field losses estimated at 0.1 million metric tons (MMT). In 2003, the disease was observed in more than 90% of the fields in Brazil, and the projected losses in Mato Grosso and Bahia alone are 2.2 MMT (US\$487.3 million). Approximately 80% of the soybean acreage in Brazil was sprayed twice with fungicides at the cost of US\$544 million. Differences in efficacy have been observed among the commercial strobilurin and triazol fungicides.

Soybeans (*Glycine max*) are infected by two species of *Phakopsora* that cause rust disease, *P. meibomiae* ("American" rust) (3,8) and the most feared, *P. pachyrhizi*, the cause of the "Asian" rust (1,6,7,9). In March 2001, a rust epidemic was recorded at Pitapó, Paraguay, causing severe yield losses (5). In May, the disease was also found on volunteer and second-crop soybeans in Paraguay and Brazil (Fig. 1). Comparative DNA tests between *P. meibomiae* and *P. pachyrhizi*, performed at USDA-ARS-NAA, Foreign Disease-Weed Science Research Unit (FDWSRU), Fort Detrick, MD, confirmed that the epidemic was caused by *P. pachyrhizi* (4). In this work, a report is given on the expansion of rust disease and the losses caused in Paraguay and Brazil from 2001 to 2003.

MATERIALS AND METHODS

Disease survey and yield loss assessments were carried out through field visits, fungicide trials, comparisons between

sprayed and unsprayed farmers' fields, and visual assessment by associating disease severity and plant growth stages. In 2001, diseased leaf samples were collected from Paraguay and Brazil and sent to USDA-ARS-NAA, FDWSRU, for species identification by DNA analysis and comparisons between known *P. meibomiae* and *P. pachyrhizi* isolates.

RESULTS

In Paraguay in 2001, rust control with fungicides resulted in 60% higher yield than the check (Fig. 2). A disease survey completed in May showed severe incidence of rust on volunteer and second-crop soybeans in all surveyed areas (4). In Brazil, rust was also found on volunteer and second-crop soybeans in the west and north of Paraná state (5,10). The genetic analysis of the two *Phakopsora* species confirmed that the rust epidemic was caused by *P. pachyrhizi*. Besides soybeans, in Paraguay, kudzu bean (*Pueraria lobata*) growing wild on the roadside and in forested areas was heavily infected and showed to be an important source of inoculum for rust.

In the 2001–02 crop season, rust was found on all soybean fields in Paraguay, from Encarnación to Troncal 3 (Cateté) (Fig. 3), but severe drought and extensive use of fungicides reduced losses. In Brazil, the rust was found on an estimated 60%

of soybean acreage, being especially serious in the south-central regions. In southern Goiás (GO) and Mato Grosso (MT) and the northeast of South Mato Grosso (MS) (Fig. 3), yield losses ranged from 30 to 75%. In Rio Grande do Sul (RS), losses on individual farms were as high as 48%. Based on government (CONAB, JUN/03) soybean production estimates for the 2001–02 crop season (41.917 million t), total grain losses due to rust reached 569,220 t (US\$125.5 million, at US\$220.50/t) (2).

In 2002–03, the soybean rust spread to almost all of Brazil, with few places not reporting cases of rust (Fig. 4). Despite the abundant rainfall, the high temperatures in the South may have restricted rust development in those areas where it was severe the previous year. The disease was most severe where it was not recorded before: the northeast (Bahia-BA) and the north-central (MT, GO, and Minas Gerais) regions. In two of the hardest hit states (MT, with 4.2 million ha, and Bahia, with 830,000 ha), the losses were estimated at 3.1 million t or the equivalent to US\$677 million. Additional losses, as high as 63%, occurred in the states of RS, GO, and MG. Total grain losses for 2003 were estimated at 3.4 million t or the equivalent to US\$759 million. Because of the rust outbreak in the previous year, about 80% (14.8 million ha) of the



Fig. 1. First rust sites in Paraguay and Brazil.

Corresponding author: J. T. Yorinori
E-mail: tadashi@cnpsa.embrapa.br
and yorinori@sercomiel.com.br

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Fig. 2. Efficacy of fungicide treatments. Photo by Wilfrido Morel.



Fig. 3. Expansion of rust in 2002.



Fig. 4. Expansion of rust in 2003.

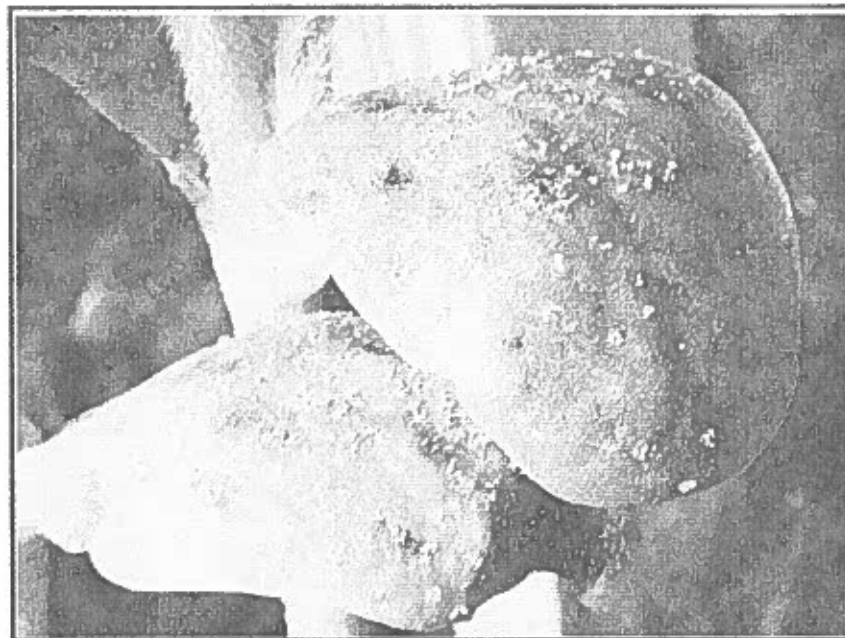


Fig. 5. Race-2003 sporulating on cotyledons.

soybean acreage in Brazil (18.534 million ha) had an average of two fungicide sprays. At the cost of US\$40.00/ha, a total US\$592 million was spent on control. Therefore, the cost of soybean rust to Brazilian farmers in 2003 reached the figure of US\$2 billion.

The rust epidemics that occurred in the northeast and north-central regions of Brazil were due to a new and highly virulent race of *P. pachyrhizi*. This race broke the resistance of all available commercial varieties, and for the first time, a severe infection and sporulation on the cotyledons was seen under greenhouse conditions (Fig. 5).

The first outbreak of rust in Bolivia was detected at San Juan de Yapacany, in the last week of July 2003, affecting the second plantings grown in the winter for seed production. A little over 100,000 ha are normally sown during this season. Although there is no rainfall during the winter, the cool weather allows for adequate moisture for a prolonged dew period. Thus, there is a potential for heavy losses due to rust. Moreover, application of foliar fungicides is not a routine practice on second-crop soybeans.

The efficiency of rust control depends upon careful and continuous field monitoring and timely application of fungicide. Currently, strobilurins and triazole fungicides are being used. It is important to note that remarkable differences exist among these compounds in their mode of action (preventive and curative). Prior knowledge of this detail is of utmost importance in choosing the fungicide and the timing of application, once the disease is established on the plant.

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