

IMPACT OF GLYPHOSATE ASSOCIATED WITH INSECTICIDE AND FUNGICIDE ON MICROBIAL ACTIVITY AND THE POTENTIAL FOR PHOSPHATE SOLUBILIZATION IN SOIL CULTIVATED WITH ROUNDUP READY® SOYBEANS¹

Impact of Glyphosate Associated with Insecticide and Fungicide on Microbial Activity and the Potential for Phosphate Solubilization in Soil Cultivated with Roundup Ready® Soybeans

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[bilingual text:] RESUMO - Objetivou-se com este trabalho avaliar a atividade microbiana e o potencial de solubilização de fosfato inorgânico em solos cultivados com soja sob diferentes manejos fitossanitários. O experimento foi conduzido em campo, em Argissolo Vermelho-Amarelo câmbico. Foram avaliados dez tratamentos em delineamento de blocos casualizados, no esquema de parcelas subdivididas, com quatro repetições. Nas parcelas, avaliou-se o efeito da aplicação ou não da mistura de inseticida (endossulfan) + fungicida (tebuconazole) e, nas subparcelas, o efeito dos métodos de controle de plantas daninhas (testemunha não capinada, testemunha capinada, aplicação única de glyphosate, aplicação sequencial de glyphosate e aplicação única de fomesafen + fluazifop-p-butyl). Amostras de solo da entrelinha da cultura foram coletadas quando as plantas atingiram o estágio R₂, para avaliação de taxa respiratória, biomassa microbiana, quociente metabólico, potencial de solubilização de fosfato inorgânico e potencial hidrogeniônico do solo. Quanto à taxa respiratória do solo, não se observaram alterações com a utilização dos diferentes manejos fitossanitários. Os herbicidas avaliados interferiram em características como o MBC e qCO₂ – variáveis diretamente relacionadas à qualidade do solo. O glyphosate aplicado em dose única ou sequencial associado ou não com endossulfan + tebuconazole apresentou os menores valores de qCO₂ (0.075 – 0.079 mg mg⁻¹ d⁻¹) e os maiores de MBC (239.64 – 312.82 mg g⁻¹), indicando menor grau de distúrbio do solo. Maiores atividades de solubilização de fosfato (425 e 472 mg L⁻¹) foram observadas nos tratamentos com aplicação única ou sequencial de glyphosate, respectivamente, sem combinação com endossulfan + tebuconazole. A aplicação dos agrotóxicos na parte aérea das plantas de soja interfere na atividade dos microrganismos associados à rizosfera.

Palavras-chave: biomassa microbiana, soja transgênica, quociente metabólico.

ABSTRACT - The objective of this work was to evaluate the microbial activity and phosphate solubilization potential of a soil cultivated with soybean under different phytosanitary management strategies. The experiment was conducted in the field in a Cambic red-yellow Argisol. Ten treatments were arranged in a randomized block design with four replications. The effect of fungicide + herbicide application (endosulfan + tebuconazole) was evaluated in the plots, while weed control management was studied in the subplots (hoed or unhoed control; single-dose or sequential glyphosate application; single-dose fomesafen + fluazifop-p-butyl application). Soil samples from the inter-row were collected when plants reached the R₂ stage and were used to measure soil respiratory rate, microbial biomass carbon (MBC), metabolic quotient (qCO₂), phosphate solubilization potential, and pH. The phytosanitary management strategies evaluated did not affect soil respiratory rates. The herbicides tested influenced soil MBC and qCO₂, variables directly related to soil quality. Glyphosate applied in a single dose or sequentially, either in combination with endosulfan + tebuconazole or not, led to lower qCO₂ values (0.075-0.079 mg μg⁻¹ d⁻¹) and higher MBC (239.64 – 312.82 μg g⁻¹), indicating less soil disturbance. Higher phosphate solubilizing activity, 425 and 472 mg L⁻¹, were observed for the treatments with single-dose or sequential application of glyphosate, respectively, in the absence of endosulfan + tebuconazole. Agrochemical application on soybean shoots affects the activity of soil microorganisms in the plant rhizosphere.

Keywords: microbial biomass, transgenic soybean, metabolic quotient.

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INTRODUCTION

Brazil is the world's largest consumer of pesticides, already having surpassed the USA; in the 2008 crop, about seven billion dollars worth of pesticides were marketed in Brazil (*Agência Nacional de Defesa Vegetal, ANDEF* [National Plant Defense Agency], 2009). Among all these pesticides, herbicides accounted for the largest volume marketed. The adoption of the glyphosate-resistant transgenic soybean crop has led to the intensive consumption of this herbicide for weed management, in view of the concomitant adoption of direct planting over conventional methods, changes in the weed population with the establishment of tolerant or resistant species and market strategies (Bonny *et al.*, 2007). However, increased herbicide consumption can lead to greater negative environmental impacts, especially those related to the soil, considering that about 60-70% of the herbicide applied post-emergence can directly or indirectly affect this environment (Law *et al.*, 2001). The application of herbicides may alter microbial activity in the soil, depending on the herbicide applied, the type of soil, the species of the plant and the microbiota and its interactions (Santos *et al.*, 2005; Reis *et al.*, 2008).

Microbial activity (measured by the evolution of CO₂) in the soil has been used as an estimator of the physiological activity of soil microbiota and the consequences of agricultural practices on ecosystems (Totola & Chaer, 2002). Microbial biomass is responsible for essential soil functions, such as the breakdown of organic matter, cycling of mineral nutrients, promotion of plant growth, biological control and degradation of compounds in the soil. It has been proposed as a parameter sensitive to the disturbances caused in this environment, especially with regard to those resulting from the application of xenobiotic substances (Wardle, 1994; Sylvia *et al.*, 2005). For more effective interpretation of soil quality, the metabolic quotient (qCO₂), which represents the relationship between the evolution of CO₂ and

the carbon of soil microbial biomass, is often used (Anderson & Domsch, 1985). This quotient is considered an indicator of soil quality and part of the assumption that, as microbial biomass becomes efficient in using resources, less carbon is lost as CO₂ by respiration. Carbon is immobilized in the microbial cells; thus, lower qCO₂ represents microbial biomass in a condition of equilibrium; i.e., an environment with a lower rate of disturbance.

In theory, glyphosate does not alter the microbial activity of the soil due to its inactivation in the soil by the binding of the phosphate group present in the molecule to iron and aluminum sesquioxides (Lancaster *et al.*, 2006; Pereira *et al.*, 2008). Respiratory rate is not always sensitive to soil disturbances, because it is a redundant activity in this environment, performed by different microorganisms. On the other hand, microbial biomass may be partially inhibited by some pesticides. However, tolerant microorganisms prevail over sensitive ones, and there is no change in total microbial biomass.

Thorough analysis of the effects of glyphosate on soil requires quantitative and qualitative studies of the microbiota present therein, such as assessments of specific activities performed by certain microbial or functional groups. Tótola & Chaer (2002) and Das *et al.* (2003) have emphasized that the activities of the microorganisms involved in biogeochemical cycles are considered satisfactory indicators of soil quality, since they directly influence the availability of nutrients and assist in clarifying changes in the functioning of the soil ecosystem.

Among the functional groups, inorganic phosphate solubilizing microorganisms (IPSM) are well suited as an indicator of soil quality, especially in tropical soils. Despite being abundant in the soil, phosphorus (P) is found in very low concentrations in soil solution: 0.1 to 1.0 mg L⁻¹ of H₂PO₄ - (Novais *et al.*, 2007). Much of the unavailable P is found precipitated with calcium (P-Ca) in less acidic soils, and with iron (P-Fe) and aluminum (P-Al) in more acidic

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soils. In order to retrieve precipitated P, plants and IPSM naturally acidify rhizospheric soil by releasing protons and organic acids (Rodríguez & Fraga, 1999; Novais *et al.*, 2007). IPSMs are responsible for releasing organic acids, such as gluconic, citric, glutamic, oxalic, lactic, fumaric, tartaric and succinic acids, which act as proton donors and chelating agents of the Ca, Al and Fe ions, favoring the solubilization of inorganic phosphate in soil (Rodríguez & Fraga, 1999). IPSMs make up about 5 to 10% of the total soil microbiota and they are found in most soils. The diversity and populations of IPSM are considerably higher in soils surrounding plant roots; that is, in rhizospheric soils (Nahas *et al.*, 1994; Nautiyal, 1999). Also, IPSMs have the ability to promote plant growth by exuding plant hormones, vitamins and antibiotics. Furthermore, the population and activity of the IPSM are related to soil management and type, being considered microbiological indicators associated with the P cycle in soil (Kucey, 1983; Nahas *et al.*, 1994; Carneiro *et al.*, 2004). It can also contribute to the adoption of agricultural practices that have a smaller negative impact on the environment. However, in Brazil there are few studies that show the potential impacts of application of herbicides on soil IPSM (Massenssini *et al.*, 2008; Reis *et al.*, 2008).

In light of the foregoing, the purpose of this study was to assess respiration and microbial biomass, the metabolic quotient of rhizospheric soil and the potential for solubilization of phosphate in soils cultivated with soybeans treated with herbicides applied in isolation or in conjunction with insecticides and fungicides.

MATERIAL AND METHODS

The experiment was conducted in the field at the Federal University of Viçosa experimental

station in Coimbra-MG (S20° 45'20" and W42° 52'40"; altitude 700 m), between December and April in the 2007/08 agricultural season, in chemically and physically characterized cambic horizon Red-Yellow Argisol (Table 1).

Fifteen days prior to sowing the soybeans, the area was chemically desiccated with glyphosate + 2,4-D (1,440 + 470 g ha⁻¹) in a mixture in a tank. Direct planting of BRS Favorite RR (Roundup Ready®) soybean was performed in the first half of December. The seeds were treated with carbendazim + thiram (0.30 + 0.70 g per kg⁻¹ of seeds) and inoculated with the SEMIA 587 and SEMIA 5019 strains of *Bradyrhizobium japonicum* (4.2 x 10⁹ viable cells per kg⁻¹ of seeds). After planting, 40 plots (10x10 m) were demarcated. A randomized-block design was used for the subdivided plots scheme, with four repetitions. In the plots, the effect, or lack thereof, of application of the insecticide + fungicide mixture was assessed and, in the subplots, the effect of weed management methods was also assessed. Applications of the insecticide endosulfan (525 g ha⁻¹) and the fungicide tebuconazole (150 g ha⁻¹), in a tank mixture were performed at 45 and 90 days after emergence (DAE) of the soybean seedlings. The weed management methods were: 1 – untrimmed control; 2 – trimmed control; 3 – single application of glyphosate (1,080 g ha⁻¹) at 15 DAE; 4 – sequential application of glyphosate (1,080 g ha⁻¹) at 15, 30 and 45 DAE; and 5 – application of fomesafen + fluazifop-p-butyl (180 + 225 g ha⁻¹) at 15 DAE. Daily rainfall, relative humidity and average temperature data were gathered at a weather station located near the plantation area throughout the entire period of the experiment (Figure 1).

Table 1 - Main physical and chemical characteristics of the cambic horizon Red-Yellow Argisol cultivated with Roundup Ready® soybeans from December/2007 to April/2008. Coimbra-MG, 2007/08

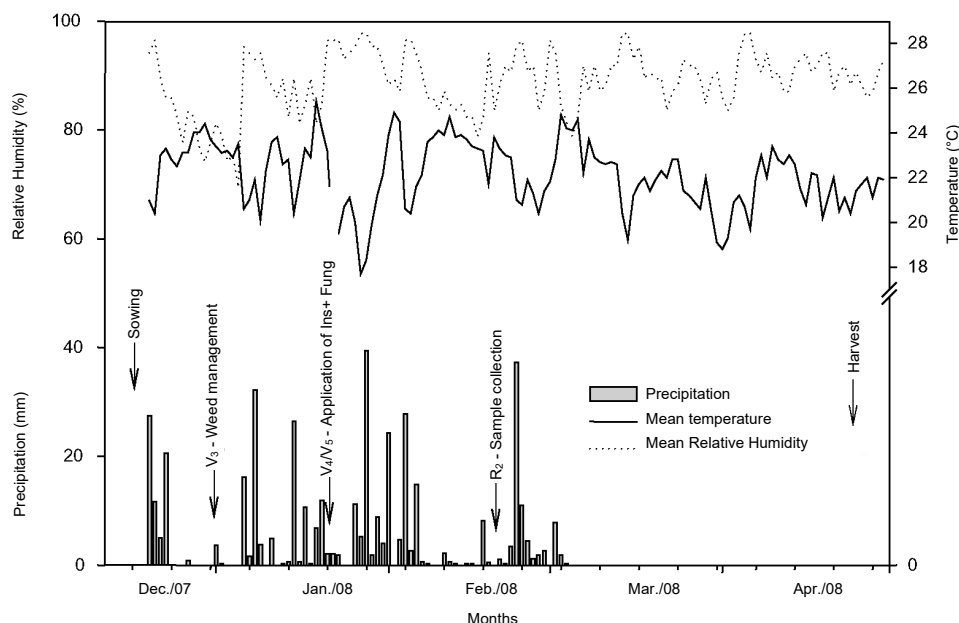
Granulometric analysis (dag kg ⁻¹)				
Clay	Silt	Fine sand	Coarse sand	Textural classification
51	13	16	20	Clayish soil



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Chemical analysis										
pH	P	K ⁺	H + Al	Al ³⁺	Ca ²⁺	Mg ²⁺	CTC _{total}	V	m	MO
(H ₂ O)	(mg dm ⁻³)		(cmolc dm ⁻³)					(%)		(dag kg ⁻¹)
5.40	10.4	138	5.78	0	2.40	0.80	9.33	38	0	1.70



*Approximate sowing dates, weed management, insecticide and fungicide applications and harvesting are marked in the period with the respective phenological stage of the soybean.

Figure 1 - Rainfall, relative humidity and daily temperature during the period from December 12, 2007 to April 20, 2008. Coimbra-MG, 2007/08

Inter-row soil samples were collected when plants reached the R₂ stage and were used to measure the respiratory rate, microbial biomass, metabolic quotient, inorganic phosphate solubilization potential and hydrogenionic potential of the soil.

For assessment of the respiratory rate, the respirometric method was used to determine the C-CO₂ that evolved from the soil, according to the methodology described by Vivian *et al.* (2006).

In order to determine microbial biomass carbon (MBC), two 20-g soil subsamples were weighed into each vial used for assessment of the respiratory rate after an incubation period. The method described by Vance *et al.* (1987), as modified by Islam & Weil (1998), was used to

estimate the microbial biomass, and the samples were subject to microwave radiation for 60 + 60 seconds.

From the values obtained for the respiratory rates and MBC of the soil, the qCO₂ was calculated by dividing the daily mean of the C-CO₂ evolved from the soil by the MBC determined in the soil (Anderson & Domsch, 1985).

In order to estimate the potential for solubilization of inorganic phosphate in liquid medium, 1 g of rhizospheric soil was transferred from the samples of each repetition (eight plants/subplot) to a test tube with NBRI liquid medium, pH 6.8-7.0, containing (g L⁻¹): glucose, 10; Ca₃(PO₄)₂, 5; MgCl₂·6H₂O, 0.5; MgSO₄·7H₂O, 0.25; KCl, 0.2; and (NH₄)₂SO₄,

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0.1 (Nautyal, 1999). After incubation at 27°C for 15 days, the liquid phase underwent centrifugation at 8,000 rpm for 20 min. In the supernatant, the amount of inorganic P was determined by the colorimetric method of the modified vitamin C at a wavelength of 725 nm (Braga & De Fellipo, 1974).

Soil pH was estimated from the soil:water solution (1:2.5), stirred for 30 minutes and rested for an additional 30 minutes (EMBRAPA (*Empresa Brasileira de Pesquisa Agropecuária* [Brazilian Agricultural Research Company]), 1997).

The data were subjected to analysis of variance ($P < 0.05$). The Tukey test was used ($P < 0.05$) to compare the treatment media.

RESULTS AND DISCUSSION

The treatments assessed did not change the respiratory rate of the soil (Table 2). Pereira *et al.* (2008) found no differences in the respiratory rates of soil after application of glyphosate on the soil microbiota cultivated with transgenic soybean. However, these authors did find that this product applied in combination with endosulfan provided greater CO₂ evolution, compared to application of glyphosate alone. On the other hand, in several studies, glyphosate alone induced higher initial respiratory rates compared to the control that had not been subject to herbicide application, indicating the potential metabolism of glyphosate by soil biota (Costa *et al.*, 1997; Moreno *et al.*, 2007).

For microbial biomass carbon (MBC), in treatments without the application of endosulfan + tebuconazole, no differences were observed between weed management methods (Table 3).

However, for treatments with the application of endosulfan + tebuconazole, the plots treated with fomesafen + fluazifop-p-butyl and the untrimmed control presented with the lowest MBC values, compared to the trimmed control and those with single or sequential applications of glyphosate (Table 3). The fomesafen + fluazifop-p-butyl mixture appears to be more harmful to the population of soil microorganisms, because the reduction in MBC was more pronounced (65.05%), compared to the trimmed control, in the situation in which fomesafen + fluazifop-p-butyl and endosulfan + tebuconazole were applied. This may be due to the potential synergistic effect between the two mixtures, thereby enhancing the negative effect of these compounds on soil microorganisms. Santos *et al.* (2005) assessed the effect of fluazifop-p-butyl and fomesafen herbicides, isolated and in combinations, on the biological quality attributes of soil cultivated with common beans (*Phaseolus vulgaris*) in a conventional cultivation and direct planting system. In both crops, there were greater reductions in the microbial biomass of the soil treated with the fluazifop-p-butyl and fomesafen mixture. Furthermore, Pereira *et al.* (2008) demonstrated that endosulfan caused a 48.64% reduction in the MBC of soil cultivated with soybeans, compared to the control without pesticides. However, no studies have been found in the literature assessing the effects of both mixtures applied simultaneously. When assessing the effect of the application of endosulfan + tebuconazole, no differences were observed between the weed management techniques (Table 3).

Table 2 - Respiratory rate of soil cultivated with BRS Favorite RR soybeans subjected to different weed management methods, with and without the application of endosulfan + tebuconazole. Coimbra-MG, 2007/08

Weed management method	Respiratory rate (mg g ⁻¹ d ⁻¹)*	
	without E+T**	with E+T
Untrimmed controls	21.75 aA ^{1/}	18.00 aA
Trimmed controls	16.77 aA	16.77 aA
Single application of glyphosate	17.51 aA	13.60 aA



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Sequential application of glyphosate	19.25 aA	16.54 aA
Single application of fomesafen + fluazifop-p-butyl	15.88 aA	11.32 aA
CV plot (%)	19.83	
CV subplot (%)	16.59	

* Soil collected when soybean plants reached the R₂ stage; i.e., 50% of full bloom. ** E + T = endosulfan + tebuconazole. ^{1/} Means followed by the same letter, lowercase in the column and uppercase in the row, for each variable, do not differ from each other in the Tukey test (P > 0.05).

Table 3 - Microbial biomass carbon (MBC) of soil cultivated with BRS Favorite RR soybeans subjected to different weed management methods, with and without application of endosulfan + tebuconazole. Coimbra-MG, 2007/08

Weed management method	Microbial biomass (g g ⁻¹ MBC)*	
	without E+T**	with E+T
Untrimmed controls	136.12 aA ^{1/}	147.27 bA
Trimmed controls	233.14 aA	237.74 abA
Single application of glyphosate	229.30 aA	239.64 abA
Sequential application of glyphosate	250.16 aA	312.82 aA
Single application of fomesafen + fluazifop-p-butyl	148.81 aA	83.05 bA
C.V. plot (%)	43.81	
C.V. subplot (%)	31.05	

* Soil collected when soybean plants reached the R₂ stage; i.e., 50% of full bloom. ** E + T = endosulfan + tebuconazole. ^{1/} Means followed by the same letter, lowercase in the column and uppercase in the row, for each variable, do not differ from each other in the Tukey test (P > 0.05).

With regard to the metabolic quotient (qCO₂) values, estimated in the R₂ stage of soybeans, it was observed that, in the absence of endosulfan + tebuconazole, the soils treated with glyphosate in single or sequential applications, fomesafen + fluazifop-p-butyl and the trimmed controls did not present any differences, and values are lower than those for the trimmed controls (Table 4). This fact may be due to the incorporation of organic matter (weeds) in the soil when the trimming is performed, because the organic material deposited in the soil will serve as a source of carbon and energy for the microorganisms, trending toward a condition of equilibrium.

However, in the treatments where herbicides were applied together with endosulfan + tebuconazole, the plots treated with single and sequential doses of glyphosate presented the lowest qCO₂ values, differing from the other treatments, especially the area treated with fomesafen + fluazifop-p-butyl and for untrimmed controls. When comparing treatments where weed management methods were applied with and without endosulfan +

tebuconazole, no differences were observed (Table 4).

For solubilized P, differences between weed management methods were observed in areas where they were implemented without application of endosulfan + tebuconazole (Table 5). Single and sequential glyphosate promoted greater phosphate solubilization, although Massenssini *et al.* (2008) reported that glyphosate in commercial formulations (Roundup NA and Transorb, Scout, Zapp QI) was detrimental to the activity of phosphate solubilizing bacteria isolated from the eucalyptus rhizosphere. However, this study was conducted *in vitro*, thereby isolating the plant-organism-soil interaction. This higher rate of phosphate solubilization may be associated with the greater radicular exudation of soybean plants treated with glyphosate, involving a greater increase of C and energy in the rhizosphere, thus consequently benefiting microbial activity. According to Kremer *et al.* (2005), application of glyphosate to soybean plants causes an increase in the quantity and changes in the composition of the exudate material, which can increase the microbial biomass associated with these plants.

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A greater increase in MBC was observed with the application of glyphosate (Table 3); therefore, there is greater evolution of CO₂, which, when released in the soil solution, reacts with H₂O, forming carbonic acid (H₂CO₃). Thus, acidification of the soil solution (Table 5) occurs consequently promoting the solubilization of phosphates. In the presence of the endosulfan + tebuconazole mixture, single and sequential application of glyphosate and the trimmed controls provided the highest phosphate solubilization values, distinguishing themselves from the untrimmed controls (Table 5).

Treatments involving application of fomesafen + fluazifop-p-butyl and mechanical trimming presented the lowest solubilized P values. The lowest phosphate solubilization value was observed in the untrimmed control plot (Table 5). The fomesafen + fluazifop-p-butyl mixture presented the lowest pH values, regardless of whether or not endosulfan + tebuconazole was present (Table 5), and there was no greater solubilization of P. This fact can be attributed to the dramatic reduction in

microbial biomass and a decrease of 65.05 and 37.18% in the MBC was observed in the plots treated with this mixture with and without endosulfan + tebuconazole, respectively (Table 3). When comparing weed management methods with or without application of endosulfan + tebuconazole, greater phosphate solubilization was observed in the plots where glyphosate was applied in single and sequential doses, without application of endosulfan + tebuconazole (Table 5).

In a study conducted by Reis *et al.* (2008), it was observed that the activity of phosphate solubilizing microorganisms was not negatively influenced by 2,4-D, ametryn, trifloxysulfuron-sodium and ametryn + trifloxysulfuron-sodium, and it was further stimulated in the presence of trifloxysulfuron-sodium and 2,4-D. Similar behavior was observed for the herbicides oxadiazon and oxyfluorfen, butachlor and basalin in studies conducted by Debnath *et al.* (2002) and Das *et al.* (2003), respectively.

Table 4 - Metabolic quotient (qCO₂) of soil cultivated with BRS Favorite RR soybeans subjected to different weed management methods, with and without the application of endosulfan + tebuconazole. Coimbra-MG, 2007/08

Weed management method	qCO ₂ (mg CO ₂ g MBC ⁻¹ d ⁻¹)*	
	without E+T**	with E+T
Untrimmed controls	0.145 bA ^{1/}	0.130 bA
Trimmed controls	0.080 aA	0.071 abA
Single application of glyphosate	0.075 aA	0.079 aA
Sequential application of glyphosate	0.078 aA	0.075 aA
Single application of fomesafen + fluazifop-p-butyl	0.113 abA	0.136 bA
CV plot (%)	29.09	
CV subplot (%)	11.99	

* Soil collected when soybean plants reached the R₂ stage; i.e., 50% of full bloom. ** E + T = endosulfan + tebuconazole. ^{1/} Means followed by the same letter, lowercase in the column and uppercase in the row, for each variable, do not differ from each other in the Tukey test (P > 0.05).

Table 5 - Solubilized phosphorus (P) and hydrogenionic potential (pH) of soil cultivated with BRS Favorite RR soybeans subjected to different weed management methods, with and without the application of endosulfan + tebuconazole. Coimbra-MG, 2007/08

Weed management method	P solubilized (mg L ⁻¹)*		pH*	
	without E+T**	with E+T	without E+T	With E+T
Untrimmed controls	74.50 cA ^{1/}	111.50 bA	6.06 aA	5.58 abB
Trimmed controls	126.00 cB	340.85 aA	5.92 abA	5.85 aA



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Single application of glyphosate	425.00 aA	318.16 aB	5.83 abA	5.70 abA
Sequential application of glyphosate	472.00 aA	373.50 aB	5.88 abA	5.47 bB
Single application of fomesafen + fluazifop-p-butyl	193.00 bA	255.00 aB	5.67 bA	5.33 bB
CV plot (%)	18.27		2.61	
CV subplot (%)	16.01		1.94	

* Soil collected when soybean plants reached the R₂ stage; i.e., 50% of full bloom. ** E + T = endosulfan + tebuconazole. ^{1/} Means followed by the same letter, lowercase in the column and uppercase in the row, for each variable, do not differ from each other in the Tukey test (P > 0.05).

pH of soil decreased with application of fomesafen + fluazifop-p-butyl in the herbicide-only treated plots (Table 5). However, in the presence of endosulfan + tebuconazole, no difference was observed between weed management methods, compared to the untrimmed controls (Table 5). Taiwo & Oso (1997) found that, in soils treated with atrazine, there was a reduction of almost one pH unit in the soil and, consequently, an addition of almost 5 mg kg⁻¹ of Pi in the soil solution.

Based on these results, it can be concluded that the weed management methods in soybeans interfered with the characteristics of MBC and qCO₂, variables directly related to soil quality, with or without application of endosulfan + tebuconazole. Single or sequential application of glyphosate associated with the endosulfan + tebuconazole mixture promoted the lowest qCO₂ values, indicating less soil disturbance. The activity of phosphate-solubilizing microorganisms was positively influenced by glyphosate in single or sequential applications in combination with endosulfan + tebuconazole.

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I, Sacha Quadrelli, hereby certify that I am competent to translate from Portuguese to English and that the attached translation is, to the best of my knowledge and belief, a true and accurate translation of the document entitled “Ex 2041 - Reis - associated treatments (002)” from Portuguese to English.



Sacha Quadrelli