

Prediction of synergistic multi-compound mixtures – A generalized Colby approach

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ABSTRACT

The investigation of chemical mixtures used to cure plant diseases has a long history but is still a field of intensive research. Not just does one have to maximize the direct effect on the disease itself but one also has to take into account the adverse effects on consumer health, the development of multiple fungicide resistance and the fate of chemicals in the environment. So far theoretical modelling using Colby's formalism was limited to the simple comparison with experimental data to verify synergistic behaviour. However, a systematic experimental investigation of all possible combinations for multi-compound mixtures is a time consuming task. Facing ever more complex diseases we show how a generalized version of Colby's formula can be used as a predictive tool for synergistic multi-compound mixtures. We also demonstrate how to address the direct chemical effect and the adverse effects on consumer health on the same footing using this generalization of Colby's formalism. We relate this to a practical example by considering a multi-compound mixture used against grey mould.

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1. Introduction

In plant protection, fungicides prevent and cure diseases which have severe adverse effects on crop yield and quality (Camoutsis et al., 2010). For instance, the efficient use of pesticides allows farmers to produce 18% of the world's food supply on 10% of the world's farmland (Croplifeamerica, 2010). This high standard is not easy to maintain since pathogen resistance is an increasing problem (Bourguet et al., 2010). Moreover, an intensive use of pesticides has adverse environmental effects (Rosenzweig et al., 2001; Buchanan et al., 2009).

As a consequence of these problems, many fungicides such as strobilurins are used as combinations or in series to destroy pathogens causing plant diseases (Suzuki et al., 1984). This innovative generation of fungicides improves the control in the key plant diseases such as downy mildew, grey mould and powdery mildew (Gilardi et al., 2008; La Torre et al., 2008; Kretschmer et al., 2009).

In this article, the joint action of fungicides in mixtures is investigated. Colby (1967) formulated a general mathematical expression for assessing synergism in mixtures of two drugs which is still used to date (Perlitz et al., 2003). For mixtures of many fungicides its predictions become unreliable. Nonetheless, an important cornerstone to the solution of the multiple resistance

problem (Elad et al., 1992) is a quantitative understanding of the effect of fungicide mixtures. Starting from Colby's original work, we show how experimental data for the efficacy of mixtures with few fungicides can be used to make better predictions for mixtures with more fungicides. This allows for an efficient quantitative identification of interesting candidates for synergistic (i.e. positively interacting) fungicide mixtures. The approach taken here is very general and may equally well be applied to insecticides, herbicides and even mixtures of chemical therapies for human diseases.

Approaching ever more complex diseases and multidrug resistance (Raposo et al., 1996; Anderson, 2005; Kretschmer et al., 2009; Sun et al., 2010), three routes can be followed. First, new chemicals can be found (Ceynowa and Lindenberg, 1997) that are not affected by the resistance. This does not only include new fungicides and pesticides but also nutrients (Dordas, 2008). Second, rules for fungicide resistance management can be established (Leroux et al., 2002; Brent and Hollomon, 2007; Kretschmer et al., 2009) to prevent resistant strains from developing. Finally, the therapies could become more complex and exploit the possibility of synergistic behaviour between compounds (Levy et al., 1986). Quite generally interactions cause the emergence of new phenomena that cannot be inferred from the single compounds alone (Anderson, 1972; Ammermann et al., 2009).

All routes need to be followed to ensure the best possible control of complex diseases. Furthermore, the best possible control does not only mean to optimise the direct biological effect but

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encompasses also the possible side effects of the chemicals employed. In particular, one has to account for the possibility of fungicide resistance (Holmes and Eckert, 1999; Leroch et al., 2011), the fate and possible negative side effects of the chemicals in the terrestrial environment (Geiger et al., 2010; Kim et al., 2011) and the effects on consumer health (Reffstrup et al., 2010).

In this work, we want to concentrate on the direct biological effect of chemical mixtures. The identification of interesting chemical mixtures may then be taken as a starting point to understand the effects on the evolution of resistance (Bourguet et al., 2010; Colbach, 2010) and the fate in the environment (Banasiak et al., 2010). We will also point out how the effects on consumer health can be addressed within our approach.

When mixtures are applied, the biological effect may be equal, greater or smaller than what might be expected from the sum of the activities when administered separately. These effects are additive, synergistic and antagonistic, respectively (Levy et al., 1986).

A widely used mathematical method (especially in patents by crop production companies) to determine synergism of multi-compound mixtures is the Colby formula (Gowing, 1960; Colby, 1967). Here, the responses of chemicals applied singly are used in calculating the expected response when they are combined. This expected response for a given combination of two chemicals can be calculated as follows.

Let P_1 and P_2 be the respective efficacies of the two compounds (in %), where by efficacy we mean the percentage of inhibition of growth of a certain fungus at a certain concentration of the fungicide. The expected response E of the combination of two chemicals assuming no interaction is then given by

$$E_{\text{Colby}} = P_1 + P_2 - \frac{P_1 P_2}{100}. \quad (1)$$

Although widely used the Colby formula is not always correct as noted (Perlitz et al., 2003). It was derived under the assumption that effect of the mixture can be described by one minus the probability of not responding to any of the compounds. In other words, the fungicides do not interact. However, two fungicides in a 3-compound mixture may interact and exhibit synergy phenomena.

For a determination of synergism the ratio SF (synergy factor) between the observed experimental efficacy of the mixture E_{measured} and the expected efficacy of the mixture is computed by

$$\text{SF} = \frac{E_{\text{measured}}}{E_{\text{Colby}}}. \quad (2)$$

A ratio SF greater or smaller than 1 indicates a deviation from the hypothesis of independent action which means that biologically the two compounds act together. If $\text{SF} > 1$, synergism is observed; if $\text{SF} < 1$, antagonism is observed.

The possible interactions are reflected by the improved Colby formula which we will derive in this article. This improved Colby formula has another advantage: finding synergistic combinations is a time consuming task since there are many possible candidates for combinations. Let n be the number of compounds to be investigated and k the number of compounds in the mixture (k -compound mixture), then $\binom{n}{k}$ possible combinations have to be tested, i.e. for the search of a synergistic 5-compound mixture out of 12 available compounds $\binom{12}{5} = 792$ possible combinations have to be investigated. The investigation of 5-compound mixtures is currently under way (Dieckmann et al., 2009). Also, the number of available components is realistic. For instance, 10 different drugs have been investigated for control of *Botrytis cinerea* (Kretschmer et al., 2009). Fig. 1 shows the dependence of the number of mixtures (trials) on

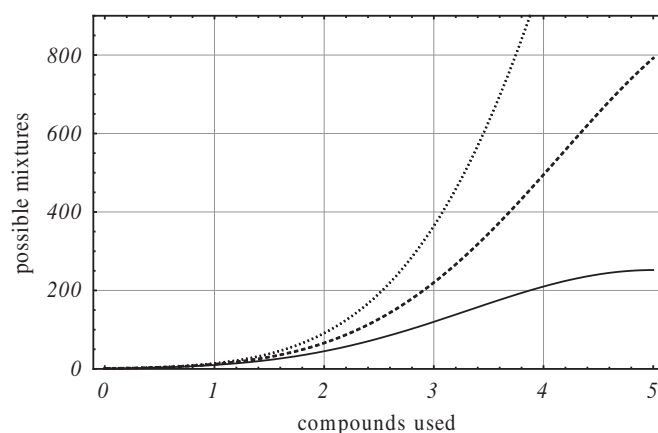


Fig. 1. Number of possible mixtures (trials) dependent on the number of compounds in one mixture for $n = 10, 12$ and 14 (solid, dashed and dotted curve) available compounds.

the number of compounds k used in one mixture given $n = 10, 12$ and 14 available compounds.

With the improved Colby formula, the number of necessary combinations to be tested can be considerably reduced as will be explained in the following sections. In this article, a new mathematical method to predict synergism of multi-compound mixtures is presented. In Section 2, the results from Colby (1967) are shortly reviewed and finally extended to a generalized Colby formula. To circumvent the above discussed disadvantage of Colby's formula (the assumption of statistically independent coincidence), an improved generalized approach is derived (see second part of Section 2). We show how to assess the risk to consumer health based on this approach. Finally, in Section 3 it is explained how to reduce the workload needed to identify interesting combinations of chemical compounds in general. Section 4 concludes and summarizes the results.

In the following, the derivation and application of the generalized Colby formula is for convenience conducted with examples only from fungicide research. However, the generalized formula is applicable in all areas working with chemical mixtures.

2. Materials and methods

In this section the efficacy of mixtures of fungicides is investigated. For each fungicide i , it is assumed that the efficacy of the single compound is known to be P_i (in %). If the mixture of two compounds is investigated and they are assumed to act independently, Colby's formula is valid. The formula as in Equation (1) is stated in terms of percentages (as often done). For a more elegant formulation, let us take the efficacies for the compounds as numbers between 0 and 1 and denote them with p_i . Then Colby's formula for the efficacy of the mixture of two fungicides i and j is

$$E_2 = p_i + p_j - p_i p_j. \quad (3)$$

This formula may be generalized to m compounds as one only has to add up the efficacies of the single fungicides and subtract the possibility of a coincident action. The set of all fungicides studied is denoted by SM. The different fungicides are numbered by indices $i_1, \dots, i_m \in \text{SM}$. The general formula is then

$$E_m = \sum_{i=1}^m p_i - \sum_{j=2}^m \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j p_{i_1} \dots p_{i_j}. \quad (4)$$

This is compact and general. For a proof by induction, we refer the reader to Appendix A. We want to illustrate the formula by considering the case of three fungicides

$$E_3 = \sum_{i=1}^3 p_i - p_1 p_2 - p_1 p_3 - p_2 p_3 - p_1 p_2 p_3. \quad (5)$$

First we add all the single efficacies. The single addition overlooks the possibility that a certain proportion of the fungi would have been killed by the application by either of two of the three possible fungicides. This overcounting is taken care of by subtracting $p_1 p_2 + p_1 p_3 + p_2 p_3$. This however subtracts also the possibility of fungicide 1, 2 and 3 acting together so that $p_1 p_2 p_3$ has to be added.

As already mentioned in Section 1, two or three fungicides in a three-compound mixture may interact and exhibit synergy phenomena. This fact was not considered in the original Colby formula. The measured value for a certain mixture of m compounds may therefore not be identical to the prediction by Colby's formula

$$E_{m,\text{measured}} \neq E_m. \quad (6)$$

Let us now investigate these deviations more closely. In the two-compound case we define the 2-fungicide correlator c_{ij} as

$$c_{ij} := p_i + p_j - E_{2,\text{measured}}. \quad (7)$$

If the measured value had been given by Colby's formula, we would have

$$(c_{ij})_{\text{Colby}} = p_i + p_j - p_i - p_j + p_i p_j = p_i p_j. \quad (8)$$

This means that the 2-fungicide correlator is a measure for correlation of the two fungicides in the experiment. It replaces the term $p_i p_j$ by a measured value that takes into account the synergistic, antagonistic or additive behaviour of the chemicals. This gives us additional information for better approximations of the efficacy for mixtures of more fungicides. Let us take the case of three fungicides with the generalized Colby formula in Equation (5). Here, we subtract from the three single efficacies the possible interactions of two fungicides and add the interactions of three fungicides. But the possible interactions of two fungicides can be obtained from experiments on two compounds alone. Therefore, we can denote an improved formula for the efficacy $\tilde{E}_3$ of three fungicides

$$\tilde{E}_3 = p_1 + p_2 + p_3 - c_{1,2} - c_{1,3} - c_{2,3} + p_1 p_2 p_3. \quad (9)$$

Analogous to Equation (7) a correlator for the interaction of three fungicides may now be defined by

$$c_{i,j,l} = E_{3,\text{measured}} - (p_i + p_j + p_l - c_{i,j} - c_{i,l} - c_{j,l}). \quad (10)$$

If the improved Colby formula in Equation (9) were correct one would obtain:

$$\begin{aligned} (c_{i,j,l})_{\text{improved,Colby}} &= p_i + p_j + p_l - c_{i,j} - c_{i,l} - c_{j,l} + p_i p_j p_l \\ &\quad - p_i - p_j - p_l + c_{i,j} + c_{i,l} + c_{j,l} = p_i p_j p_l. \end{aligned}$$

As for the 2-compound case $c_{i,j,l}$ replaces the term $p_i p_j p_l$ by a measured value. Again this result can be used for mixtures with more fungicides. Looking at the generalized Colby formula in (4) we also see an interesting property of the correlators. Since all p_i 's are numbers smaller than one the higher terms like $p_{i_1} p_{i_2} p_{i_3} p_{i_4}$ tend to zero very fast. For simplicity let us take $p_i = p$ for all fungicides. Fig. 2 shows the behaviour of the correlators $p_{i_1} \cdots p_{i_j}$ for $j = 2, \dots, 6$

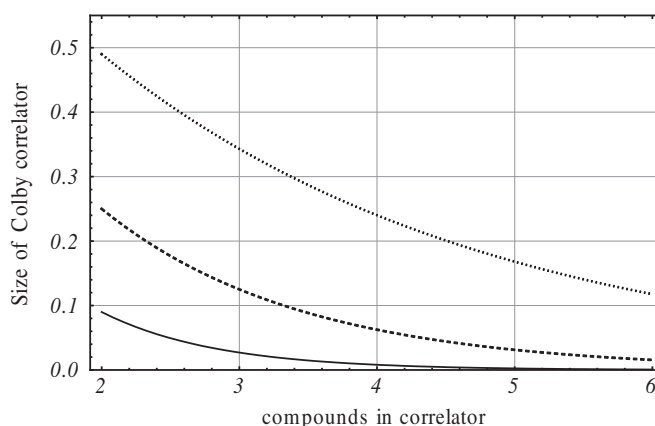


Fig. 2. Size of the correlator in the Colby formula for $p = 0.3$ (lower), $p = 0.5$ (middle) and $p = 0.7$ (upper) as a function of the compounds in the correlator.

compounds. The interesting correlators are the ones with few compounds and their changes need to be recorded. The higher correlators are inherently small and can therefore be well approximated by the Colby result, so just $p_{i_1} \cdots p_{i_j}$.

This is intuitively clear since interactions that require e.g. four or five compounds are rare compared to those requiring two or three. Nonetheless, mixtures of five compounds might be very beneficial, if there are many sets of two or three of its ingredients that exhibit strong synergism. For completeness we should also define the higher correlators mathematically.

Let SM again be the set of used fungicides in a mixture then the following formula

$$\begin{aligned} c_{i_1, \dots, i_m} := & (-1)^m \left[(p_{i_1} + \dots + p_{i_m}) - \sum_{j=2}^{m-1} \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j c_{i_1, \dots, i_j} \right] \\ & - (-1)^m E_{m,\text{measured}}. \end{aligned}$$

calculates the general correlator for m fungicides. This also enables a definition of the generalized improved Colby formula

$$\tilde{E}_m := \sum_{i=1}^m p_i - \sum_{j=2}^m \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j c_{i_1, \dots, i_j} - (-1)^m p_1 \cdots p_m. \quad (11)$$

This formula allows including information previously obtained in experiments on fewer fungicides to make better predictions for the mixture of many fungicides.

The generalized Colby formalism can also be compared to a method for risk assessment of mixtures of pesticides (Reffstrup et al., 2010). For the assessment the US EPA has recommended to assume simple dissimilar action of the chemicals (US EPA, 2000) meaning that the probability of an adverse effect from a mixture of two fungicides is equal to the sum of the individual responses (Könemann and Pieters, 1996; ATSDR, 2004). They use the following equation to estimate the risk for the mixture

$$\text{Risk} = \sum_{i=1}^n \text{Risk}_i = \sum_{i=1}^n d_i B_i, \quad (12)$$

where Risk_i is the risk estimate for the i th compound, d_i is the dose and B_i is a potency parameter (US EPA, 1986). Normally one

considers the risk for an individual to develop cancer so that B_i refers to the potency parameter of the i th carcinogen. According to the US EPA the equation is appropriate when the risks of the individual compounds are less than 0.01 and the sum of the individual risks is less than 0.1 (US EPA, 1989, 2000; ATSDR, 2004).

Considering the case of two fungicides in (3) we see that following the original Colby formula the efficacy of a mixture is not the sum of the single efficacies but a reduced number. The risk given by (12) for $n = 2$ is the sum of the single risks. For illustration let us assume to have two fungicides with equal risk $Risk_1$ and equal efficacy p so that also their relative risk $Risk_1/p$ is equal. If the mixture follows Colby's formula we have

$$Risk/E_{Colby} = \frac{Risk_1}{p(1-p/2)} > \frac{Risk_1}{p}, \quad (13)$$

Therefore, mixtures that follow Colby's formula relatively enhance consumer risk. However, in a measurement, the correlator $c_{1,2}$ in (7) may turn out negative. This means that the relative consumer risk in (13) may be reduced due to the strong synergistic behaviour. Therefore, a well defined measure for interesting fungicide combinations is a negative correlator.

3. Results

3.1. Experimental procedure

Before testing new active ingredients in greenhouse trials or field trials they have to be adapted to the specific environment (formulated). Doing so means that substances are preserved, and made insensitive to light and/or to oxygen. Moreover, appropriate detergents have to be chosen and added to the substances to increase the surface tension of the final solution which provides strong adhesion effects of the active ingredients on the plant surfaces.

In parallel to this formulation process, trial plants have to be prepared showing the required growth phase for trials. When in right growth stage plants are sprayed with the active ingredients. A final rating decides the true activity of the chemical substances. Both the preparation of the chemical substances and the preparation of the trial plants (final greenhouse trials as well as field trials) are time consuming.

3.2. Reduced experimental effort

In recent trials, measured efficacy values from two-compound mixtures being much larger than the expected efficacy values computed via Colby formula could be observed (Schelberger et al., 2003). If from two-compound-measurements three compounds can be observed such that $\tilde{E}_3 > 1$ holds, these compounds represent a promising candidate for a three-compound trial which will be proven in the following paragraph:

From two-compound mixtures with

$$E_{2,measured} >> E_{Colby} \quad (14)$$

synergism follows. This implies a small or even negative two-compound correlator c_{ij} . For a combination of three fungicides such that all $c_{1,2}$, $c_{1,3}$ and $c_{2,3}$ fulfil this requirement $\tilde{E}_3 >> E_3$ is likely to be obtained. Hence, $\tilde{E}_3 >> E_3$ holds if the three compound mixture is likely to show synergistic effects.

This derivation has important practical influence. Promising three compound mixtures can be found by investigating results from two compound mixtures without – and that is the important part – doing experiments with three compounds. As already mentioned above n available compounds and the search for

synergistic two compound mixtures require at least $\binom{n}{2}$ experiments – assuming only one given concentration, otherwise the number of experiments would be even higher. Similarly, looking in an experimental way for synergistic three compound mixtures given n available compounds requires $\binom{n}{3}$ experiments. However, the method described above, namely just checking if $\tilde{E}_3 >> E_3$ holds for some synergistic compounds tested in previous experiments, reduces the experimental effort. A prediction of likely synergistic compounds is possible only with experimental data from two compound studies. Thus, experiments with three compound mixtures are – for a first prediction of synergism – redundant. Consequently, the experimental effort can be reduced from $\binom{n}{3}$ to $\binom{n}{2}$ or to $3/n-2$ times the experiments needed before.

In Fig. 3, we show the function $3/n-2$ for n between 7 and 14. It illustrates the scaling of the time/money saved by studying only the two-compound mixtures instead of all possible three-compound mixtures. Of course, the interesting mixtures of three compounds need to be tested afterwards leading to more experiments that need to be performed. However, in most cases the experiments for two compounds are already done so that only a small fraction of the two-compound experiments actually need to be performed so that the function should give a correct qualitative argument. Addressing the case of four compounds that one may also predict from the 2-compound studies, the experimental effort is reduced by $3 \cdot 4/(n-2)(n-3)$ what is also shown in Fig. 3.

The presented method can successively be applied to n -compound studies. However, the higher the number of compounds tested in a trial, the more important the correlators of three fungicides will become. Hence, for a prediction of synergistic effects in $n = 5, 6$ compound mixtures, experimental data from three-compound trials should be available.

To relate this discussion to a practical example, we want to consider grey mould caused by *B. cinerea* (see Fig. 4). In the patent (Dieckmann et al., 2009) the synergistic behaviour of insecticides from the group of abamectin, fungicides from the group of carboxamides and an insecticidal component from clothianidin with a strobilurin fungicide and fludioxinil have been investigated. Binary mixtures of strobilurin fungicides and clothianidin have been shown to exhibit synergistic behaviour in Krohn et al. (2008). The same has been shown for clothianidin and fludioxinil in Erdelen et al. (1999), for clothianidin and carboxamides in Yoshio and Shinichi (1991) and for clothianidin and abamectin in

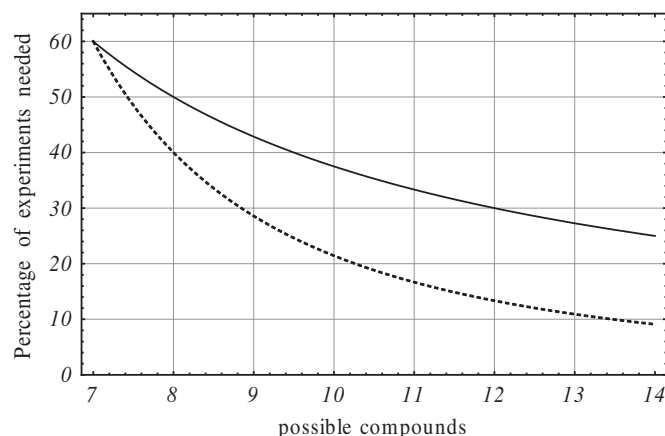


Fig. 3. Percentage of experiments needed for the study of 2-compound mixtures compared to the number of experiments needed for all three-compound (upper) and four-compound (lower) mixtures as a function of the possible compounds that can be used.



Fig. 4. Grey mould on strawberries.

Andersch et al. (2003). Therefore, the combined synergistic effect could have been predicted from the generalized Colby formula using improved binary correlators.

4. Discussion

The present work was intended to shed some light on the computation of synergistic effects of multi-compound mixtures. Complex diseases and occurrence of drug resistance require more and more complex chemical therapies such as multiple drug therapies. The experimental process of finding out which drug combinations show only additive or antagonistic or even synergistic effects is often tedious and time consuming. However, fast progressive diseases such as AML of type 3 and potato blight require short development times of new multiple drug combinations.

Additionally the problem is complicated by the need to consider the effect of mixtures on the development of resistant strains, the fate of chemicals in the soil and possible adverse effects on consumer health.

In this article, a new method is described which reduces the experimental effort of finding synergistic multi-compound mixtures. This theoretical method presented here predicts from previous experiments promising candidates for synergistic multi-compound mixtures.

In a first step, the general Colby formula has been derived that can be used to predict the efficacy of chemical mixtures from the single efficacies. We showed how experimental data from studies with few chemical compounds can be used to make reliable predictions for mixtures with multiple compounds. In Section 4 the tedious experimental way of finding synergistic multi-compound mixtures is described. Additionally it is shown how this experimental effort can be reduced by the improved Colby formula. Colby (1967) mentioned that there are obviously practical limitations in using mathematical formulas in predicting the responses for multi-compound mixtures in the field of herbicide research. He indicated that his derived Colby formula is only approximate but it represents an improvement over no attempt to predict responses. The authors showed that Colby's formalism can be extended to allow for a reliable prediction of synergism.

The theory of isobole diagrams developed by Tammes in the field of pesticides (Tammes, 1964) is another famous method of finding synergism of multi-compound systems. With this method the degree of synergism can be determined graphically from the dose response curve. Contrarily the Colby formula is based on the effect of a chemical and independent of the dose. The effect and the dose are however interchangeable (Chou, 2006) and therefore

a combination of Colby's and Tammes approach should be possible in face of the work done on the prediction of the dose–response curve (Chou and Talalay, 1984). A combination of both approaches would deepen the understanding of theoretical synergy computations. We believe that the approach presented here is feasible to clarify these questions with the help of further systematic investigations of both approaches.

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Appendix A. Proof of the general formula

The general formula (4) can be proven by induction. Let us take $\tilde{m} = m + 1$ and the last fungicide to be $\tilde{m}$. Then we know E_m and by Colby's formula we know for the mixture of the m first fungicides with fungicide $\tilde{m}$

$$\begin{aligned} E_{\tilde{m}} &= E_m + p_{\tilde{m}} - E_m p_{\tilde{m}} \\ &= \sum_{i=1}^m p_i + p_{\tilde{m}} - \sum_{j=2}^m \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j p_{i_1} \dots p_{i_j} - \sum_{i=1}^m p_i p_{\tilde{m}} \\ &\quad + \sum_{j=2}^m \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j p_{i_1} \dots p_{i_j} p_{\tilde{m}}, \\ &= \sum_{i=1}^{\tilde{m}} p_i - \sum_{j=2}^m \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^j p_{i_1} \dots p_{i_j} - \sum_{i=1}^m p_i p_{\tilde{m}} - \sum_{j=2}^m \\ &\quad \times \prod_{\substack{i_1, \dots, i_j \in \text{SM} \\ i_1 < \dots < i_j}} (-1)^{j+1} p_{i_1} \dots p_{i_j} p_{\tilde{m}} \end{aligned}$$

The set $\overline{\text{SM}}$ of the $\tilde{m}$ fungicides is the mixture of m compounds with compound $\tilde{m}$. This means that $\overline{\text{SM}}$ contains the combinations $i_1, \dots, i_j \in \text{SM}$, the combinations $i_1, \tilde{m}$ with $i_1 \in \text{SM}$ and the combinations $i_1, \dots, i_j, \tilde{m}$ with $i_1, \dots, i_j \in \text{SM}$. Therefore the last three terms may be recast into the formula

$$E_{\tilde{m}} = \sum_{i=1}^{\tilde{m}} p_i - \sum_{j=2}^{\tilde{m}} \prod_{\substack{i_1, \dots, i_j \in \overline{\text{SM}} \\ i_1 < \dots < i_j}} (-1)^j p_{i_1} \dots p_{i_j}$$

This proves the general formula.

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