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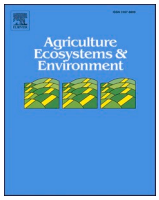

The effects of sequential herbicide applications on phosphorus cycling and mycorrhization in soybean: A two-year field study

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ABSTRACT

Herbicide use has evolved from single pre-sowing applications to the use of tank mixtures applied at higher rates both pre-emergence and in-crop. Understanding the effects of these practices on non-target organisms is critical for protecting soil functions and regulating pesticide use. This work examines the impact of a commonly used herbicide application scheme in soybean production on non-standardized endpoints related to the phosphorus cycle, under field conditions over two years. Specifically, total arbuscular mycorrhizal colonization, arbuscule and vesicle formation in soybean roots, phosphorus concentration and total content in shoots were evaluated at the vegetative and reproductive stages of the plants. Nine treatments were assessed: a control without herbicides, a full herbicide scheme at label recommended rate comprising preplant, preemergence and early post emergence applications; all seven partial scheme variations; and a worst-case scenario, full scheme at twice the label-recommended rate. Total and arbuscular colonization showed non-linear dose-response to herbicide load (number and rate of active ingredients), not directly related to specific modes of action. Treatments with only the preplant and only the preemergence herbicide application (T2 and T3, respectively) had lower total and arbuscular colonization than the control in the second year at the vegetative stage. The treatment with the preplant and preemergence herbicide applications (T5) had lower total colonization than the control in the first year at the reproductive stage. Vesicle formation was the most sensitive endpoint, with a significant stimulatory response to herbicide-induced stress compared to the control in the reproductive stage of year 1. Phosphorus concentration increased in T3 treatment compared to the control the first year in the reproductive stage. The remaining treatments were not statistically different from the control. The low herbicide levels detected in soil under no-tillage management, with over 95 % of soil covered by straw, could explain the absence of negative effects in the evaluated soil functions under the full sequence of herbicides. Overall, the results suggest mild yet significant counterproductive effects on agroecosystems, and the need for better risk assessment frameworks, pre- and post-registration of pesticides.

1. Introduction

Pesticides play a crucial role in modern agriculture, and their global use continues to grow despite increasing awareness of potential adverse effects (FAO, 2024). In particular, herbicides represent 52.6 % of the global pesticide use in agriculture (FAOSTAT, 2022). This is reflected in the increasing reports of detections in soil worldwide (Geissen et al., 2021; Pérez et al., 2021; Riedo et al., 2021; Rose et al., 2022; Silva et al., 2023; Knuth et al., 2024), along with growing literature of their effects on key soil functions (Raj and Syriac, 2017; Meena et al., 2020; Jakobsen et al., 2021; Pinheiro et al., 2023).

Due to the high persistence of some pesticides in soil, the agro-ecosystem can become a hotspot for pesticide contamination (Vryzas, 2018; Zhang et al., 2022). Knuth et al. (2024) found pesticide residues in fields converted to organic farming five years prior to sampling. Accumulation occurs when input exceeds dissipation, being more likely when

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there is a combination of high frequency of application, high pesticide application rate, and high persistence of active substances (Primost et al., 2017). In particular, the increase of herbicide-resistant weeds has led to the widespread use of herbicide mixtures (Bain et al., 2017) and the implementation of sequential application strategies at short intervals to ensure overlap and prolong pre-emergence control (Chahal et al., 2018; de Sanctis et al., 2021). These practices have intensified the co-occurrence of herbicide residues in soils, raising concerns about their cumulative and long-term effects on soil ecological risks (Li et al., 2024).

In light of these concerns, exhaustive assessments on the fate and risks of active substances and plant protection products are conducted before being approved in the European Union market; in other regions, as Latin America and the Caribbean, current and emerging regulatory frameworks remain less restrictive (Vryzas et al., 2020). Despite the stringent EU regulation (EC, 2009), the analysis of the current European risk framework emphasizes the necessity for a transition to a system-based approach (Sousa et al., 2022). Relevant authorities have recognized the importance of revising the guidance document for soil organisms exposed to pesticides and have initiated the process of updating the existing guidelines (EFSA, 2017; Carrasco Cabrera et al., 2023; European Commission, 2024). These improvements suggest testing pesticide mixtures, assessing both standard and native species in mono- and multi-species setups, and exploring a wider range of effect indicators. The study of the multiple-residues impact on the plant-soil system remains very limited and highly challenging.

One approach to contribute to a more comprehensive risk assessment is to address key soil functions due to their important role in providing ecosystem services. In this line, Arbuscular Mycorrhizas Fungi (AMF) evaluations have been suggested as complementary endpoint to classical environmental risk assessment (EFSA, 2017). This symbiotic process acts as an extension of the crop's root system, allowing the roots to explore a larger soil volume. This association increases the plant mineral nutrition- especially phosphorus-, improves water uptake, and contributes to overcome abiotic stress (Feng et al., 2020). Pesticides can affect the AMF either directly, before root colonization, by damaging hyphae or spores present in soil (Druille et al., 2013), or indirectly by affecting the host-plant and consequently the quality and quantity of carbohydrates available for the symbiosis (Druille et al., 2013; Hage-Ahmed et al., 2019).

Previous studies have focused on the impact of individual active ingredients on AMF. In particular, the herbicides studied in this work have shown varying effects on mycorrhizal colonization; see a comprehensive overview on existing data on García Carriquiry et al. (2024). In brief, and as example, Santos et al. (2006) observed no significant effect of fomesafen at 250 g. ha^{-1} on mycorrhizal colonization in *Phaseolus vulgaris* roots, grown under no-till field conditions and evaluated at 12 and 51 days after application. Pot experiments evaluating glyphosate effects on AMF colonization showed no significant effects in GMO soybean testing up to 1920 g ha^{-1} of active ingredient sprayed over the plants in the vegetative stage (Mujica et al., 1999; Savin et al., 2009) nor on phosphorus content in shoot (Savin et al., 2009). Malty et al. (2006) tested up to 3600 g ha^{-1} of glyphosate applied to the soil in a pot experiment and found no effect on AMF colonization of GMO soybean roots. However, Zaller et al. (2014) found negative effects on AMF colonization in sprayed over-clover at a dose equivalent to 12, 700 g ha-1 of glyphosate in a mesocosm experiment.

This study continues a previous investigation (García Carriquiry et al., 2024) that evaluated the impacts of increasing doses of a sequence of two herbicide mixtures (glyphosate + dicamba + clethodim, and flumioxazin + S-metolachlor) on AMF in soybean roots, from a non-genetically modified cultivar, in a pot experiment. These results showed a decrease in total AMF colonization, arbuscules formation, phosphorus concentration and content in shoots in the vegetative stage of the plants. The effects on total AMF colonization at twice the label recommended rate persisted in the reproductive stage of the plants, and vesicle formation also decreased (García Carriquiry et al., 2024).

This research advances knowledge, which is, as presented above, limited to single substances effects and mixture information under semi-controlled conditions, by assessing mixtures-AMF interactions. Also, by providing new insights into herbicide-induced disruptions to the phosphorus cycle under realistic environmental conditions, reproducing common farming practices. Specifically, the objective of this research was to evaluate the impact of a current herbicide application scheme - composed by a sequence of three herbicide applications - on total arbuscular mycorrhizal colonization, arbuscule and vesicle formation on soybean roots, and the phosphorus content in those plants. This was assessed in a 7-months field experiment, repeated over two consecutive years, spanning from herbicide application during fallow period to the end of the soybean crop cycle, which is the most widely produced oilseed worldwide (USDA, 2025).

2. Materials and methods

2.1. Experimental design

The experimental design is described in detail in (García Carriquiry et al., 2025), who addressed endpoints related to the nitrogen cycle. The present study shares the same overarching objective but focuses on effects related to the phosphorus cycle, providing complementary insights. Briefly, the field experiment was conducted in Estación Experimental M. A. Cassinoni in Paysandú, Uruguay, in two consecutive growing seasons (2020–2021 and 2021–2022) using two contiguous sites to avoid possible carryover effects. The experiment was located in a Typic Argiudol soil, in a temperate region with mean temperature of 20.5 °C, and 670 mm and 850 mm rainfall during the 7 months of each experiment for the first and second year, respectively. Nine treatments were tested following a complete randomized block design, with three blocks/replicates. A total of 54 plots was monitored, accounting for the two years of the study. Each plot had 60 m^2. The evaluation of two time points of the plant life-cycle was useful to address medium-term effects of herbicide applications: at the initial stage of plant growth and the critical moment of soybean crop for determining yield.

Herbicide treatments were applied at preplant, preemergence, and early postemergence stages, following a typical soybean agricultural practice. At preplant, a mixture of glyphosate + dicamba + clethodim was applied in specific treatments as described below. It is currently used to desiccate the cover crop and start preparing the seedbed for soybean crop at least 30 days before sowing. The preemergence application that included glyphosate + flumioxazin + S-metolachlor, is usually used around four days before sowing, so the crop starts growing without weed interference. Finally, the early postemergence application, when soybean plants had approximately four fully developed leaves, included fomesafen only. It is usually used to control small weeds and to extend the herbicide residual effect during the crop season. These herbicides were applied as commercial products (table in supplementary material C of García Carriquiry et al., (2025), at the label recommended rates of active ingredient: 1080 g ha^{-1} for glyphosate, 192 g ha^{-1} for dicamba, 192 g ha^{-1} for clethodim, 72 g ha^{-1} for flumioxazin, 1152 g ha^{-1} for s-metolachlor, and 300 g ha^{-1} for fomesafen.

The nine treatments were defined according to the herbicide load applied: 0) control without herbicide applications, 1) three herbicide applications at the label recommended rate, 2) only one application at preplant, 3) only one application at preemergence, 4) only one application at early postemergence, 5) two applications at preplant and preemergence, 6) two applications at preemergence and early post emergence, 7) two applications at preplant and early postemergence, and 8) the three applications at twice the recommended rate. The experimental outline is summarized in Table 1. Weeds were removed by hand periodically to keep the plots weed-free, to evaluate the herbicide impacts without weed interference. Environmental conditions were monitored by a weather station located at 50 m from the experimental site. The plants were not fertilized with phosphorus, which was at

Table 1
Experimental outline: application moments, herbicide mixtures, mode of action, persistence, and treatment description. Sampling time and number of replicates.

Timing	Active Ingredient	Mode of action	DT50	Treatments								
				0	1	2	3	4	5	6	7	8
Application 1, preplant (~40 d before sowing)	Glyphosate	Inhibition of EPSP synthase	16.1									x2
	Dicamba	Synthetic auxin	9.62									
	Clethodim	Acetyl CoA carboxylase inhibitor	0.55									
Application 2, preemergence (~4 d before sowing on average)	Glyphosate	Inhibition of EPSP synthase	16.1									x2
	S-metolachlor	Inhibition of VLCFA (inhibition of cell division)	90									
	Flumioxazin	Inhibits protoporphyrinogen oxidase (PROTOX)	21.9									
Plant Vegetative sampling (~33 d after sowing)				n=10	n=10	n=10	n=10					n=10
Application 3, early postemergence (~42 d after sowing)	Fomesafen	Inhibits protoporphyrinogen oxidase (PROTOX)	86									x2
Plant Reproductive sampling (80 d after sowing)				n=5	n=5	n=5	n=5	n=5	n=5	n=5	n=5	n=5

restricting levels for plant growth, to avoid interaction with mycorrhizal colonization. The phosphorus measured levels are shown in supplementary materials A1, while the reported requirement of phosphorus for soybean crop in the pampas region is 13 ppm (García et al., 2007).

2.2. Soil property determinations

Soil samples were collected at different times and for specific purposes. At the beginning of each experimental year, composite samples of the top 10 cm were randomly taken from 5 sites per plot. These samples were sieved through a 2 mm mesh, and organic residues were then carefully removed. The samples were oven-dried at 40°C to constant mass, and subsequently divided for analysis. Subsamples of 0.5 g was used to determine organic matter using Nelson and Sommers (1996) method, and 60 g was used to determine soil texture using Bouyoucos (1962) method. Across experimental plots, soils exhibited an average texture of 27 % sand, 32 % clay, and 41 % silt, with 4 % organic matter content.

Before sowing, composite soil samples were collected from the upper 20 cm of the top soil layer to determine bioavailable phosphorus content, per block in the first year, and per plot in the second year for more accuracy. The Bray 1 method (Bray and Kurtz, 1945) was employed at this depth since it allows for comparing phosphorus levels to regional levels and to reference values (Hoffman, 2013).

Separate soil samples were collected within 48 hours after herbicide application to assess herbicide residues. These composite samples were randomly taken from ten sites from the top 5 cm per plot, homogenized by hand, mixed and frozen at −20 °C for 48 hours. Subsequently, they were lyophilized and stored in darkness at room temperature until herbicide quantification. From each sample, 5 g were taken to analyse glyphosate, AMPA and dicamba desmethyl by ionic chromatography coupled to tandem mass spectrometry (Niell et al., unpublished results), and 10 g to analyse the remaining herbicides and metabolites (dicamba, flumioxazin, fomesafen, clethodim, clethodim sulfoxide, clethodim sulfone, and metolachlor ethane sulfonic acid) by a QuEChERS adaptation method (Anastassiades et al.,2007).

Soil samples collected from all experimental treatments after preplant application were also analysed through a general screening on pesticide residues (presented in supplementary material A3 in García Carriquiry et al., (2025), to evaluate background contamination on each plot and ensure that no cross-contamination occurred due to potential drift. Only glyphosate and AMPA were detected as background contamination, on average glyphosate was 162 ± 54 μg kg^{-1} in the first year and 213 ± 54 μg kg^{-1} in the second year, while AMPA levels averaged 237 ± 89 μg kg^{-1} in the first year and 492 ± 110 μg kg^{-1} in the second year, (results present at García Carriquiry et al., (2025).

Soil samples collected from all experimental treatments after preemergence and early postemergence applications were used as a reference for the vegetative exposure of the plants, and for the reproductive exposure of the plants, respectively. The periods of exposure ranged from 34 to 41 days, from soil residue determination to plant sampling timepoint.

2.3. Plant and mycorrhizas determinations

Plant destructive samples were taken in two timepoints of each crop season. First, when plants in the control had four developed leaves, corresponding to V4 in the Fehr and Caviness (1977) method. Ten plants per plot of the treatments 0, 1, 2, 3 and 8 were randomly selected and sampled by digging a 20 cm square hole around each plant, deep enough to remove the entire root. The remaining treatments were not sampled at this timepoint because they did not represent a different herbicide load

by that time. Then, when plants in the control were beginning pod formation, i.e., R3 stage, five plants per plot of all treatments were randomly sampled, as described above. This adjustment to five plants was made for logistical reasons given the large number of samples to be taken and processed. From now onwards, these sampling time points will be referred as vegetative and reproductive respectively.

After collecting the plants, their roots were rinsed with tap water. Roots fresh weight was determined, and then more than 30 pieces of 1 cm long of the finest roots were stored in alcohol 70 % at 4 °C for a maximum period of four months until arbuscular mycorrhizal fungi (AMF) quantification. Then, the shoots were dried for 48 hours at 60 °C, weighted and grinded. Murphy and Riley (1962) method was used to quantify phosphorus content per plant, obtaining the phosphorus concentration in the shoot. Then total phosphorus in the shoot was calculated by multiplying phosphorus concentration and shoot biomass per plant.

The pieces of root stored were stained using Wardhani et al. (2019) method to make fungi structures visible. Next, arbuscular mycorrhizal colonization was quantified following Trouvelot et al. (1986) method. From each plant, two samples composed by 15 1-cm-pieces of root were randomly taken to ensure representativity. In total, the roots of 570 plants were analysed, being 300 plants from vegetative stage (10 plants per plot, 5 treatments, 3 replicates per treatment and 2 years), plus 270 plants from reproductive stage (5 plants per plot, 9 treatments, 3 replicates per treatment and 2 years); and 1140 samples (2 subsamples per plant) were observed in the microscope. The presence and absence of hyphae, arbuscules and vesicles were registered on 100 microscope fields on each sample.

2.4. Statistical analysis

The herbicide effects on AMF and phosphorus in shoot were analysed with Generalized Linear Mixed Models (GLMM), using the fixed and random effects shown in Table 2. The fixed effects of these statistical models included the interaction between treatment and year to account for the expected differences of treatments response within each year. The interaction was nested within sampling timepoint to examine separately the vegetative and reproductive stage of the plants. These statistical models included "Plot identity" as a random effect to account for variability between different plots.

The package fitdistrplus (Delignette-Muller and Dutang, 2015) was used to assess the appropriate probability distributions to model each response variable. All AMF-related variables were converted to proportions that were modelled using the Beta distribution and a logit link function. AMF-related variables were transformed using the formula (Y*(n − 1)+ 0.5)/n (Smithson and Verkuilen, 2006), where Y represents the original values of the response variable and n is the total number of observations. This transformation is required because the logit link function is undefined for both Y= 0 and Y01. The goodness of fit of statistical models was evaluated with residual analyses (see Inchausti 2023). The overall models' performance was assessed by calculating marginal and conditional R2 values (Nakagawa and Schielzeth, 2013) with the MuMin package (Bartoń, 2023).

Post-hoc tests were conducted on the models for each sampling timepoint to detect differences between means. For the vegetative stage, marginal means were calculated for treatments 0, 1, 2, 3 and 8 across the two years, orthogonal contrast matrices were calculated and p-values of the pairwise comparisons were adjusted using the false discovery rate method (Inchausti, 2023). In the reproductive stage, where all treatments were sampled, the marginal means, contrasts and comparisons were calculated for the nine treatments (0−8). R.4.4.1 software was used for the statistical analysis (R Core Team, 2024), the glmmTMB package version 1.1.9 (Brooks et al., 2017) was used to fit the statistical models, and emmeans version 1.10.3 (Lenth et al., 2024) was used for post-hoc tests.

Table 2
Statistical models and distributions used for AMF and content of phosphorus in plant analysis.

Response variable	R function	Model	Distribution
Total colonization	glmmTMB	~ POM + ppmP+ Timepoint/(Treat*Year) + (1|Plot)	beta_family (link = "logit")
Arbuscular colonization	glmmTMB	~ POM + ppmP+ Timepoint/(Treat*Year) + (1|Plot)	beta_family (link = "logit")
Vesicles colonization	glmmTMB	~ POM + ppmP+ Timepoint/(Treat*Year) + (1|Plot)	beta_family (link = "logit")
Phosphorus in shoot (%)	glmmTMB	~ POM + ppmP+ Timepoint/(Treat*Year) + (1|Plot)	lognormal(link = "log")
Total Phosphorus in shoot	glmmTMB	~ POM + ppmP+ Timepoint/(Treat*Year) + (1|Plot)	Gamma(link = "log")

* POM, percentage of organic matter; ppmP, phosphorus content in soil; Timepoint, sampling timepoints; Treat, Treatments; Year, experimental year

3. Results

3.1. Soil characterization

Organic matter and phosphorus soil content per plot and treatment are presented in supplementary materials Table A1., and were included in the statistical models, as shown in Table 2.

3.2. Arbuscular mycorrhizal colonization and phosphorus content in plant

The results are presented in a logical sequence, beginning with the analysis of deviance to assess the effects of the explanatory variables on the response variables, and followed by detailed treatment-specific differences in AMF colonization and plant phosphorus content.

The analyses of deviance for all statistical models concerning AMF (total colonization, arbuscular colonization and vesicle formation) and phosphorus content in plant is presented in supplementary materials B. These results indicate significant influence of timepoint, treatment and year, and their interactions on the response variables. The marginal and conditional r^2 values show that these models explain a large proportion of the existing variance on each response variable. The goodness of fit for the models concerning AMF and phosphorus content in plant successfully met the assumptions assessed by residual analysis.

The effects of herbicide treatments on AMF colonization did not show the expected association with the quantity or type of herbicides applied, with the exception of vesicle formation during the first experimental year (Fig. 1). In the vesicles case, the roots in the control plots had lower vesicle formation than those in the herbicide-treated plots, regardless of the moment of application, and the type and amount of herbicide applied. However, in the second year, no statistically significant differences were found among treatments.

In the vegetative stage of the plants during the first experimental year, AMF quantification showed no statistically significant differences between treatments and the control, nor among treatments themselves. In the second year, treatments 2 and 3, treated with only the preplant and only the preemergence herbicide application respectively, showed lower total and arbuscular colonization than the control, even lower than the treatments under both herbicide applications and rates (treatments 1 and 8).

In the reproductive stage, only treatment 5 in the first year presented lower total colonization than the control and the treatment 1 (applied with the three herbicide applications). In the second year, no statistically significant differences were observed between the control and the applied treatments in total and arbuscular colonization.

In another regard, the results revealed a lower initial mycorrhizal colonization in the second experimental year compared to the first. This difference might be related to a higher precipitation, 17 vs 45 mm, in the

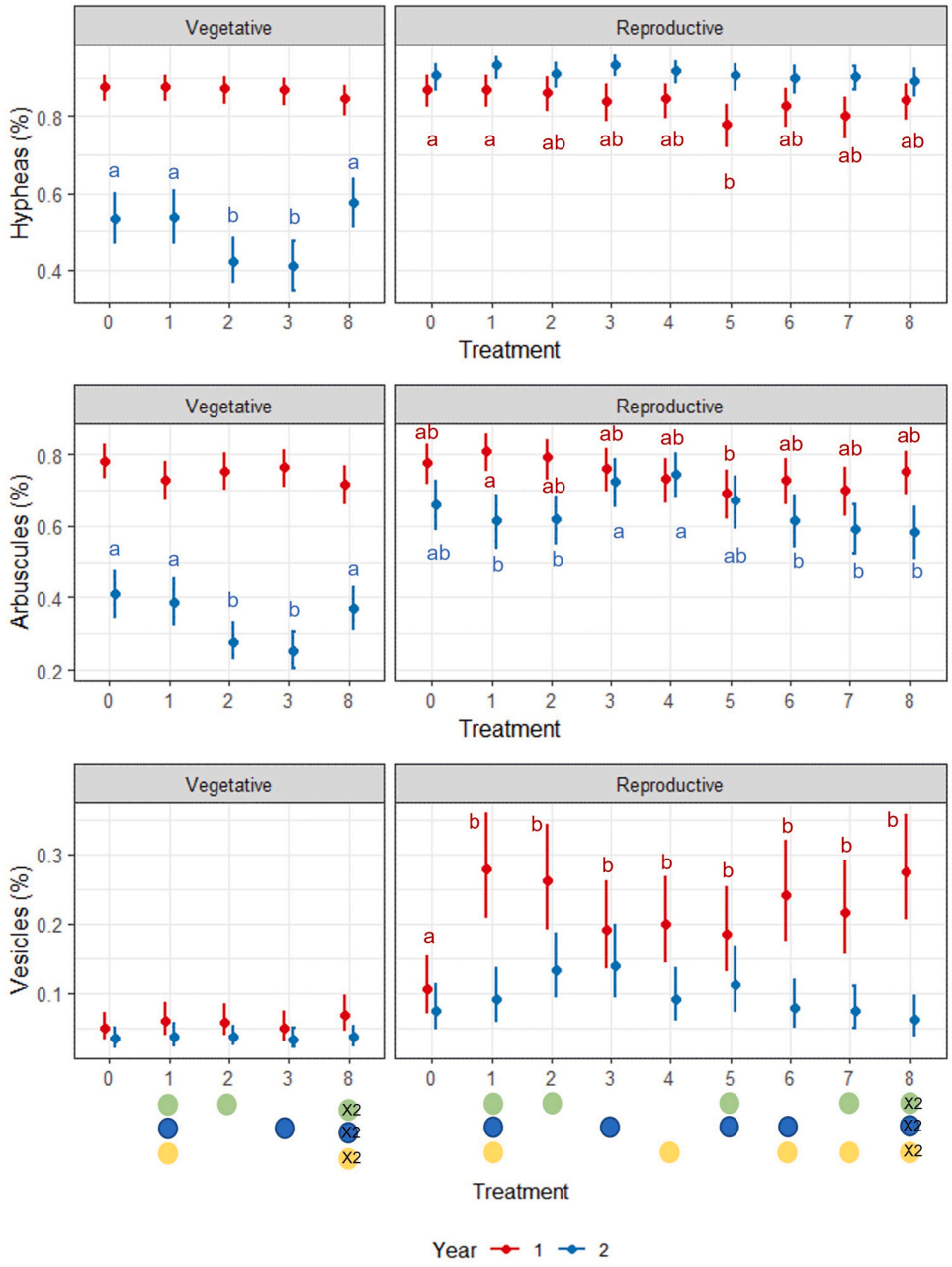

Fig. 1. Marginal means of total colonization, arbuscular colonization and vesicle formation per treatment and year, and their confident intervals. Treatments are represented by green circle: glyphosate + dicamba + clethodim, blue circle: glyphosate + S-metolachlor + flumioxazin, yellow circle: fomesafen, and X2: twice the recommended rate. Different letters represent significant differences ($p < 0.05$) within each timepoint and year (red for Year 1, blue for Year 2). The coloured dots at the end of the figure are meant to facilitate its read, with information on pesticide scheme per treatment, number of treatments (see Table 1) at the x axis.

first and second year, respectively, registered between the sowing time and the vegetative sampling. The efficiency of AMF colonization increases with soil moisture (Karasawa et al., 2000).

No statistically significant differences were found among treatments in the shoot phosphorus concentration in the vegetative stage, nor in the reproductive stage in the second experimental year (Fig. 2). However, in the first year, plants in treatment 3 that received only the preemergence herbicide mixture, showed higher phosphorus content than plants in the control, while the other treatments were not different from the control.

In this line, no statistically significant differences were found in the mean total phosphorus in shoot among treatments in the vegetative stage of the plants. In the reproductive stage of the plants, none of the herbicide-applied treatments showed significant differences compared to the control. Some statistically significant differences were detected among different treatments but not directly related to the total amount or type of herbicide applied (Fig. 2). It is worth noting that certain herbicides, such as glyphosate, contain phosphorus in their composition. Although the contribution of phosphorus from these herbicides is expected to be minimal, it may hinder the real effect, further complicating the study of their impact in phosphorus deficient context.

3.3. Herbicide residues

Fig. 3 shows the sum of total herbicide residues in soil and the corresponding metabolites that reflect the plant and microbiome exposure. The first panel shows the vegetative exposure, determined approximately 34 days before the vegetative sampling point. The soil sample was taken after the second application moment, so it may contain

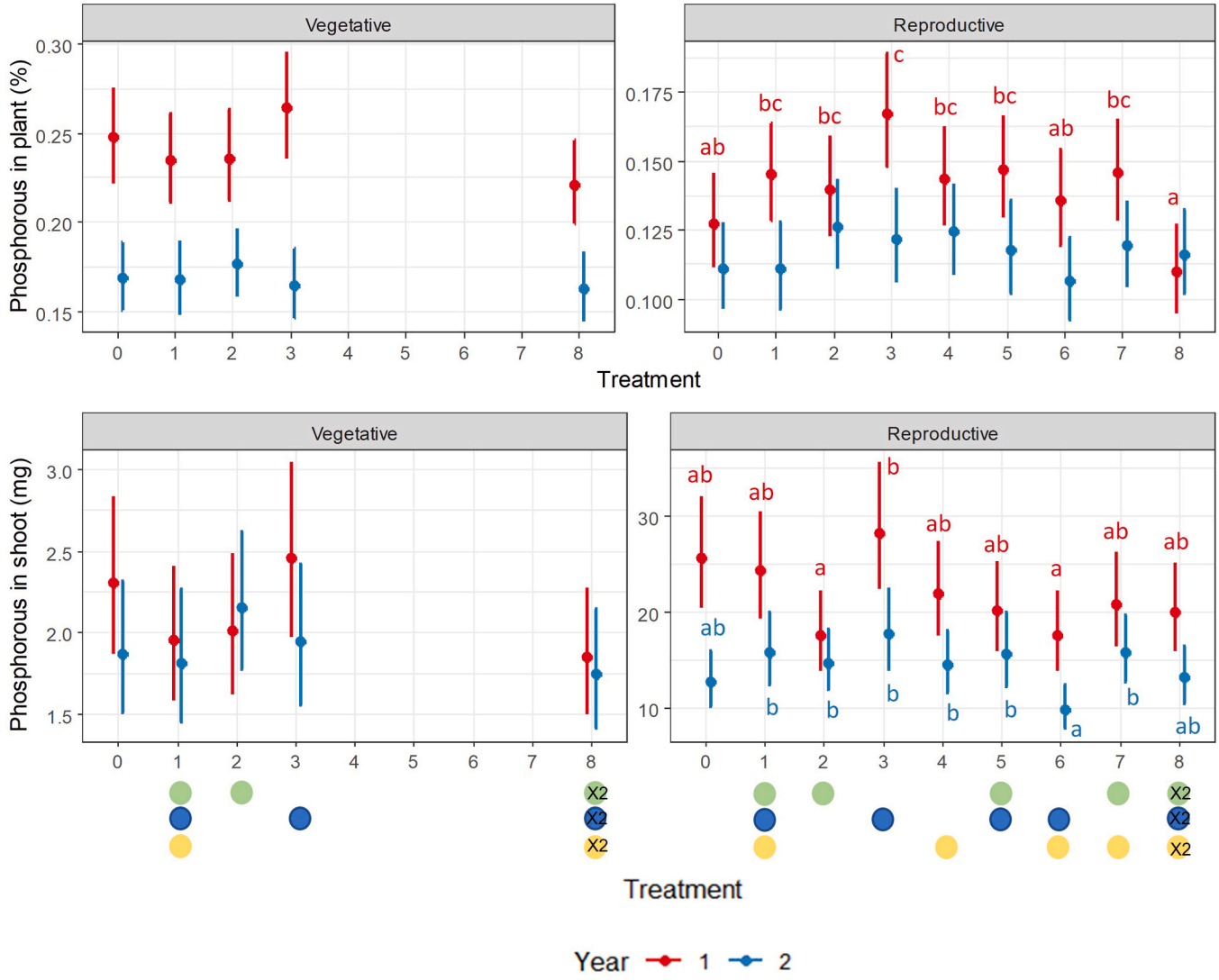

Fig. 2. Marginal means of phosphorus concentration in shoot (%) and total phosphorus in shoot (mg) per treatment and year, and their confident intervals. Treatments are represented by green circle: glyphosate + dicamba + clethodim, blue circle: glyphosate + S-metolachlor + flumioxazin, yellow circle: fomesafen, and X2: twice the recommended rate. Different letters represent significant differences ($p < 0.05$) within each timepoint and year.

residues from the first two herbicide mixtures. Dicamba and clethodim, involved in the first mixture applied preplant, and their metabolites were no longer detected at this timepoint due to their fast degradation rate.

The second panel of Fig. 3 shows the reproductive exposure, determined approximately 41 days before the plant reproductive sampling point. The soil sample was taken after the third application moment, early postemergence, so it may contain residues from the three applications, depending on the treatment. More detailed information about compounds separately, determined after each application moment, can be consulted in García Carriquiry et al., (2025). Important differences can be observed among the first and second experimental years on each exposure phase, mainly explained by the rainfall magnitude after the herbicide applications that could move the herbicide residues from the aboveground straw to the topsoil layer accordingly.

4. Discussion

The results of the field experiment showed that the full sequence of the herbicide applications for weed control in soybean crop, applied at recommended or twice the recommended rate, did not affect the endpoints related to phosphorus cycle under study. Specifically, no effects were observed on AMF colonization and arbuscules formation of the local community, total phosphorus, or phosphorus concentration in the shoots for those treatments. Moreover, the type of herbicides tested in mixtures (glyphosate + dicamba + clethodim; glyphosate + s-metolachlor + flumioxazin; and fomesafen) did not show a direct response of any of the combinations on these endpoints. Overall, these results suggest that these mixtures and sequence of herbicide applications did not pose severe risks on these endpoints at the field level, under realistic field management practices. However, nuances and uncertainties should be further explored before generalizing these results to other contexts.

In a previous pot experiment, using a different soil and soybean variety but testing similar mixtures of herbicides, the plants under twice the recommended herbicide rate presented significantly lower total and arbuscular colonization in the vegetative stage (García Carriquiry et al., 2024). Overall, the residues of glyphosate and AMPA for this treatment were similar in both experiments. However, the residues of metolachlor and flumioxazin were 2 to 15 times lower in the field than in the pot experiment, which led to a lower exposure of the AMF. This is probably the main reason for the different results. As mentioned before, the straw aboveground intercepted and retained a substantial portion of the applied herbicides. While it is possible that herbicide washed off from the straw to the topsoil, this is highly dependent on the rainfall quantity

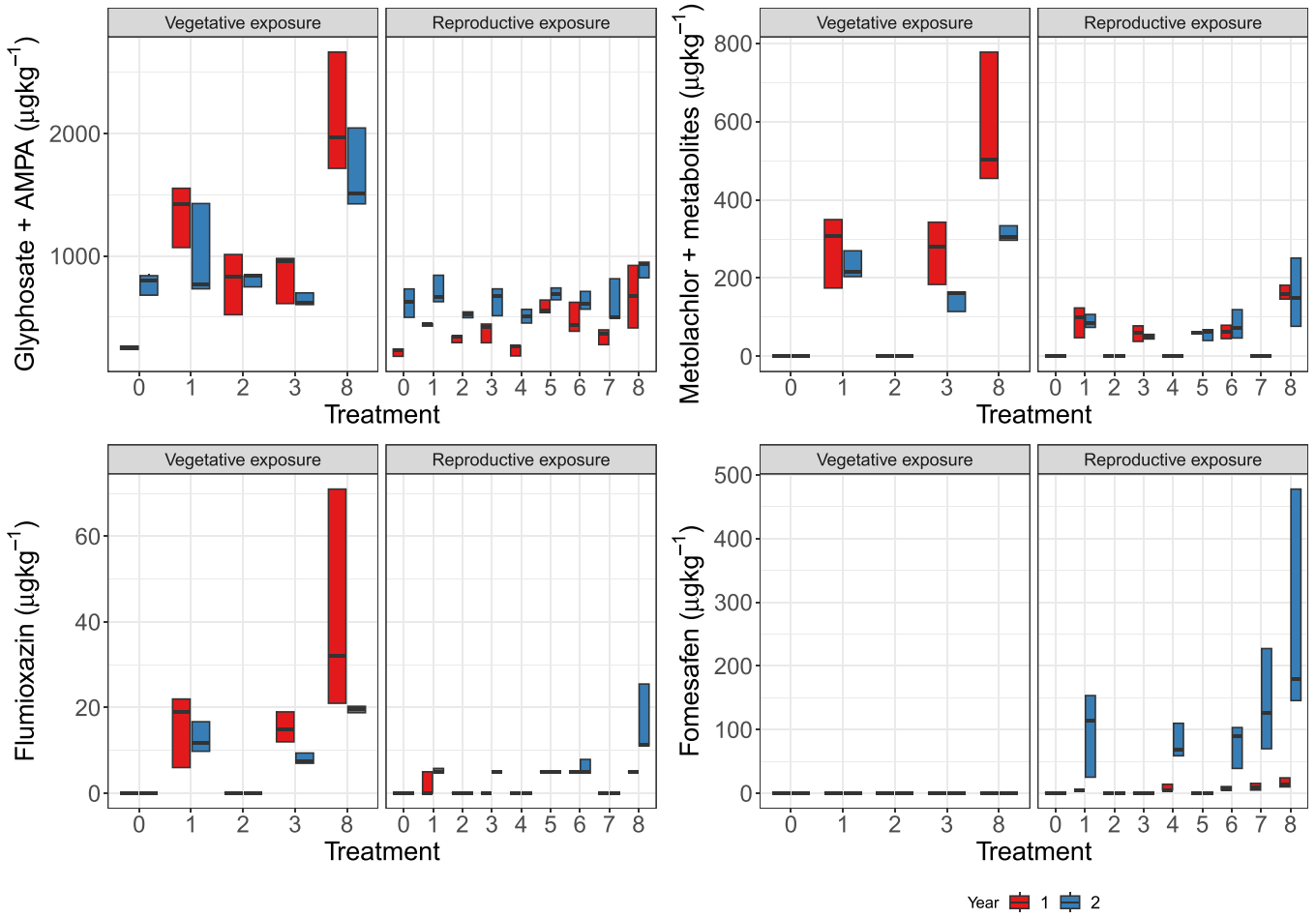

Fig. 3. Herbicide residues plus corresponding metabolites in soil 34–41 days prior to vegetative and reproductive sampling timepoints, per year. Herbicides that were not detected at these timepoints are not represented. Refer to Fig. 1 for treatment descriptions.

and intensity after the applications. The causes of the different herbicide concentrations in the soils were thoroughly discussed by García Carriquiry et al., (2025). These field experiments support the evidence that the total and arbuscular mycorrhizal colonization is not significantly affected at low concentrations of these mixtures.

Cause and effects are hard to link accurately in field experiments. In this regard, the results observed in treatments 2 and 3 (single preplant and single preemergence applications respectively) in the vegetative stage of the plants in the second year appear inconsistent, since the total and arbuscular colonization were lower than treatments with both applications, the preplant and the preemergence mixtures, at the recommended and twice the recommended rate. Moreover, this lower colonization rate in treatments 2 and 3 did not have an impact on phosphorus concentrations and content in shoots. It is possible that some weed growth might have occurred in plots with incomplete herbicide treatments, which may have influenced the results. Maintaining 100 % weed-free conditions in 60 m^2 plots is empirically challenging, especially considering that priority was consistently given to the control plots. This subtle discrepancy between weed growth and weeding efforts did not affect soybean plant growth but may have influenced the water regime, which was already generally deficient. The same could have occurred with total colonization of treatment 5 that received the preplant and the preemergence herbicide mixtures, in the reproductive stage, the second experimental year. Further studies should be conducted to explain the lower colonization observed in treatments 2 and 3 in the vegetative stage and in treatment 5 in the reproductive stage, preferably under more controlled conditions that would allow for a direct connection between active ingredient and herbicide rates and the observed effects on AMF colonization.

The vesicle formation of AMF is linked to a nutrient-reservoir function (Smith and Read, 2008). In the vegetative stage no differences were observed among treatments and vesicle formation was generally low, which is consistent with the pot experiment results. In the reproductive stage, the pot experiment showed a significant decline in vesicle formation under the treatment with twice the recommended herbicide rate, while no statistically significant effects were observed for lower-rate treatments (García Carriquiry et al., 2024). However, the field experiment in the first experimental year revealed significantly higher vesicle formation in all treatments compared to the control in the reproductive stage. Based on the results from both experiments and the levels of herbicide exposure, the findings from the first year of the field experiment could be explained by a hormetic response, where a low concentration of herbicide stimulated vesicle formation, while higher concentrations - as seen in the second year and the mesocosm experiment - did not induce such a response, or even resulted in a decrease. This phenomenon is described as the stimulus in response to a very low pesticide concentration (stressor), and can be also expected in AMF exposed to low pesticide levels (Jakobsen et al., 2021).

In this line, the reproductive exposure to herbicide and metabolites residues in soils was lower in the first experimental year compared to the second experimental year. In the second year, the immediate rainfall that followed the application washed off the herbicide retained in the straw to the topsoil, increasing its concentration in soil compared to the first year. Despite the immediate rainfall mentioned, the overall environmental conditions were similar in both years for the period between third application early postemergence, and R3 sampling point, so no

strong effects on vesicles were expected among years in this regard. According to these findings, there are no effects on vesicle formation at low concentrations of these herbicide mixtures, but at very low concentrations of the entire sequence or separate mixtures, it could result in an hormetic response. The stimulus in vesicle formation cannot be attributed to any specific compound because the effect was observed in all the applied treatments, including different compounds and mixtures. Instead, it may be associated with the low concentrations of exposure at the reproductive stage on year 1, and possibly with the medium-term changes (legacy effects) on the microbiome.

Phosphorus concentration in shoots was significantly higher in treatment 3 (glyphosate, metolachlor and flumioxazin applied at pre-emergence) than control in the reproductive stage of the first experimental year. The herbicide residues measured in this treatment, 40 days before the reproductive sampling point, showed an exposure of glyphosate (190–343 μg kg^{-1}), AMPA (< LOQ) and metolachlor and its metabolites residues (ranging 10–35 μg kg^{-1} each one). Instead, the control plots presented among 80–139 μg kg^{-1} of glyphosate at that timepoint. The herbicides and metabolites present in treatment 3 plots may have caused an hormetic response (Jakobsen et al., 2021). This result was not observed in the second year, which may be explained by a higher exposure of flumioxazin, AMPA, and metolachlor resulting from different environmental conditions immediately after the herbicide applications. It should be noticed that the phosphorus content in the soil was included in the statistical models to avoid the confounding effects of the different background levels of phosphorus among years and treatments. It was also observed that total phosphorus content in shoot was not significantly different between the control and treatment 3, which is basically explained by a higher plant biomass in the control.

Based on the pot experiment that studied similar mixtures, it was expected a decrease in phosphorus concentration in shoots under treatment 8, with twice the recommended rate of herbicides, in the vegetative stage. However, pesticide analyses showed that the exposure levels of the different compounds in this treatment and timepoint under field conditions were generally lower than in the pots, reaching up to 15 times difference for certain compounds. This low pesticide concentrations in the field experiment may explain the absence of significant effects on phosphorus content at this stage. Moreover, the time for degradation was also lower in the pots than in the field soil, with approximately 15 days difference from the preemergence application to the reproductive stage. Both experiments conclude that there are no significant effects on total phosphorus content in plant at low herbicide concentration of the studied herbicide sequences in soil.

4.1. Strengths and limitations

The experimental design was useful for studying the effects of the herbicide mixtures and the sequence of application on arbuscular mycorrhizal fungi and phosphorus in shoots, while also complementing studies on other endpoints to provide holistic view of their impact on soil health under field conditions.

The experimental design was driven by some of the major current challenges around pesticides and risk assessment namely i) the limited data around the effects of mixtures, addressed here via tank mixtures and the spray series, ii) the limited data from real setups, for validation/calibration of data obtained under laboratory and semi-controlled setups, and iii) the need of more holistic and new indicators on health assessments. Using twice the recommended rate was expected to inform on more pesticide intense schemes. Results highlighted non-linear answers from exposure to mixtures of herbicides, different sensitivity of different endpoints, and major impact that soil management practices (e.g., surface crop residue) may have on pesticides levels in soil and subsequently on nutrient cycling.

However, this study entails three main limitations. First, the treatments were based on theoretical herbicide rates, that resulted in ranges of herbicide and metabolite concentrations in the plots. Although this variability of concentrations within the treatments was measured, it could not be included in the statistical analysis because these concentrations change in time, and analysing the compounds separately or summing up the different compound concentrations is not ecotoxicologically relevant. From an ecotoxicological and regulatory point of view, it would be important to link the responses to the measured herbicide concentrations in the soil, however, the experimental design did not allow to obtain dose-response relations and existing ecotoxicological information on this endpoint is rather limited. Reference ecotoxicological databases such as the Pesticide Properties DataBase (PPDB, 2024) or Pesticide Chemical Search (EPA, 2025), cover individual compound data, and provide standard ecotoxicological endpoints, mainly acute, for terrestrial ecosystems including mammals, birds, earthworms and honeybees, and occasionally other species. Risk quotient analyses could be performed, provided that there is enough endpoint specific data in ecotoxicological databases/literature, but underlying additivity rationale could underestimate the real risk, as it excludes interaction of pesticides in the mixture, and potential indirect cascade effects linked with their co-occurrence (Panico et al., 2022). It is also noteworthy to highlight that this experimental design did not allow to weight the effects of herbicides individually on the response variables. An example of this, which was also highlighted in García Carriquiry et al., (2025), is that in the statistical analysis the model solution considered treatments as fixed effects, while in fact, there is a gradient of herbicides on each one. For instance, the control plots in year 2 presented higher levels of glyphosate plus AMPA than the control plots in year 1, and similar levels of these compounds than the other treatments. However, both were equally considered as controls.

This example leads to the second limitation, which was the absence of a true negative control, as control plots in both experimental years had some residual levels of glyphosate plus AMPA. The presence of glyphosate and AMPA in soil is rather a normal condition where non-organic soybean is produced under no-till systems. Considering that the frequency of glyphosate application is higher than the degradation rate of glyphosate and AMPA, Primost et al. (2017) suggested to re-categorize glyphosate from non-persistent to pseudo-persistent compound.

A third limitation identified is that the analysis of arbuscular mycorrhizas colonization did not account for the impact on the structure of the fungal community, including the detection of changes in the species or family compositions. This primary study focused on AMF colonization starting from propagules of local communities already present in soil. To further understand the impacts on AMF, future research should explore AMF community composition and diversity, since these responses can be affected even when herbicides do not impact colonization rates (Sheng et al., 2012). Herbicides could affect the balance of different fungi species, where some species could be stimulated covering the niche of depressed species in response to the herbicide treatments (Pagano et al., 2023). Then, there may be underlying effects that warrant further investigation through genetic and functional analysis of AMF communities.

Building on these results, future studies should investigate dose-dependent responses of individual herbicides on arbuscular mycorrhizal colonization in order to establish a reference under controlled conditions. This endpoint, although non-traditional, is gaining recognition for its ecological relevance, as evidenced by the development of a new standardized protocol under the ERAMYC (2025) (project financed by the German Federal Environment Agency). Moreover, further studies should also consider higher field exposure scenarios. Factors such as reduced straw cover or immediate rainfall following application may result in still real and significantly high exposure levels, potentially allowing for the observation of the complete biphasic response. For instance, the stimulus of vesicle formation at low herbicide residues and the significant decrease at high herbicide concentration.

5. Conclusions

The results of this study indicate that the full sequence of herbicides at field-level concentrations did not significantly affect total AMF colonization, arbuscule formation in soybean roots, or plant phosphorus content - key variables in the phosphorus cycle. However, it is important to note that significant negative effects were observed in specific treatments within the general application scheme. Specifically, individual treatments corresponding to only the preplant and only the preemergence herbicide applications resulted in reduced total and arbuscular colonization compared to the control in year 2 at the vegetative stage. In addition, the combined preplant and preemergence treatment led to a significant decrease in total colonization in year 1 at the reproductive stage. Among the mycorrhizal structures, vesicle formation emerged as the most sensitive indicator, showing a marked increase under herbicide-related stress in the reproductive stage of year 1. Furthermore, phosphorus content in plant tissue increased under the only preemergence applied treatment compared to control conditions.

It is essential to evaluate the cumulative effects of full pesticide sequences commonly used in soybean crops to effectively understand and protect the key functions of arbuscular mycorrhizae in soil.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109754.

Data availability

Data will be made available on request.

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