University of São Paulo
“Luiz de Queiroz” College of Agriculture

Fungicide soybean seed treatment: interactions with inoculant, soil, and plant

Felipe Fadel Sartori

Thesis presented to obtain the degree of Doctor of Science. Area: Crop Science

Piracicaba
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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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1. *Glycine max* 2. Ureídeo 3. Pré-inoculação 4. Translocação 5. Radiomarcado 6. *Phytophthora sojae* I. Título

DEDICATION

To my father and my mother. For all their efforts and support along my academic life, but most importantly, for their constant example of kindness and gratitude. Every time an unfortunate life event tried to push them back, they stood up and thrived, and I will always carry this lesson in my life.

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CONTENT

RESUMO

Fungicidas no tratamento de sementes de soja: interações com inoculante, solo e planta

O tratamento de sementes se tornou parte do sistema de produção de soja no Brasil, a ponto de que quase não se encontraram áreas que não utilizem essa modalidade de aplicação. Não obstante, apesar da sua sólida presença comercial, dúvidas aparecem safras após safra sobre questões que envolvem a interação dos produtos aplicados via sementes, e dos mesmos com o ambiente de produção. Assim, os objetos do presente estudo foram: (i) determinar como o tratamento de sementes com fungicidas associado à pré-inoculação de *Bradyrhizobium elkanii* em soja pode prejudicar a fixação biológica de nitrogênio (FBN) e a produtividade; (ii) como os fungicidas aplicados via tratamento de sementes são absorvidos e translocados nas plântulas, e como interagem com o solo e suas propriedades; (iii) e a longevidade da ação dos fungicidas aplicados via tratamento de sementes ao longo do desenvolvimento das plantas. Para investigar os objetivos propostos pelo projeto, quatro experimentos de campo e sete em casa de vegetação foram executados ao longo dos anos 2016, 2017, 2018 e 2019. Os experimentos de campo tiveram como propósito avaliar o efeito de fungicidas somado à pré-inoculação de *B. elkanii* em sementes de soja, tanto na FBN como na produtividade, avaliando-se a concentração de ureídeos, a eficácia da FBN e os componentes de produtividade da cultura. Já os experimentos em casa de vegetação buscaram elucidar o padrão de absorção e translocação de diferentes fungicidas aplicados via tratamento de sementes em plântulas de soja por meio do uso de moléculas radiomarcadas, bem como a longevidade do tratamento de sementes na eficácia do controle de *Phytophthora sojae*, avaliando-se a severidade de doença nas raízes e biometria das plantas. Os resultados mostram que tanto o tratamento de sementes como a pré-inoculação afetam a FBN, não acarretando, contudo, em impactos na produtividade. Ademais, o tratamento de sementes não resultou em produtividade superior ao controle com apenas inoculante em nenhum dos experimentos. Quanto à absorção e translocação de fungicidas aplicados via tratamento de sementes em plântulas de soja, nota-se que a maior parte dos produtos, quando absorvidos, concentram-se nos cotilédones das plantas, e que o teor de matéria orgânica do solo pode influencia na absorção dos produtos. Por último, em relação a longevidade de ação de fungicidas ao longo do desenvolvimento das plantas, notou-se que o limite de eficácia satisfatória no manejo de *Phytophthora sojae* foi de até 14 dias após a semeadura.

Palavras-chave: *Glycine max*, Ureídeo, Pré-inoculação, Translocação, Radiomarcado, *Phytophthora sojae*

ABSTRACT

Fungicide soybean seed treatment: interactions with inoculant, soil, and plant

Seed treatment has become part of the soybean production in Brazil and, currently, almost a hundred percent of the areas use this type of pesticide application. Nevertheless, despite its solid commercial presence, questions regarding the interaction of pesticides with biological products and the environment arise year after year. Thus, the objectives of the present study were: (i) to determine how soybean seed treatment with fungicides associated with *Bradyrhizobium elkanii* pre-inoculation can be detrimental to the biological nitrogen fixation (BNF) and yield; (ii) how fungicides applied via seed treatment are absorbed and translocated in seedlings, and how they interact with the soil and its properties; (iii) and the longevity of fungicide action along plant development. In order to investigate these goals, four field and seven greenhouse experiments were carried out over the years 2016, 2017, 2018 and 2019. The field experiments aimed to evaluate the effect of fungicides plus pre-inoculation with *B. elkanii* on soybean seeds, both on BNF and yield, evaluating the concentration of ureides, the BNF efficiency and the productivity components of the crop. The greenhouse experiments sought to elucidate the absorption and translocation pattern of different fungicides applied via seed treatment in soybeans using radiolabels active ingredients, as well as the longevity of seed treatment efficacy on controlling *Phytophthora sojae*, assessing severity of root rot and plant development. The results showed that both seed treatment and pre-inoculation affect BNF, however, did not cause impacts on yield. In addition, seed treatment did not have superior yield than control with inoculant alone in any of the experiments. Regarding the absorption and translocation of fungicides applied via seed treatment in soybean, it was observed that most products, when absorbed, were concentrated in the cotyledons of plants, and that the soil organic matter content can influence the absorption of the products. Finally, regarding the longevity of action of fungicides during plant development, it was shown that the satisfactory efficacy in the management of *Phytophthora sojae* was up to 14 days after planting.

Keywords: *Glycine max*, Ureides, Pre-inoculation, Translocation, Radiolabelled, *Phytophthora sojae*

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INTRODUCTION

On 2018/2019 crop season, almost 98% of soybean seeds were treated with fungicides in Brazil (Richetti and Goulart, 2018). Specifically for soybean seeds, the first official recommendation for fungicide treatment was made by Embrapa Soja in 1981 (Henning et al., 1981). The treatment of soybean seeds with fungicides, which in the 1991/92 crop season did not reach 5% of the area (Henning et al., 2010), now approaches the 100%.

Currently, many different groups of fungicides have been used in soybean seed treatment to control both seed and soilborne pathogens (Dorrance et al., 2003; Broders et al., 2007; Ellis et al., 2010). Among these, benzimidazoles (which affects mitosis and cell division), strobilurins (which affects respiration), and phenylamides (which affects protein production) (Oliver and Hewitt, 2014) represent important molecules for fungicide seed treatment. Benzimidazoles (e.g., thiabendazole, carbendazim, and thiophanate-methyl), are effective against a wide range of ascomycetes and basidiomycetes, but not against oomycetes. On the other hand, phenylamides (e.g. metalaxyl and mefenoxam) are effective exclusively against oomycetes. Strobilurins (e.g. pyraclostrobin, azoxystrobin, and trifloxystrobin) are effective against some members of ascomycetes, basidiomycetes and oomycetes (Oliver and Hewitt, 2014). In addition to the mode of action of each active ingredient, other physicochemical characteristics may contribute to the classification of fungicides, such as the log K_{ow} (also known as log *P*), which indicates the partition coefficient between n-octanol and water and measures hydrophobicity/hydrophilicity of the molecule (Oliver and Hewitt, 2014).

Phytophthora sojae is one of the most important soybean pathogens across the world. This soilborne disease is essentially monocyclic. *P. sojae* produces sexual oospores in root tissue following infection, which can survive in the soil (Schmitthenner, 1985; Dorrance et al., 2007), and as a result, plants grown in fields infested with *P. sojae* are at constant risk. The primary strategies to manage Phytophthora root and stem rot are resistant cultivars (Rps genes and quantitative resistance), and seed treatment with systemic fungicides (Schmitthenner and Dorrance, 2015; Dorrance, 2018). The use of fungicides to control Phytophthora root and stem rot in fields with high disease pressure is a useful technique for both moderate susceptible and resistant cultivars, when compared to the non-treated control (Dorrance and McClure, 2001; Dorrance et al., 2009) thus soybeans grown in these high disease-risk environments should be treated with at least one active ingredient available in the market against oomycetes in their seed treatment mix (Dorrance, 2018).

More recently, seed producers have implemented a practice that has been very well accepted by soybean growers: Industrial Seed Treatment (IST). This technology relies on the use of special and highly sophisticated equipment, which combines the application of fungicides, insecticides, nematicides, micronutrients, biological products and so on, with high rate accuracy (França-Neto et al., 2015).

In Brazil, *Bradyrhizobium* sp. inoculants are commonly applied in seed treatment or in furrow, the former being the most common. When applied via seed treatment, the inoculant is part of packages with chemicals such as fungicides, insecticides, nematicides, micronutrients and biostimulants, which sometimes are incompatible with the rhizobial survival (Campo et al., 2009). The possibility to deliver the inoculant along with the chemicals in the seed treatment triggered the development of the well-known “long life inoculants”, which, in theory, would allow seed pre-inoculation for periods of up to 60 days.

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1. SOYBEAN SEED TREATMENT AND PRE-INOCULATION WITH *BRADYRHIZOBIUM* ON BIOLOGICAL NITROGEN FIXATION AND YIELD

Abstract

Biological nitrogen fixation (BNF) plays a key role in soybean production worldwide. As a result of symbiosis between the host and *Bradyrhizobium* sp., nitrogen becomes available to the plant. Although highly effective, this process may be affected by several factors, such as soil temperature, moisture, flooding and xenobiotics. The objective of this study was to determine the influence of pesticide soybean seed treatment and pre-inoculation with *Bradyrhizobium elkanii* prior to planting on BNF and yield. Four field experiments during the crops seasons of 2016/17 and 2017/18 were carried out using the same cultivar. Ureides, BNF efficiency, yield and thousand seed weight were assessed at vegetative and reproductive stages. Both pre-inoculation and seed treatment affected BNF even when there was a high population of *Bradyrhizobium* sp. present in the soil. However, this fact did not result in a detrimental effect on crop yield or thousand seed weight in most cases. Moreover, none of the seed treatments had a superior yield when compared to non-treated control with only *Bradyrhizobium* sp. inoculant. Therefore, pre-inoculation associated with pesticide seed treatment did not affect soybean yield.

Keywords: *Glycine max*, Ureide, Fungicides, *Bradyrhizobium*

1.1. Introduction

Soybean is the main source of plant protein for human and animal feed. The high level of protein in its seeds implies a high demand for nitrogen estimated to be 80 kg per metric ton of seeds, of which 60 kg are allocated in the seeds and the remaining in the plant residues (Salvagiotti et al, 2008; Bender et al., 2015). Thus, when considering the total soybean produced worldwide in 2019, 20.8 million tons of N would be needed. Nevertheless, a considerable part of this demand is met by biological nitrogen fixation (BNF) with *Bradyrhizobium*, which eliminates the need for N fertilizers. The independence of N fertilizers, which are reliant on fossil fuel for manufacturing, provides both economic and environmental benefits, making BNF a strategically sustainable option for protein production.

The BNF process with *Bradyrhizobium* in nodules converts the atmospheric N_2 into NH_3 (Mulder et al., 2002; Baral et al., 2012; Baral et al., 2014) and, in exchange, the host plant provides dicarboxylic acids (e.g.: malate) (Udvardi and Day, 1997) as source of carbon and energy. In soybeans, the final products of BNF are transported to the shoots as allantoin and allantoic acid, both belonging to the ureide class (Baral et al., 2016).

The low C:N ratio of allantoin (1:1) results in N transport in the plant at a minimum carbon cost. Once ureides accumulate in uninfected nodule cells, they move into the xylem, and are translocated via transpiration stream (Werner and Witte, 2011), and finally accumulate mainly in expanded leaves (Baral et al., 2012, 2014). In mature leaves, ureides are then hydrolyzed by a metabolic pathway involving Mn^{2+}-dependent enzymes and, to a lesser extent, Co^{2+} and Ni^{2+}, resulting in glyoxylate and ammonium (Serventi et al., 2010). In soybean, the amount of ureides increase during the plant cycle, reaching a peak between R_3 and R_5 (Fehr et al., 1971; Zapata et al., 1987; Osborne and Riedell, 2011).

The level or concentration of ureides in nodules and xylem is considered an indicator of the BNF efficiency (Duran and Todd, 2012). Thus, the relative abundance of ureides ([ureide-N / ureide-N + nitrate-N] × 100) in plants shoots (stem + petiole) is a measure of the efficiency of BNF in soybean (Herridge, 1982).

The annual application of *Bradyrhizobium* inoculants (reinoculation) is more common in South America than in the United States (Perticari, 2015; Graham et al., 2004). According to Leggett et al. (2017), comparing the use of inoculants in the US and Argentina, inoculation of seed showed the highest increase in yield in areas of lower potential in both Argentina and the US , 9.5 and 14.0%, respectively , compared with the control relying on natural inoculum. For high yield potential areas the differences were 3.5% and 0.6% in Argentina and US, respectively. The inoculation also significantly increased yield by 1.67% in the US and 6,39% in Argentina, on average. In Brazil, research results point to a yield increase by 8% with annual inoculation of *Bradyrhizobium* sp. (Hungria and Mendes, 2015), and by 16% with co-inoculation with *Bradyrhizobium* sp. + *Azospirillum* sp. (Hungria et al., 2013).

In Brazil, inoculants are commonly applied in seed treatment or in furrow, the former being the most common. When applied via seed treatment, the inoculant is part of packages with chemicals such as fungicides, insecticides, nematicides, micronutrients and biostimulants, which sometimes are incompatible the rhizobial survival (Campo et al., 2009).

Commercial formulations of fungicides, in general, include multiple active ingredients for controlling various pathogens, e.g.: metalaxyl-M and other phenylamides to control oomycetes (Dorrance and McClure, 2001; Dorrance et al., 2009), while benzimidazoles, fludioxonil and strobilurins are used to protect against *Fusarium*, *Phomopsis* and *Rhizoctonia* (Dorrance et al., 2003; Broders et al., 2007; Ellis et al., 2010).

From 1996 to 2013, the use of fungicide-treated seeds in the US increased from 8 to 75% (Munkvold, 2009; Munkvold et al., 2014). This trend may be related to changes in cropping practices in the production system, such as early sowings (Dorrance et al., 2009; Esker and Conley, 2012). In Brazil , the treatment of soybean seeds with fungicides was less than 5% in 1991/92, but currently reached more than 98%, from which approximately 26% are made by industrial seed treatment (IST) (Richetti and Goulart, 2018). IST uses fungicides, insecticides, nematicides, micronutrients, among other products, with high dosing accuracy. This type of treatment has gained market, and most of the seed companies treat the seed in the seed processing unit or at the moment of delivery to the farmers (França-Neto et al., 2015).

The possibility to deliver the inoculant along with the chemicals in the seed treatment triggered the development of the well-known "long life inoculants", which, in theory, would allow seed pre-inoculation for periods of up to 60 days. However, there are only few studies reporting the effect of pre-inoculated seeds with chemicals and stored for up to 30 days on the *Bradyrhizobium* survival and the BNF process. Araujo et al. (2017), using specific technologies for IST inoculated with pyraclostrobin + methyl thiophanate + fipronil, observed a reduction of 6.7×10^7 colony forming units (CFU) per seed at zero storage day to 2.3×10^3 CFU seed^{-1} at 28 days. However, in field experiments, such reduction did not decrease the number of nodules in three out of four experiments when compared with inoculation in the sowing day. Interestingly, in the three areas where there was no significant difference, two of them had the most probable number (MPN) of rhizobia in the order of 10^4 cells g^{-1}, while only one of them had 10^1 cells g^{-1}. In addition, in one of the experiments with MPN of 10^4 cells g^{-1}, both the 0 and 30 days treatments were statistically superior to the non-inoculated treatment.

The objective of this study was to evaluate under greenhouse and field conditions the effect of pre-inoculation with *Bradyrhizobium* of soybean seeds for up 30 days prior to sowing, with and without chemicals used for seed treatment.

1.2. Material and Methods

1.2.1. Experiments

Four field experiments were conducted over two cropping seasons. In the 2016/2017 crop season, two field experiments were conducted, one in Ponta Grossa (PR), named PG 16/17, and other in Piracicaba (SP), named PI 16/17. The experiment PG 16/17 was sown on 2016/11/05 with 50 kg[P] ha^{-1} and 50 kg[K] ha^{-1} in a soil classified as Rhodic haplustox soil containing 2.9% of organic matter, previously cropped with soybeans in the last 10 seasons. The experiment PI 16/17 was sown on 2016/11/20 with 50 kg[P] ha^{-1} and 25 kg[K] ha^{-1} in a soil classified as Rhodic kandiustoc soil containing 1.8% of organic matter, without soybean in the last 4 crop seasons. In 2017/2018, other two field experiments were conducted, both in Piracicaba (SP), named PI-1 17/18 and PI-2 17/18. The experiments were sown on 2017/12/02 with 50 kg[P] ha^{-1} and 25 kg[K] ha^{-1} in a soil classified as Rhodic kandiustoc soil containing 1.4% of organic matter. All field trial soils had 10^5 rhizobia cells g^{-1}. The cultivar

TMG7062 IPRO RR2 was used in all field experiments, without application of N-fertilizer throughout the crop cycle. The PG 16/17 was the only one that was rain fed, while the others were irrigated via a central pivot.

1.2.2. Treatments and experimental design

Pre-inoculation and pesticides used in seed treatment were the two fixed effects considered in the experimental design. The first was related to the storage time of pre-inoculated seeds, which were 0 and 30 days. The second factor was the type of seed treatment: i) pstc0 (control without pesticide application); ii) pstc1 (pyraclostrobin 0.050 g kg^{-1}[seeds] + thiophanate-methyl 0.450 g kg^{-1}[seeds] + fipronil 0.500 g kg^{-1}[seeds]); iii) pstc2 (thiabendazole 0.188 g kg^{-1}[seeds] + fludioxonil 0.031 g kg^{-1}[seeds] + metalaxyl-M 0.025 g kg^{-1}[seeds]) and; iv) pstc3 (carbendazim 0.300 g kg^{-1}[seeds] + thiram 0.700 g kg^{-1}[seeds}). All experiments were conducted in a 2 × 4 factorial arrangement, except in the PG 16/17 experiment, in which there was no pstc0 treatment (control without pesticides). In all experiments, a randomized complete block design with five replications was used.

In the greenhouse experiment and in the PG 16/17, PI 16/17 and PI-1 17/18, PI-1 18/19 and PI-2 18/19 experiments, *Bradyrhizobium elkanii* formulated as a peaty inoculant (5×10^9 colony forming units[CFU] g^{-1}; rate of 4 g kg^{-1}[seeds]) was used, while in the PI-2 17/18 experiment a double rate was applied. In addition, colorant polymer (Polyplus® Forquímica – rate of 3 mL kg^{-1}[seeds]), osmoprotectant polymer (S30® BASF – rate of 3 mL kg^{-1}[seeds]) and powder-drier (Alldry® Forquímica – rate of 4 g kg^{-1}[seeds]) were added in all treatments in the following order: first, the pesticides were mixed with the spreader and osmoprotectant polymers, applying the resulting slurry to the seeds. Subsequently, with the seeds still wet, the inoculant was added. Finally, after mixing the treated seeds with the inoculant, the powder-drier was added.

1.2.3. Evaluations

1.2.3.1. Ureide and nitrate concentrations in plants

After drying, petioles and stems were grinded in a Wiley mill (Herridge and Peoples, 1990). For determination of ureides and nitrate, 0.1 g of the processed samples was placed in 15 mL Falcon vials, added 10 mL of distilled water, and placed in a water bath for 1 h at $45^{o}C$ (Teixeira et al., 2018). The suspension was centrifuged at 15,344 x G and the supernatant transferred to new 15 mL Falcon vials. The determination of ureide was performed according to Young and Conway (1942), adapted by Teixeira et al. (2018). Nitrate determination was performed only for the field experiments using the salicylic acid method proposed by Cataldo et al. (1975), adapted by Teixeira et al. (2018).

1.2.3.2. Efficiency of biological nitrogen fixation

Ureides and nitrate concentrations were used to calculate the efficiency of BNF (EF_{BNF}) in the field experiments, as proposed by McClure et al. (1980), Herridge (1982), and Herridge and Peoples (1990), using equation 1, where EF_{BNF} is given as a percentage, and ureides and nitrate are given in mM g^{-1}[dry matter of stem and petioles]. Constant 4 refers to the ratio of nitrogen atoms in an allantoin (ureide) molecule compared with a nitrate molecule, which is 4:1.

Eq.1
$$\mathrm{EF}_{\mathrm{BNF}} = \frac{4\times[ureides]}{(4\times[ureides])+[nitrate]}$$

1.2.3.3. Yield components

Seed yield was determined at the end of the crop cycle, harvesting 5.4 m^2 (4 meters in length from the three central rows) of each plot. Seeds were cleaned and weighed, and the yield estimated based on 13% moisture.

The seeds were also assessed for the thousand seed weight by measuring the mass of 100 grains randomly separated five times from the total sample of each plot.

1.2.4. Data analysis

Statistical analysis was performed using the software R Studio (R Core Team, 2013). Generalized linear mixed model were used through the *lme4* package (Bates et al. 2015) to compare the storage of pre-inoculated seeds and pesticides, which were considered fixed effects of the model. When the fixed effect factors showed difference or interaction between them ($p < 0.1$), the analysis was unfolded and compared using the least-squares means through the *emmeans* package (Searle et al. 1980, Lenth et al. 2019). Pairwise comparisons were made using Tukey method at 90% confidence level.

1.3. Results

1.3.1. Ponta Grossa (PR), crop season 2016/17 (PG 16/17)

The average yield in Ponta Grossa (PR) in the 2016/17 crop season was 4505 kg ha^{-1} and ranged from 4175 to 4802 kg ha^{-1}. Yield was not affected by the storage of pre-inoculated seeds or pesticide seed treatments. However, there was effect of storage on the thousand seed weight. The seeds originated from plants inoculated in the day of sowing were 3.6% heavier than those inoculated and stored for 30 days (Table 1).

Table 1. Effect of seed treatment with different pesticides and pre-inoculation before sowing on yield and thousand seed weight of soybean in Ponta Grossa (PR) in the 2016/17 crop season. Bold numbers represent the average of each treatment within the seed storage after pre-inoculation or pesticide seed treatment.

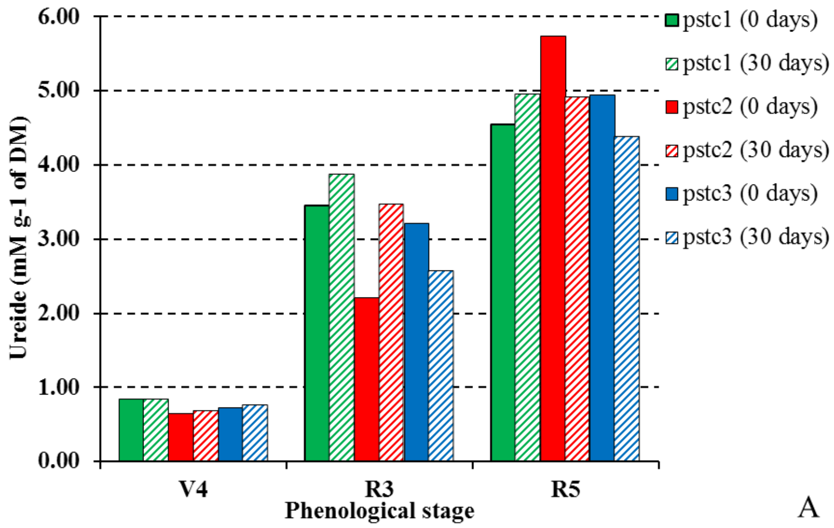
Pesticide•	Pre-inoculation		
	Yield (kg ha^{-1})		
	0 day	30 days	**Mean**
pstc1•	4285	4445	**4365**^{ns}
pstc2	4802	4569	**4686**
pstc3	4175	4754	**4464**
Mean	**4421**^{ns}	**4589**	
P values	Pstc (PS)=0.3037	Pre-inoc (PI)=0.3250	PS × PI=0.3441
	Thousand seed weight (g)		
	0 day	30 days	**Mean**
pstc1	217.7	211.0	**214.4**^{ns}
pstc2	225.4	207.4	**216.4**
pstc3	219.6	220.6	**220.1**
Mean	**220.9a***	**213.0b**	
P values	Pstc (PS)=0.5061	Pre-inoc (PI)=0.0421	PS × PI=0.1489

•pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram)
*Means followed by the same letter do not differ by the Tukey method (α=0.1)
^{ns}no significant difference

The concentration of ureides in V_4 ranged from 0.65 to 0.84 mM g^{-1}[dry matter], when plants from seeds treated with pstc1 (pyraclostrobin + thiophanate-methyl + fipronil) had higher concentration than pstc2 (thiabendazole + fludioxonil + metalaxyl-M). These values increased in R_3 and R_5 but did not differ among treatments (Figure 1A).

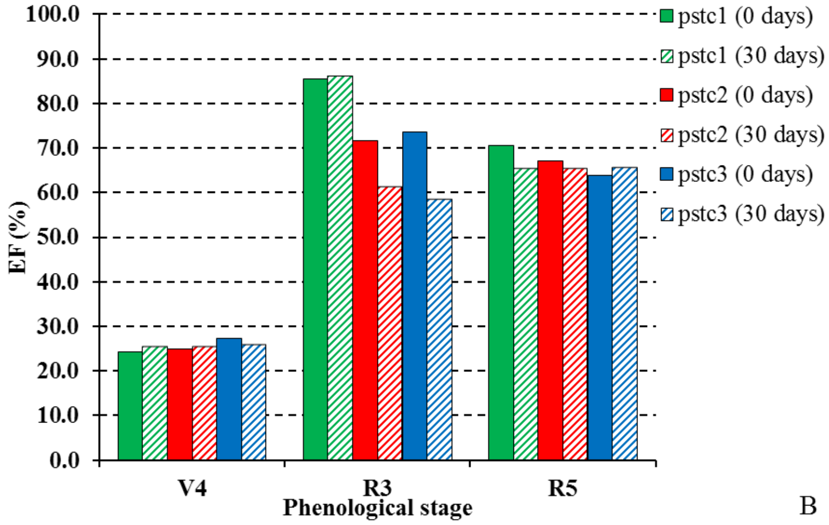
For the efficiency of BNF, the variation was 24.3 to 26.0% in V_4, 58.6 to 86.2 in R_3, and 63.8 to 70.6 in R_5 (Figure 1B). Differences between pesticides or pre-inoculation were observed only in R_3, when plants from seeds treated with pstc1 (pyraclostrobin + thiophanate-methyl + fipronil) had higher BNF efficiency than pstc2 (thiabendazole + fludioxonil +

metalaxyl-M) and pstc3 (carbendazim + thiram). Regarding the pre-inoculation, plants from seeds pre-inoculated 30 days before sowing had significantly lower BNF efficiency at R_3.



A

V_4: Pstc (p=0.0807); Pre-inoc (p=0.7071); Pstc x Pre-inoc (p=0.9572)
R_3: Pstc (p=0.3724); Pre-inoc (p=0.5188); Pstc x Pre-inoc (p=0.3527)
R_5: Pstc (p=0.5773); Pre-inoc (p=0.5649); Pstc x Pre-inoc (p=0.6427)



B

V_4: Pstc (p=0.6852); Pre-inoc (p=0.9380); Pstc x Pre-inoc (p=0.8209)
R_3: Pstc (p=0.0046); Pre-inoc (p=0.0946); Pstc x Pre-inoc (p=0.3958)
R_5: Pstc (p=0.4401); Pre-inoc (p=0.5261); Pstc x Pre-inoc (p=0.4703)

Figure 1. Concentration of ureides (mM g^{-1}) and BNF efficiency - EF (%) of soybean plants in the field experiment carried out in Ponta Grossa (PR) in the 2016/17 crop season, considering different seed treatment with inoculant and chemicals, for 0 or 30 days before sowing. Results represent the average of five plants per replication at the phenological stage V_4, and three plants at the phenological stages R_3 and R_5. pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram).

1.3.2. Piracicaba (SP), crop season 2016/17 (PI 16/17)

The average yield in Piracicaba (SP) (2016/17 crop season) was 3027 kg ha^{-1}, and ranged from 2634 to 3590 kg ha^{-1}, and there was significant interaction between pre-inoculation and pesticides. At 0 day of storage after inoculation, plants from seeds treated with pstc1 (Pyraclostrobin + thiophanate-methyl + fipronil) resulted in higher yield than those treated with pstc2 (thiabendazole + fludioxonil + metalaxyl-M) and pstc3 (carbendazim + thiram), although none differed from the pstc0 (control without chemicals). Plants from seeds pre-inoculated for 30 days associated to the chemical treatment pstc1 had significantly lower yield compared with the seeds treated in the day of sowing (Table 2). There was no effect of treatments on the thousand seed weight.

Table 2. Effect of seed treatment with different pesticides and pre-inoculation before sowing on yield and thousand seed weight of soybean in Piracicaba (SP) in the 2016/17 crop season. Bold numbers represent the average of each treatment within the seed storage after pre-inoculation and pesticide seed treatment.

Pesticide•	Pre-inoculation		
	Yield (kg ha^{-1})		
	0 day	30 days	**Mean**
pstc0•	3288abA**	3239aA	**3264**
pstc1	3590aA	2781aB	**3186**
pstc2	2634bA	2727aA	**2681**
pstc3	2888abA	3073aA	**2981**
Mean	**3100**	**2955**	
P values	Pstc (PS)=0.0277	Pre-inoc (PI)=0.3055	PS × PI=0.0669
	Thousand seed weight (g)		
	0 day	30 days	**Mean**
pstc0	176.5	167.2	**171.8^{ns}**
pstc1	167.9	167.2	**167.6**
pstc2	171.2	166.0	**168.6**
pstc3	157.2	167.1	**162.1**
Mean	**168.2^{ns}**	**166.9**	
P values	Pstc (PS)=0.6483	Pre-inoc (PI)=0.8097	PS × PI=0.6342

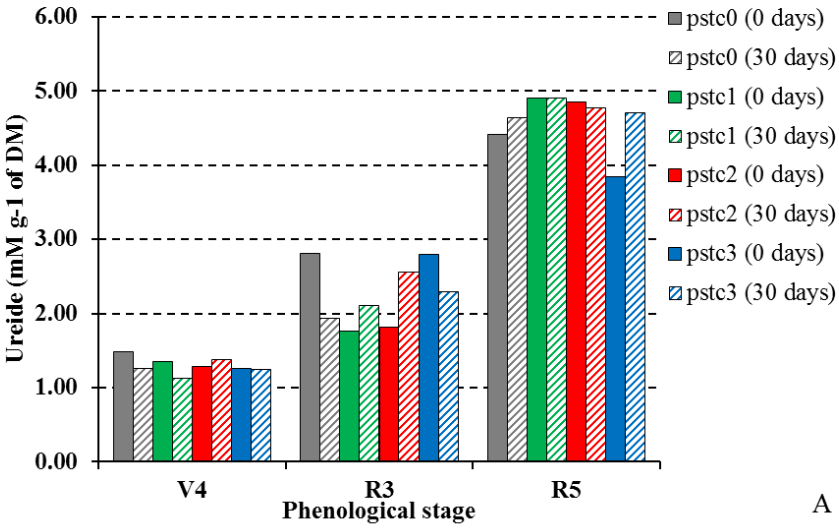
•pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram)

**Lowercase letters indicated statistical differences in the column, while uppercase letters in indicate it in the row. Means followed by the same letter do not differ by the Tukey method (α=0.1)

^{ns}no significant difference

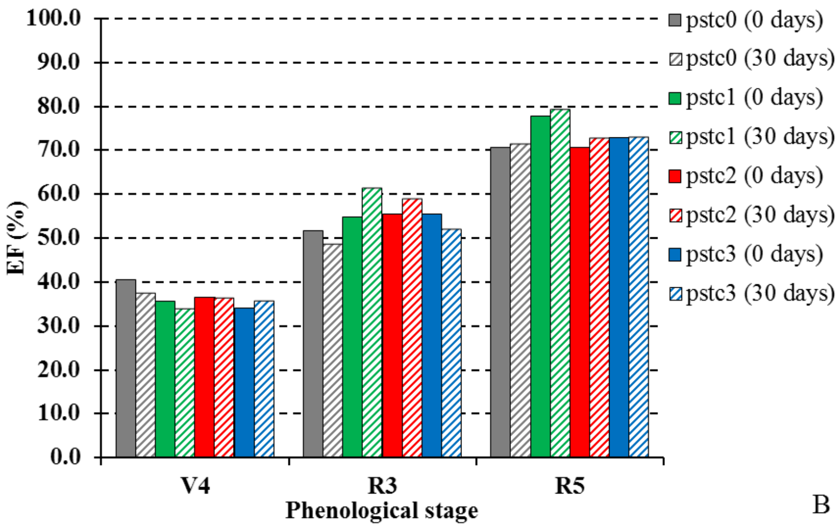
The concentration of ureides at V_4 ranged from 1.13 to 1.49 mM g^{-1}[dry matter], and increased in R_3 and R_5 ranging from 1.77 to 2.82 and 3.84 to 4.91, respectively (Figure 2A). There was no effect of treatments at V_4 and R_5, but interaction between factors occurred at R_3, when pre-inoculation for 30 days before sowing decreased the concentration of ureides when associated to seed treatments pstc0.

For the FBN efficiency, the variation was from 33.9 to 40.4% at V_4, 48.5 to 61.4 at R_3, and 70.6 to 79.2 at R_5 (Figure 2B). Significant differences were observed only at R_3. At this stage, ureides concentration in pstc1 was inferior to the treatment with inoculant alone (pstc0) in the storage period of 0 days, but not in 30 days (Figure 2).



A

V_4: Pstc (p=0.7167); Pre-inoc (p=0.3330); Pstc x Pre-inoc(p=0.5754)
R_3: Pstc (p=0.1760); Pre-inoc (p=0.7210); Pstc x Pre-inoc(p=0.0252)
R_5: Pstc (p=0.4903); Pre-inoc (p=0.4285); Pstc x Pre-inoc (p=0.7090)



B

V_4: pesticide (p=0.6455); pre-inoculation (p=0.7561); interaction (p=0.9261)
R_3: pesticide (p=0.3165); pre-inoculation (p=0.8213); interaction (p=0.6329)
R_5: pesticide (p=0.1391); pre-inoculation (p=0.6468); interaction (p=0.9934)

Figure 2. Concentration of ureides (mM g^{-1}) and BNF efficiency - EF (%) of soybean plants in the field experiment carried out in Piracicaba (SP) in the 2016/17 crop season, considering different seed treatment with inoculant and chemicals, for 0 or 30 days before sowing. Results represent the average of five plants per replication at the phenological stage V_4, and three plants at the phenological stages R_3 and R_5. pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram).

1.3.3. Piracicaba (SP), crop season 2017/18 with commercial rate of inoculant (PI-1 17/18)

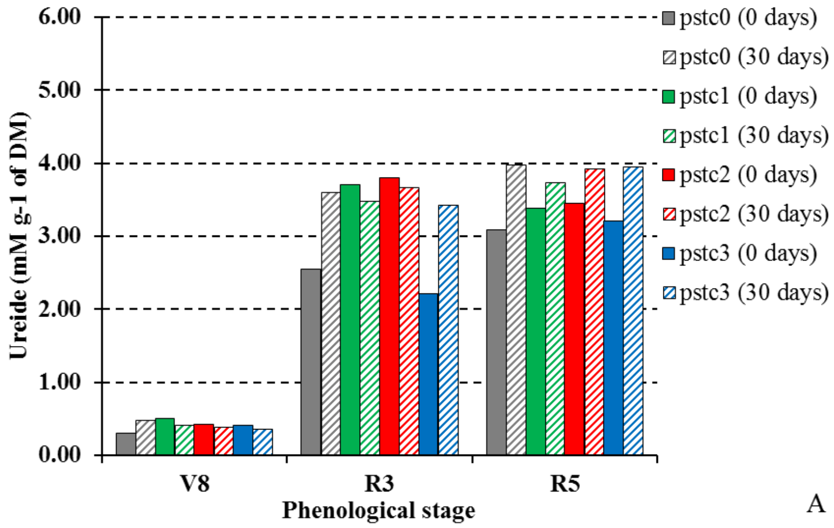
In the first experiment in the 2017/18 crop season in Piracicaba (SP), the average yield was 3481 kg ha^{-1}, ranging from 3137 to 3737 kg ha^{-1}. However, there was no effect of treatments on yield and thousand seed weight (Table 3).

Table 3. Effect of seed treatment with different pesticides and pre-inoculation before sowing on yield and thousand seed weight of soybean in Piracicaba (SP) in the 2017/18 crop season, with commercial rate of inoculant. Bold numbers represent the average of each treatment within the seed storage after pre-inoculation and pesticide seed treatment.

Pesticide•	Pre-inoculation		
	Yield (kg ha^{-1})		
	0 day	30 days	**Mean**
pstc0•	3737	3610	**3674^{ns}**
pstc1	3350	3405	**3378**
pstc2	3415	3680	**3548**
pstc3	3511	3137	**3326**
Mean ¤	**3505^{ns}**	**3458**	
P values	Pstc (PS)=0.1679	Pre-inoc (PI)=0.6956	PS × PI=0.2840
	Thousand seed weight (g)		
	0 day	30 days	**Mean**
pstc0	221.0	231.9	**226.4^{ns}**
pstc1	224.8	224.3	**224.6**
pstc2	223.8	222.8	**223.3**
pstc3	237.7	223.5	**230.6**
Mean	**226.8^{ns}**	**225.6**	
P values	Pstc (PS)=0.5563	Pre-inoc (PI)=0.7543	PS × PI=0.1644

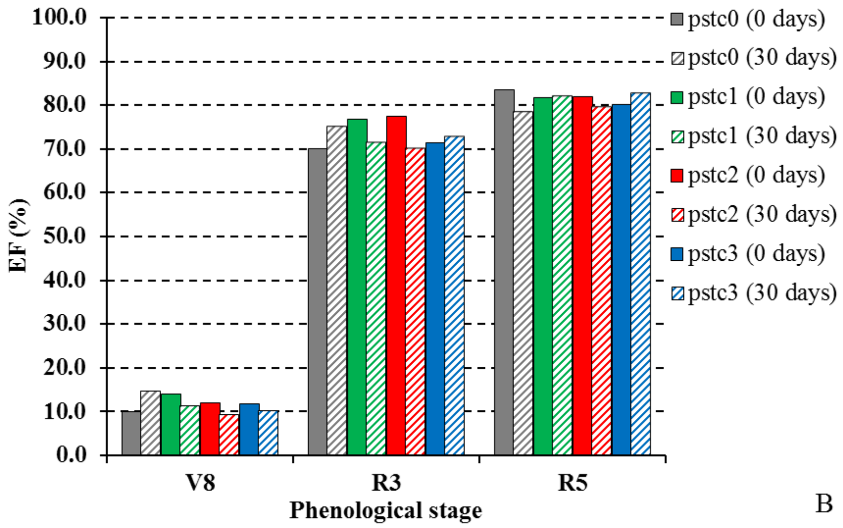
•pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram)
^{ns}no significant difference

The concentration of ureides at V_8 ranged from 0.31 to 0.50 mM g^{-1}[dry matter], and increased at R_3 and R_5, with values ranging from 2.22 to 3.80 and 3.08 to 3.97, respectively (Figure 3A). Significant differences were observed only at R_5, when the 30 days pre-inoculation of seeds resulted in higher concentrations of ureides than seeds just treated and sown (0 day), irrespectively of the chemical associated or the control without chemicals. For the efficiency of BNF, the variation ranged between 9.4 to 14.6% at V_8, 70.1 to 77.5 at R_3, and 79.5 to 83.5 at R_5 (Figure 3B). There was no effect of treatments on the efficiency of BNF.



A

V_8: Pstc (p=0.6419); Pre-inoc (p=0.9650); Pstc x Pre-inoc (p=0.1674)
R_3: Pstc (p=0.2783); Pre-inoc (p=0.2170); Pstc x Pre-inoc (p=0.3784)
R_5: Pstc (p=0.9752); Pre-inoc (p=0.0259); Pstc x Pre-inoc (p=0.8762)



B

V_8: Pstc (p=0.9098); Pre-inoc (p=0.4576); Pstc x Pre-inoc (p=0.2125)
R_3: Pstc (p=0.9098); Pre-inoc (p=0.4576); Pstc x Pre-inoc (p=0.2125)
R_5: Pstc (p=0.9823); Pre-inoc (p=0.5392); Pstc x Pre-inoc (p=0.5892)

Figure 3. Concentration of ureides (mM g^{-1}) and BNF efficiency - EF (%) of soybean plants in the field experiment carried out in Piracicaba (SP) in the 2017/18 crop season, with commercial rate of inoculant, considering different seed treatment with inoculant and chemicals, for 0 or 30 days before sowing. Results represent the average of five plants per replication at the phenological stage V_4, and three plants at the phenological stages R_3 and R_5. pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram).

1.3.4. Piracicaba (SP), crop season 2017/18 with twice the commercial rate of inoculant (PI-1 17/18)

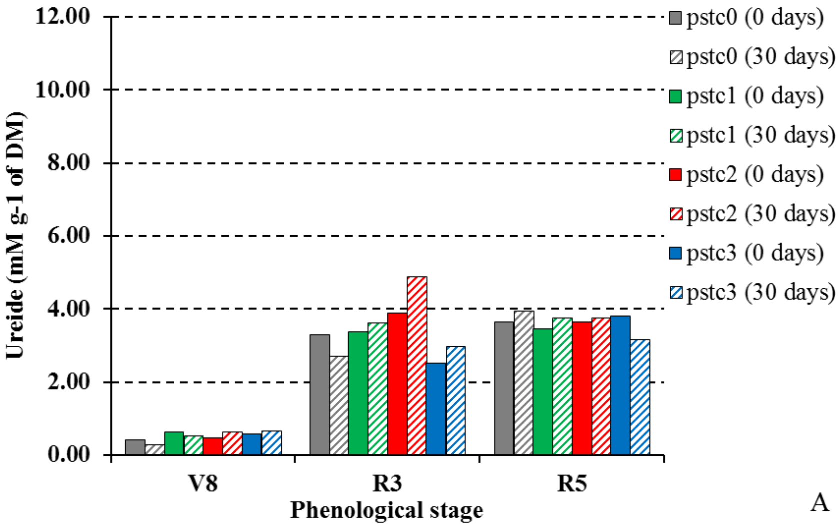
In the second experiment carried out in 2017/18 crop season in Piracicaba (SP), the average yield was 3371 kg ha^{-1}, ranging from 3001 to 3676 kg ha^{-1}. However, treatments had no effects on yield or thousand seed weight (Table 4).

Table 4. Effect of seed treatment with different pesticides and pre-inoculation before sowing on yield and thousand seed weight of soybean in Piracicaba (SP) in the 2017/18 crop season, with twice the commercial rate of inoculant. Bold numbers represent the average of each treatment within the seed storage after pre-inoculation and pesticide seed treatment.

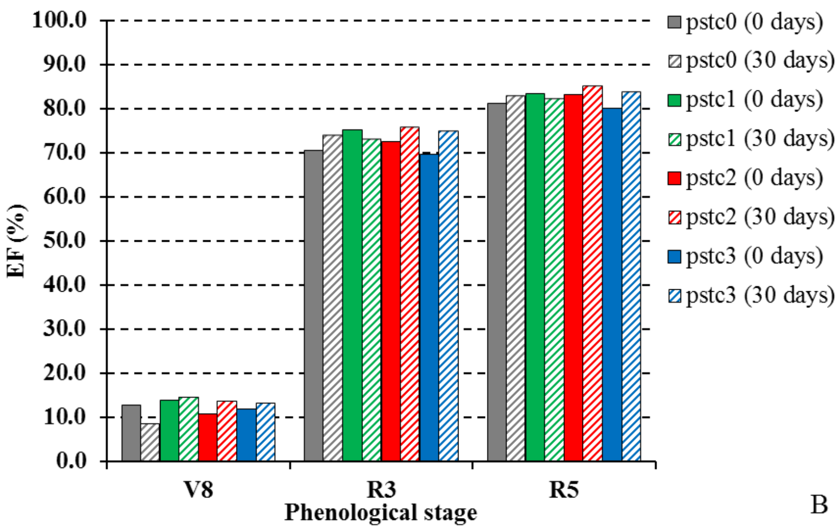
Pesticide•	Pre-inoculation		
	Yield (kg ha^{-1})		
	0 day	30 days	**Mean**
pstc0•	3676	3291	**3484**^{ns}
pstc1	3617	3291	**3454**
pstc2	3009	3265	**3137**
pstc3	3368	3449	**3409**
Mean	**3418**^{ns}	**3324**	
P values	Pstc (PS)=0.1398	Pre-inoc (PI)=0.4155	PS × PI=0.1512
	Thousand seed weight (g)		
	0 day	30 days	**Mean**
pstc0	219.4	227.4	**223.4**^{ns}
pstc1	239.8	222.8	**231.3**
pstc2	228.5	233.7	**231.1**
pstc3	230.0	237.2	**233.6**
Mean	**229.4**^{ns}	**230.3**	
P values	Pstc (PS)= 0.4049	Pre-inoc (PI)= 0.8502	PS × PI=0.1621

•pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram)
^{ns}no significant difference

The concentration of ureides at V_8 ranged from 0.28 to 0.66 mM g^{-1}[dry matter], and increased at R_3 and R_5 to ranges between 2.53 to 4.89 and 3.17 to 3.96, respectively (Figure 4A). There was effect of seed treatments in the assessments at V_8, when pstc0 was lower than all the treatments with chemicals, and at and R_3, when pstc2 was superior to the treatment with inoculant alone (pstc0) and associated with pstc3, irrespectively of the pre-inoculation (Figure 4A). For efficiency of BNF, the variation ranged between 8.6 to 14.6% in V_8, followed by an increase in R_3 and R_5, with ranges between 69.6 to 75.6, and 80.2 to 84.9, respectively (Figure 4B).



V_8: Pstc (p=0.0033); Pre-inoc (p=0.8537); Pstc x Pre-inoc (p=0.1360)
R_3: Pstc (p=0.0059); Pre-inoc (p=0.3974); Pstc x Pre-inoc (p=0.3799)
R_5: Pstc (p=0.7699); Pre-inoc (p=0.9352); Pstc x Pre-inoc (p=0.3870)



V_8: Pstc (p=0.4795); Pre-inoc (p=0.9005); Pstc x Pre-inoc (p=0.4382)
R_3: Pstc (p=0.8571); Pre-inoc (p=0.2670); Pstc x Pre-inoc (p=0.6373)
R_5: Pstc (p=0.7395); Pre-inoc (p=0.3731); Pstc x Pre-inoc (p=0.7047)

Figure 4. Concentration of ureides (mM g^{-1}) and BNF efficiency - EF (%) of soybean plants in the field experiment carried out in Piracicaba (SP) in the 2017/18 crop season, with twice the commercial rate of inoculant, considering different seed treatment with inoculant and chemicals, for 0 or 30 days before sowing. Results represent the average of five plants per replication at the phenological stage V_4, and three plants at the phenological stages R_3 and R_5. pstc0 (control without pesticide); pstc1 (pyraclostrobin + thiophanate-methyl + fipronil); pstc2 (thiabendazole + fludioxonil + metalaxyl-M); pstc3 (carbendazim + thiram).

1.4. Discussion

The average yield of the experiments ranged from 2947 to 4503 kg ha^{-1}, which represents a difference of -10 to + 34% in relation to the national averages for the respective crop seasons (Conab, 2019). The highest yield was observed in the experiment carried out in

Ponta Grossa (PR), a region that historically has the best national soybean yields because of a more favorable environment for soybean production (Franchini et al., 2016).

In three out of four experiments where three phenological stages were evaluated (PI 16/7, PI-1 17/18 and PI-2 17/18), R_5 presented the highest efficiency of BNF. These results corroborate previous reports describing the BNF peak between the R_3 and R_5 phenological stages (Zapata et al., 1987; Osborne and Riedell, 2011).

Yield is the main evaluated variable for field experiments, as it represents the final product of all factors that interact with the crop along its development in the field. Comparing the yields of plants which seeds were treated with inoculants, with or without chemicals (psct0, psct1, pstc2 and pstc3), at 0 or 30 days of storage before sowing for experiments carried out in Piracicaba (SP), the seed treatments with chemicals resulted in equal or less yields than the control without pesticides (pstc0) (Tables 2, 3 and 4). The yield was particularly hampered in the treatment with pyraclostrobin + thiophanate-methyl + fipronil (pstc1) due to storage of pre-inoculated seeds (Table1). Golden et al. (2016) observed that, in some cases, soybean yield inoculated with nitrogen-fixing bacteria may be higher with the use of pesticide-treated seeds when compared with the control without pesticide. The authors also conclude that the interaction between fungicides and inoculants is inconsistent and that it is difficult to define a pattern. However, in most cases, incompatible combinations between inoculant and chemicals in the seed treatment may decrease the plant yield potential because of negative effects on the BNF (Campo et al., 2009), especially when the treated seeds are stored before sowing.

Despite the fact of being an excellent management strategy for several diseases and pests (Dorrance and McClure, 2001; Urrea et al., 2013), the cost-effectiveness of using soybean seed treatment is a topic of increasing discussion, especially because of the large variations in yield results (Bradley, 2008). Rossman et al. (2018), testing combinations of fungicides, fungicides + insecticides, and insecticides + fungicides + nematicides over two crop seasons in seven different environments observed that the chemicals increased the plant stand in V_C/V_1 when compared with the control without any seed treatment. However, only fungicide + insecticide showed an increase on yield. The authors further demonstrated that although yield is correlated with plant stand ($r=0.16$, $p<0.0001$), the increase in plant stand resulted in increased yield in only one location and crop season when the control treatment plant population fell below 247,000 plants per hectare. This study showed statistical gain in yield comparing seed treatment with and without fungicides in only two out of 21 production environments studied (7 sites x 3 years). The results shown in Tables 2, 3 and 4 agree with the ones afore mentioned, where the control treatment without seed treatment was not statistically superior to the ones with seed treatment in any of the experiments. Nevertheless, it is also important to highlight that the fields where the experiments were carried out had no significant infestation of soilborne pathogens such as *Fusarium* sp. *Rhizoctonia* sp., or *Phytophthora* sp., which may be the cause for the lack of difference between seed treatment and the control without seed treatment.

There were differences between the factors or interaction between them regarding the yield components in experiments of the 2016/17. In addition, the results of yield and thousand seed weight were not consistent between treatments as the BNF-related assessments performed in this study. This is not surprising and has been often reported in studies related to BNF in soybean. Nodule number and yield are complementary measures in studies on BNF. For example, inoculation influenced soybean nodulation, but not yield (Sanginga et al., 2000). Conversely, in Brazil, inoculation increased yield but did not affect the nodulation parameters (Hungria et al., 1998). Even in situations where there are linear responses in the number and mass of nodules to increased inoculant rates, there may not be a corresponding effect on yield (Hungria et al., 2017) because several environmental factors will affect the final yield (Franchini et al., 2016).

Based on the results for yield components, no differences were observed for yield and thousand seed weight at the same time. In PG 16/17 (Table 1), there was a reduction in the thousand seed weight due to storage of pre-inoculated seeds for 30 days, but there was no reduction in yield. On the other hand, in PI 16/17 (Table 2) the storage of inoculated seeds for 30 days before sowing with pyraclostrobin + thiophanate-methyl + fipronil (pstc1) decreased yield. Araujo et al. (2017) found no differences in yield comparing the use of inoculant associated with pyraclostrobin + thiophanate-methyl + fipronil (pstc1) and storage of treated and pre-inoculated seeds for 0 and 30 days in four experiments carried out in soils with *Bradyrhizobium* population varying from 0 to 10^4 per g of soil. In addition, there was no reduction in the number and mass of soybean nodules in the vegetative phase. Although no differences were found on yield in the field trials, there was a reduction from 6.70×10^7 to 2.31×10^3 CFU seed^{-1} with the storage for 30 days before sowing, which would be technically below the recommended level to ensure good symbiotic performance under Brazilian conditions (Hungria et al., 2017).

1.5. Conclusion

This study demonstrated that pre-inoculation of soybean seeds for 30 days associated with pesticides used in seed treatments may negatively affect the BNF in soybean even in areas with an established population of *Bradyrhizobium* spp. Nevertheless, seed treatment and storage reduced yield only in one out of four experiments, and in this case only for pyraclostrobin + thiophanate-methyl + fipronil (PI 16/17). Furthermore, seed treatment did not increase yield in any of the experiments when compared to control treatment without pesticides.

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2. SOYBEAN SEED TREATMENT: HOW DO FUNGICIDES TRANSLOCATE IN PLANTS?

Abstract

Soybean seed treatment with fungicides is a well-established disease management strategy. However, the movement of these fungicides within seedlings is not always well characterized. Thus, the objectives of this study were to determine the pattern of translocation of three fungicides with different modes of action applied as a seed treatment, and the effect of soil type on translocation. Most of the absorbed radioactivity was concentrated in the cotyledons and the maximum sum of the rates of absorption by roots, stems, and leaves of the plants was 15%. In most cases, absorption by roots, stems, and leaves were lower than 5% for 14C-pyraclostrobin and 14C-metalaxyl, and 1.6% for 14C-carbendazim. Fungicides absorbed by the roots and the whole seedlings were higher when plants were grown in soil with lower organic matter content. Fungicides in the cotyledons are unlikely to be redistributed and are lost when cotyledons fall off the plants. Cotyledons are the part of the plant where fungicides are most absorbed, regardless of the fungicide. Soil type affects the absorption of fungicides, and in this study it was most likely caused by soil organic matter. These data improve knowledge of the movement of seed treatment fungicides in soybean seedlings and may help the development of seed treatment chemistry to manage seed and soilborne pathogens.

Keywords: Radiolabeled, *Glycine max*, Disease management, Translocation, K_{ow}

2.1. Introduction

Fungicide seed treatment is a disease management practice used to protect germinating seeds from seedborne and soilborne pathogens. The first systemic fungicide seed treatment, carboxim, was introduced in United States (U.S.) only in the 1970's (Crop Life Foundation, 2013). Fungicide seed treatment reduces infection of both seed borne and soil borne pathogens of seed and seedlings, including *Diaporthe* sp., *Fusarium* sp., *Rhizoctonia* sp., and *Phytophthora* sp. and thereby mitigate stand loss and protect the health of seedlings (Dorrance and McClure, 2001; Bradley, 2008; Ellis et al., 2011; Urrea et al., 2013; Weems et al., 2015; Prochazka et al., 2015; Rossman et al., 2018). Phytophthora root rot, for example, was estimated to cause more than two million ton in yield losses in the eight top soybean producing countries around the globe, while damping-off caused by *Rhizoctonia* sp. was estimated over one and a half million tons (Wrather et al., 2010).

Currently, many different groups of fungicides have been used in soybean seed treatment, to control both seed and soilborne pathogens. Among these, benzimidazoles (which affects β-tubulin assembly, and affects mitosis and cell division), strobilurins (which inhibits complex III in the mitochondrion [bcI complex], and affects respiration), and phenylamides (which inhibits RNA synthesis, and affects protein production) (Oliver and Hewitt, 2014) represent important molecules for fungicide seed treatment. Benzimidazoles, for example, thiabendazole, carbendazim, and thiophanate-methyl, are effective against a wide range of ascomycetes and basidiomycetes, but not against oomycetes. While, phenylamides (e.g. metalaxyl and mefenoxam) are effective exclusively against oomycetes, strobilurins (e.g. pyraclostrobin, azoxystrobin, and trifloxystrobin) are effective against some members of ascomycetes, basidiomycetes and oomycetes (Oliver and Hewitt, 2014). In addition to the mode of action of each active ingredient, other physiochemical characteristics may contribute to the classification of fungicides, such as the log K_{ow} (as known as log *P*), which indicates the partition coefficient between n-octanol and water and measures hydrophobicity/hydrophilicity of the molecule (Oliver and Hewitt, 2014).

Although seed treatments have increased in use in the past decade (Munkvold, 2009; Munkvold et al., 2014; França-Neto et al., 2015), especially with adoption of newer and more accurate seed treatment application machinery, there is limited information on how the fungicides applied to the seed translocate in the plants, the same is also true for how fungicides may interact with the soil. The soil component, such as organic matter and clay minerals, may directly affect the amount of pesticides absorbed by plants (Singh et al., 1989; Stougard et al., 1989; Locke and Bryson, 1997; Wang et al., 1999 Guimarães et al., 2018). Notably, most of research to date has been focused on herbicides, which directly impacts the recommendation of which active ingredient to use based not only in weed control, but also on molecule characteristics (e.g. K_{ow}), soil properties and precipitation (Ross and Fillols, 2017). Such information could lead to a better understanding of how seed treatments reduce infection from soil borne pathogens and allow for their targeted usage in a sustainable manner.

The translocation of pesticides in plants can be done measuring directly the active ingredient or, indirectly, spraying the molecule in one part of the plant and assessing the development of pathogen in another part of the same plant (He et al., 2017). Direct methods involve the extraction of the active ingredient for plant tissues and quantification through spectrometry, which can be time consuming (Camargo et al., 2019). Direct measures of the translocation of fungicides in plants can also be made using the radiolabeled technique (Jablonkai and Dutka, 1986; Alsayeda et al., 2008; Liu et al., 2018). The method consists in the use of an active ingredient which one of the carbons (usually the most stable one) is the radioactive isotope 14C.

Pesticide seed treatment absorption and translocation has been poorly studied along the past decades when compared to foliar applications. O'Neill et al. (1979), studying the absorption and translocation of 14C-ethazol in soybean seedlings, showed that most of the fungicide absorbed was concentrated in the cotyledons, and a possible translocation of the fungicide absorbed by them over time. Similar results were found by Stamm et al. (2016), studying three radiolabeled neonicotinoid insecticides. Moreover, the authors also showed that total absorption of insecticide seed treatment by the plants was lower than 25%.

The objectives of the present study was to determine i) the pattern of translocation of three fungicides, carbendazim, pyraclostrobin and metalaxyl, in soybean seedlings when applied as a seed treatment and ii) if soil organic matter can limit the absorption and movement of these fungicides.

2.2. Material and Methods

In order to assess the pattern of translocation of fungicides applied as seed treatment in soybean seedlings, three experiments using radiolabeled molecules were performed at the Center for Nuclear Energy in Agriculture (Cena), University of São Paulo (USP), Piracicaba, São Paulo State (SP), Brazil.

2.2.1. Experiment I – Movement of pyraclostrobin in a commercial formulation

In the first experiment, soybean seeds treated with 14C-pyraclostrobin were planted into sandy soil (10 g dm^{-3} of organic matter). The radiolabeled molecule of pyraclostrobin diluted in methanol, and radioactivity purity equal to 95%, was added to the commercial product Standak Top® (pyraclostrobin + thiophanate-methyl + fipronil) plus colorant polymer (Polyplus®). The mix of 14C-pyraclostrobin + Standak Top® + Polyplus® was applied to fifty soybean (cv. TMG 7062 RR2 IPRO) seeds placed in a 250 mL high density polyethylene flask by gently mixing until all seeds were uniformly covered with the treatment.

A day after the treatment, one seed per 500 mL pot was planted for a total of ten pots o and grown in greenhouse exclusively dedicated for radioactive materials, with sprinkler irrigation with deionized water. To determine the maximum amount of radiation that each plant could absorb, the radioactivity of seven seeds was measured using the method described below. Additionally, the testa and embryo from another 7 seeds was evaluated separated so radioactivity could be determined in both parts. Fourteen days after planting (DAP), three plants were dug up, had their roots washed, and dried in a continuous hot and dry air flow chamber for 72 h at 45^{o}C. The radioactivity from roots, hypocotyl + cotyledons, epicotyl and leaves of each plant was determined after burning them in a biological oxidizer (OX 600 Harvey Instruments Crop. Hillsdale, NJ, USA), which traps 14C in plants into a scintillation solution vial. The vials were then placed in a scintillation counter (Packard 1900 TR) and values were expressed in Becquerel (Bq).

2.2.2. Experiment II – Comparison of fungicides

A second experiment compared the translocation of the three fungicide molecules used in seed treatment. 14C-pyraclostrobin (k_{ow}=3.99; H_2O solubility=1.9 mg L^{-1}; radiochemical purity=95%) diluted in methanol, 14C-carbendazim (k_{ow}=1.52; H_2O solubility=8 mg L^{-1}; radiochemical purity=94%) diluted in methanol, and 14C-metalaxyl (k_{ow}=1.65; H_2O solubility= 8,400 mg L^{-1}; radiochemical purity=94%) diluted in acetonitrile, were added to commercial products for seed treatments (Standak Top® - pyraclostrobin + thiophanate-methyl + fipronil; Maxim Advanced® - thiabendazole + fludioxonil + metalaxyl; Derosal Plus® - carbendazim + thiram, respectively) and colorant polymer (Poliplus®). In this manner, each 14C-fungicide was mixed to its correspondent commercial seed treatment. The mix of 14C-fungicides + commercial seed treatment + Polyplus was applied to a hundred soybean (cv. TMG 7062 RR2 IPRO) seeds placed in a 250 mL high density polyethylene flask by gently mixing until all seeds were uniformly covered with the treatment.

A day after treatment, for each radiolabeled fungicide, 32 treated seeds were planted in 32 150 mL pots (1 seed per pot) filled with an organic substrate (71 g dm^{-3} of organic matter) and grown in greenhouse exclusively dedicated for radioactive materials all at the same time. The pots were placed in containers with deionized water, so that the water demand was supplemented according to the evapotranspiration of each pot. Pots were organized in a completely randomized design. For each radiolabeled fungicide, ten seeds were evaluated for the maximum amount of radioactivity as described above.

For each radiolabeled molecule, five treated plants were removed from the pots and the roots were washed at 16 DAP, and dried in a continuous hot and dry air flow chamber for 72h at 45^{o}C. The radioactivity from roots, cotyledons, unifoliate leaves, trifoliate leaves, and stem of each plant was determined after burning them in a biological oxidizer (OX 600 Harvey Instruments Crop. Hillsdale, NJ, USA), which traps 14C in plants into a scintillation solution vial. The vials were then placed in a scintillation counter (Packard 1900 TR) and values were expressed in Bq.

2.2.3. Experiment III – understanding the relationships between soil organic matter and fungicides

The third experiment evaluated if two different soils with different levels of organic matter could affect the distribution of two fungicides with different K_{ow}. Soybean (cv. TMG 7062 RR2 IPRO) seeds from experiment II and treated with 14C-pyraclostrobin and 14C-metalaxyl were planted in

500 mL styrofoam cups (1 seed per pot) filled with organic substrate (71 g dm^{-3} of organic matter) or sand (5 g dm^{-3} of organic matter), and grown in greenhouse exclusively dedicated for radioactive materials all at the same time, with sprinkler irrigation with deionized water. The experiment followed a 2x2 factorial scheme with two fungicides and two types of substrate, with 7 replications in a completely randomized design.

For each combination of fungicide vs. substrate, seven treated plants were removed from the cups and roots were washed at 18 DAP, and dried in a continuous hot and dry air flow chamber for 72 h at 45^{o}C. The radioactivity from roots, cotyledons, unifoliate leaves, trifoliate leaves, and stem of each plant was determined as described in experiment II, with values expressed in Bq.

2.2.4. Data analysis

Data from experiment II and III were analyzed using the free software R Studio (R Core Team, 2013). Values of Bq where transformed as percentage based on the seed radioactivity for each fungicide, and then transformed using the function asin(√(x/100)). Generalized linear mixed model was used through the *lme4* package (Bates et al. 2019). When differences inside or between factors were significant, results were analyzed by the least-squares means using the *emmeans* package (Searle et al. 1980, Lenth et al. 2019). Pairwise comparisons were made using Tukey method at 90% confidence level.

2.3. Results and Discussion

The first experiment evaluated the effect of one fungicide pyraclostrobin in a commercial formulation planted in sand soil. The amount of 14C-pyraclostrobin added to Standak Top® + Poliplus® was enough to result in a total of 1667 Bq per seed. However, after treating the seeds inside the high density polyethylene flask, the mean value from seven seeds was 596 Bq per seed. The seed treatment was concentrated on seed testa (seed coat), and only insignificant values of radiation was recovered from seed embryo (cotyledons +

embryonic axis) (Table 1). The results from the 14 DAP plants on experiment I indicated that most of the radiation recovered was concentrated on the cotyledons and less than 50% of the radiation was recovered from the whole plant (Table 1).

Table 5. Mean percentage of radiation (100%=596Bq) from 14C-pyraclostrobin* recovered from seeds (mean of 7 seeds) immediately following treatment and plants (mean of 3 plants at 14 DAP in sandy soil) on Experiment I.

seed		plant				
testa	embryo	roots	hypocotyl + cotyledons	epicotyl	leaves	total
96.9%	3.1%	1.5%	44.9%	0.4%	0.7%	47.5%

*14C-pyraclostrobin + Standak Top® + Poliplus®

In the second experiment, the translocation between three fungicides (carbendazim, pyraclostrobin and metalaxyl) was compared. All parts of the plants showed significant differences among the fungicides, except the roots (Table 2). As in the first experiment, cotyledons had the highest levels of radiation. Similarly, less than 50% of radiation absorbed was detected in the plant when compared to initial values from the seed. The amount of 14C-pyraclostrobin fungicide mixture absorbed in the whole plant was higher than 14C-metalaxyl and 14C-carbendazim.

Table 6. Mean percentage of radiation of 14C- carbendazim (100%=832 Bq), 14C-metalaxyl (100%=1417 Bq) and 14C-pyraclostrobin (100%=689 Bq) recovered from plants (mean of 5 plants at 16 DAP in soil mix).

Fungicide	root[1]	stem[2]	cotyledon[3]	unifoliate leaves[4]	1st trifoliate leaf[5]	total[6]
carbendazim	0.4%a	0.7%b*	10.0%b	0.4%c	0.1%b	11.6%b
metalaxyl	0.5%a	0.7%b	11.2%b	1.2%a	0.2%a	13.8%b
pyraclostrobin	0.9%a	1.3%a	29.5%a	0.8%b	0.2%a	32.6%a

[1] $p=0.1298$
[2] $p=0.0003504$
[3] $p=0.0003433$
[4] $p=0.0003902$
[5] $p=0.0221$
[6] $p= 0.0003018$
* Means followed by the same letter do not differ by the Tukey method ($\alpha=0.1$)

The last experiment compared how soil types may influence the amount of fungicide absorbed by the plants, using pyraclostrobin and metalaxyl. An increase in soil organic matter affected fungicide absorption for all parts of the plants but the first trifoliate leaf (Table 3). Roots of plants grown in sand absorbed more 14C-pyraclostrobin than 14C-metalaxyl, but there was no difference for those grown in organic substrate with a higher content of soil organic matter. Similar to both previous experiments, cotyledons had higher levels of fungicide compared to other parts of the plant. Nevertheless, pyraclostrobin had a reduction on the amount of radiation in the cotyledons when soil organic matter content increased. Unifoliate leaves of plants grown in sand had a greater amount of radiation regardless the fungicide. The first true leaves also had a higher value of 14C-metalaxyl than 14C-pyraclostrobin, which differs from the result shown for the first trifoliate leaf. Similar to roots, stem + petioles of plants grown in sand absorbed more 14C-pyraclostrobin than 14C-metalaxyl, but no difference was found when plants were grown in organic substrate with a higher content of soil organic matter. Finally, for the whole plant, 14C-pyraclostrobin had higher rates than 14C-metalaxyl regardless the soil type. Moreover, soil type affected the amount of 14C-pyraclostrobin absorbed by the whole plants, but did not affect 14C-metalaxyl (Table 3).

Table 7. Percentage values of radiation of 14C-metalaxyl (100%=1417 Bq) and 14C-pyraclostrobin (100%=689 Bq) recovered from plants (mean of 7 plants at 18 DAP) on Experiment III.

roots[1*]				cotyledons[2*]			
	sand	substrate			sand	substrate	
metalaxyl	1.4%bA	0.9%aB		metalaxyl	15.7%bA	14.8%bA	
pyraclostrobin	7.0%aA	0.9%aB		pyraclostrobin	48.8%aA	32.8%aB	
first trifoliate leaf[3•]				unifoliate leaves[4•]			
	sand	substrate			sand	substrate	
metalaxyl	0.2%	0.2%	**0.2%b**	metalaxyl	1.8%	1.1%	**1.5%a**
pyraclostrobin	0.5%	0.4%	**0.4%a**	pyraclostrobin	0.9%	0.5%	**0.7%b**
	0.4%ns[◊]	**0.3%**			**1.4%a**	**0.8%b**	
stem + petioles[5*]				total[6*]			
	sand	substrate			sand	substrate	
metalaxyl	1.1%bA	1.3%aA		metalaxyl	20.3%bA	18.4%bA	
pyraclostrobin	3.3%aA	1.5%aB		pyraclostrobin	60.5%aA	36.0%aB	

[1]molecule ($p=1.819e^{-06}$); soil ($p=3.639e^{-08}$); molecule x soil ($p=2.001e^{-06}$)
[2]molecule ($p=1.873e^{-07}$); soil (p=0.02788); molecule x soil (p=0.03816)
[3]molecule (p=0.001323); soil (p=0.459234); molecule x soil (p=0.375040)
[4]molecule ($p=3.403e^{-07}$); soil ($p=1.371e^{-05}$); molecule x soil (p=0.9521)
[5]molecule (p=0.0003237); soil (p=0.0458875); molecule x soil (p=0.0060907)
[6]molecule (p=3.111e-07); soil (p=0.003622); molecule x soil (p=0.010052)
[*]Numbers in bold are the mean of each level in each factor. Values followed by the same letter were not statistically different by LSD test (α=0.1)
[•]Values followed by the same lowercase letter were not statistically different in the columns by the Tukey test (α=0.1); values followed by the same uppercase letter were not statistically different in the lines by the Tukey test (α=0.1)
[◊]ns=no significant difference

These results reveal some of the characteristics about the application of products in soybean seeds.

The majority of the radioactivity absorbed by the plants for all the molecules evaluated was concentrated in the cotyledons (Tables 1, 2 and 3). These results also agree with Gupta et al. (1985), which showed that most part of 14C-metalaxyl was retained by the cotyledons when applied by seed treatment in soybeans. In that same study, soil drench and seed treatment application were compared. Results show that, although soil drench allowed the roots to absorb higher percentages of 14C-metalaxyl, the total amount of radiation recovered by the plants was lower than those with seed treatment. Another fungicide well-known for oomycetes management as a soybean seed treatment, ethaboxam (k_{ow}=2.89; H_2O solubility=12.4 mg L^{-1}) also has the same pattern of translocation showed by the fungicides used in this study, with most of the active ingredient concentrated in the cotyledons of soybean plants 14 days after planting (D. McDuffee, personal communication, February 26, 2019). This is of concern for two reasons. Firstly, the root tissues are the primary target for soilborne pathogens thus the fungicide is not predominantly in the tissues that need protection. In most cases, less than 2% of the fungicides were found in the roots (Tables 1, 2 and 3). Secondly, the cotyledons senesce from the seedlings very early in their growth, soon after the first true leaves emerge and thus the fungicide would be lost. It is also possible that the molecules were absorbed by the cotyledons at the time of seed imbibition, not through transpiration flow. Further work is needed to investigate this possibility.

All fungicides in this study had absorption values by the plants lower than 50% in most cases (Tables 1, 2 and 3). Thus, it is expected that some of the products would have remained in the soil close to the seed and also in the testa. In experiment I, we were able to recover testa fragments from the soil where up to 29.2% of 14C-pyraclostrobin was found, while in experiment III, pieces of testa with up to 8.5% of 14C-pyraclostrobin was found.

Moreover, the distribution of 14C labelled fungicides in soybean roots was different than expected, since the fungicides were concentrated in the cotyledons and hypocotyl regions. This suggests that protection of root tissues may not be as efficient as previously thought. Additionally, a higher soil organic matter content may decrease the amount of fungicides on the roots, with higher reduction rates for fungicides with higher K_{ow} values (Table 3).

The difference in absorption of fungicides into the roots in in two different soils suggests that a better understanding about the interactions of these products with the soil is definitely an important step to improve seed treatment. In this study, we believe that a difference in soil organic matter content (5 g dm^{-3} in sand to 71 g dm^{-3} in organic substrate) may be the main responsible for the differences found between the two soils for both fungicides tested. Considering the interaction of these molecules with other components of the soil, few studies showing interactions of fungicide seed treatments with clay minerals are available (Liu et al., 2018). This knowledge could improve the control of soilborne pathogens.

When soil organic matter content was low (sand - 5 g dm^{-3} of organic matter), pyraclostrobin was more absorbed by the roots of the plants than metalaxyl. The later active ingredient is known for increasing its mobility on soils with low organic matter content (Sharom and Edginton, 1982). Therefore, metalaxyl was probably leached from the root zone, differently from pyraclostrobin, which has a higher K_{ow} and bounds stronger to soil organic matter than metalaxyl, and thus have a higher soil adsorption (Shareef and Hamadamn, 2009; Cabrera et al., 2014).

2.4. Conclusion

This study was able to show that the total amounts of fungicides absorbed by seedlings are frequently less the half of what is present on the seed, and most of the amount absorbed was concentrated in the cotyledons, not in seedling roots. Moreover, soil type affects the amount of fungicide absorbed by the plants for both lipophilic and hydrophilic molecule, and this effect includes the amount of fungicides in the roots.

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3. FUNGICIDE SEED TREATMENT LONGEVITY AND CONTROL OF *Phytophthora sojae*

Abstract

Phytophthora root and stem rot, caused by *Phytophthora sojae,* has been a constant threat to soybean production worldwide. The management of this pathogen relies in a combination of tools, such as genetic resistance and seed treatment. Fungicides applied on the seed assure plant emergence and protect it on the beginning of its development. Nevertheless, the exact amount of days which fungicide seed treatment is an effective weapon against Phytophthora root and stem rot. This way, the objective of this study was to determine the effectiveness of different fungicides seed treatment along soybean development. In order to do so, two greenhouse and two growth chamber experiments were carried out. Soybean was inoculated with zoospores of *Phytophthora sojae* at different moments of plant development, beginning on planting day until 21 days after planting. Plant emergence, root rot score and plant biomass were evaluated. Plant emergence was only affected when zoospores were inoculated at the planting day. Overall, all fungicides were effective on controlling *Phytophthora sojae*. A new fungicides tested, herein called compound A, showed the best efficiency on controlling the pathogen due to its low rate of usage. Moreover, fungicides were had a satisfactory control of the pathogen until 14 days after planting.

Keywords: *Glycine max*, Phytophthora root and stem rot, Zoospores, Pesticides

3.1. Introduction

Phytophthora root and stem rot of soybeans, caused by *Phytophthora sojae* (Kaufmann and Gerdemann [synonyms: *Phytophthora megasperma var. sojae*, *P. megasperma f. sp. glycinea*, and *P. sojae f. sp. glycines*]) is a common problem in soils that are poorly drained and have a tendency to stay saturated for long periods of time. This disease strikes soybean in areas all around the world. In the US, losses of 100% loss have been reported, and it is estimated that the pathosystem may be responsible for a loss over 1 million tons of soybean around the world during years with higher than average rainfall (Wrather and Koenning, 2009; Schmitthenner and Dorrance, 2015; Allen et al., 2017; Dorrance, 2018).

This soilborne disease is essentially monocyclic. *Phytophthora sojae* produces sexual oospores in root tissue following infection, that can survive in the soil (Schmitthenner, 1985; Dorrance et al., 2007), and as a result, plants grown in fields infested with *P. sojae* are at constant risk. Under saturated soil conditions, oospores germinate and form sporangia. Sporangia may directly infect the roots, or through the motile asexual zoospores. The zoospores are chemically attracted to the roots, encyst and form a germination tube and an appressorium prior to the infection and colonization of the root (Morris and Ward, 1992; Erwin and Ribeiro, 1996).

Symptoms of this disease are reduced plant population early in the season through pre and post emergence damping off. At later growth stages, the characteristic diagnostic symptom on soybean is a dark brown lesion beginning in the root that can extends up the stem. Consequently, the plants wilt and die (Schmitthenner, 1985; Dorrance et al., 2009; Dorrance, 2015).

The primary strategies to manage Phytophthora root and stem rot are resistant cultivars (Rps genes and quantitative resistance), and seed treatment with systemic fungicides (Schmitthenner and Dorrance, 2015; Dorrance, 2018) Numerous pathotypes of *P. sojae* have been reported so far (Kaitany et al., 2001; Dorrance et al., 2003, 2016) , and therefore, many Rps genes has been shown ineffective depending on the populations of P. sojae (Schmitthenner and Bhat, 1994; Yang et al., 1996; Abney et al., 1997; Leitz et al., 2000; Dorrance et al., 2003; Dorrance et al., 2016). Thus, fungicide seed treatment is necessary even with resistant cultivars. The use of fungicides to control Phytophthora root and stem rot in fields with high disease pressure is a useful technique for both moderate susceptible and resistant cultivars, when compared to the non-treated control (Dorrance and McClure, 2001; Dorrance et al., 2009) thus soybeans grown in these high disease-risk environments should be treated with at least one active ingredient available in the market against oomycetes in their seed treatment mix (Dorrance, 2018).

Systemic fungicides are absorbed actively or passively by roots, stems, leaves, and flowers, and can be translocated to other parts of the plants. The translocation may be through the leaf (translaminar), new tissues in the upper part (apoplastic) or lower part (symplastic) of the plants. Most fungicides translocate trough the transpiration stream in the xylem (Oliver and Hewitt, 2014).

The development of systemic fungicides to control oomycetes began in the 1970’s (Cohen and Coffey, 1986; Oliver and Hewitt, 2014) with cymoxanyl, followed by metalaxyl, furalaxyl, ofurace, oxadixyl, and fosetyl-AL (Erwin and Ribeiro, 1996).

Metalaxyl and its enantiomer, mefenoxam (also known as metalaxyl-M), have probably been the most studied fungicide for oomycetes management on soybeans. Guy et al. (1989) have shown that, when applied as an in-furrow or seed treatment, the addition of metalaxyl improved stands and increased yields of susceptible soybean cultivars when P. sojae was present and the environmental conditions favored the disease. Metalaxyl, which belongs to the phenylamide group, is a ribosomal RNA inhibitor and interrupts the protein synthesis. The initial phases of Phytophthora root and stem rot (germination and early infection) present low sensitivity to this group of fungicides because zoospores have enough ribosomes which consequently allow the initial development of the pathogen (Muller and Gisi, 2007; Oliver and Hewitt, 2014).

To a lesser extent, pyraclostrobin has also been shown to be effective against oomycetes (Radmer et al., 2017). Pyraclostrobin belongs to the quinone outside inhibitor group, which inhibits the electron transport on complex III (complex bc1) of the mitochondrial electron transport chain (Oliver and Hewitt, 2014).

More recently, two new fungicide seed treatments have been shown also effective against oomycetes, ethaboxam (Dorrance et al., 2012; Radmer et al., 2017) and oxathiapiprolin (Miao et al., 2016). Ethaboxam is a benzamide, which affects the microtubes and consequently the cellular division exclusive in oomycetes (Oliver and Hewitt, 2014). Oxathiapiprolin inhibits an oxysterol binding protein (OSBP) homologue. Oxysterol binding proteins are implicated in the movement of lipids between membranes, among other processes. Inhibiting OSBP may disrupt other processes in the fungal cell, such as signaling, maintaining cell membranes, and the formation of more complex lipids that are essential for the cell to survive (FRAC, 2019).

The objective of this study was (i) to compare the efficacy of a new compound A to control *Phytophthora sojae* to other fungicides; (ii) to assess the longevity of each fungicide seed treatment from seed to early growth stages of soybean plants.

3.2. Material and Methods

3.2.1. Zoospore production

One isolate of *P. sojae* (OH25) from the collection of the Soybean Pathology Laboratory at The Ohio State University was used. Long-term storage of isolates was in 10% sterile glycerol in cryovials in liquid nitrogen (Tooley, 1988). Zoospores were produced via a method previously described by Qutob et al. (2000). Briefly, five plugs (6 mm) of 3 days old culture were transferred onto non-clarified V8-juice agar plates. Four days later plates were flooded with 15 ml of sterile deionized water (pH 6 to 7) for approximately 14 to 18 hours. Water in plates was then removed and fresh water added every 30 min for a total of 5 times, with a final incubation for 3 h at 26°C. Zoospores were collected and counted with a hemacytometer.

3.2.2. Experiments

Four experiments were carried during December 2018 through March 2019, two in greenhouse (experiment I and II) and two in growth chamber (experiments III and IV).

For experiment I, II and III, 10 soybean seeds were planted in coarse vermiculite (Therm-O-Rock East, New Eagle, PA) in 250 mL styrofoam cups. Seed treatments in this study included untreated control and five fungicides, mefenoxam (0.0113 mg a.i./seed), compound A (0.0038 mg a.i./seed), pyraclostrobin (0.0118 mg a.i./seed), ethaboxam (0.0120 mg a.i./seed) and oxathiapiprolin (0.0120 mg a.i./seed). To obtain seedlings of different ages for inoculation, pots were planted in a sequence of 0, 3, 7, 14, and 21 days. Plants of the soybean cv. Williams of different ages were then inoculated on the same day with a 10 mL zoospore suspension (1×10^4) and were kept flooded for 12 h. Data was collected 14 days after inoculation (DAI) for plant emergence, shoot weight, root weight, whole plant fresh weight and root rot score. A root rot score of 1 to 5 was assigned to the ten seeds on each cup using the ordinal scale: 1 = a healthy root system with no visible signs of lesions or rot; 2 = small lesions on the lateral roots with approximately 1-20% of the root having visible lesions; 3 = rot on lateral roots; visible signs of rot beginning on the main tap root with 21-75% of the roots having visible symptoms; 4 = both lateral roots and main tap root have visible signs of rot; 76-100% of the roots are infected; 5 = no germination/complete colonization of the seed. Pots were organized in a randomized complete block design with five replications for greenhouse trials (experiment I and II), and three replications for growth chamber trial (experiment III).

For experiment IV, 10 soybean seeds were planted in coarse vermiculite (Therm-O-Rock East, New Eagle, PA) in 2 L plastic pots. Seed treatments in this study included untreated control and the five fungicides of experiments I, II and III, inoculated with P. sojae zoospores and non-inoculated. In this study, only 21 days old plants of the soybean cv. Williams were inoculated with a 60 mL zoospore suspension (1×10^4) and kept flooded for 12 h. Evaluations followed the same as described in experiments I, II and III. Pots were organized in a completely randomized design with three replications.

3.2.3. Data analysis

Root rot score was rank transformed as suggested by Shah and Madden (2004). A generalized linear mixed model analysis followed by a least-squares means analysis was performed for all variables using the R studio (R Core Team, 2013) with the packages glme4

(Bates et al. 2019) and emmeans (Searle et al. 1980, Lenth et al. 2019), respectively. Pairwise comparisons were made using Tukey method at 90% confidence level.

3.3. Results and Discussion

Experiment I (Table 1) had differences (p=0.0560) on germination only, while none of the other variables had differences when inoculation happened on the day of planting. In this case, pyraclostrobin had a higher number of plants than the non-treated control. Differences for root rot score were found on all times of inoculations. At 0 DAP, pyraclostrobin had a lower root rot score than non-treated control (p=0.0886), indicating control of P. sojae. At 3 DAP non-treated control had a higher score than all other treatments (p<0.0001). At 7 DAP, compound A and oxathiapiprolin were lower than pyraclostrobin, ethaboxam and the non-treated control (p=0.0015). At 14 DAP, compound A and oxathiapiprolin were lower than non-treated control (p=0.0210). At 21 DAP, oxathiapiprolin was lower than compound A and pyraclostrobin (p=0.0663). Root fresh weight had differences between treatments at 0, 3 and 7 and 21. At 0 DAP, non-treated control had a higher value than ethaboxam (p=0.0893). At 3 DAP, pyraclostrobin and oxathiapiprolin was higher than non-treated control (p=0.0887). At 7 DAP, compound A was higher than pyraclostrobin, ethaboxam and non-treated control (p=0.0073). At 21 DAP, oxathiapiprolin had a higher root fresh weight than mefenoxam (p=0.0887). Shoot fresh weight had differences only at 3 DAP, where compound A was higher than non-treated control and mefenoxam (p=0.0003). The whole plant fresh weight had differences only at 3 DAP, where non-treated control was lower than all other treatments (p=0.0053).

Experiment II (Table 2) had differences on germination only at 0 days after planting (DAP), where ethaboxam and oxathiapiprolin had a higher number of plants than the non-treated control (p=0.0528). Differences on root rot score were found among seed treatments at 0, 7, and 14 DAP. At 0 DAP, ethaboxam had a lower score than non-treated control (p=0.0636). At 7 DAP, non-treated control had a higher score than all other treatments but mefenoxam (p<0.0001) and oxathiapiprolin was lower than mefenoxam, ethaboxam, pyraclostrobin and the non-treated. At 14 DAP, oxathiapiprolin was lower than mefenoxam, ethaboxam, and the non-treated control (p=0.0184). At 21 DAP, pyraclostrobin had a lower root rot score than mefenoxam (p=0.0962). Root fresh weight had differences between treatments at 0 and 14 DAP, where non-treated control was higher than compound A, pyraclostrobin and oxathiapiprolin (p=0.0343) on the first case, and oxathiapiprolin was higher than only mefenoxam (p=0.0804) in the second case. Shoot fresh weight had differences only at 7 DAP, where oxathiapiprolin was higher than non-treated control and mefenoxam (p=0.0003). The whole plant fresh weight had differences at 0, 7 and 14 DAP. At 0 DAP, non-treated control was higher than oxathiapiprolin (p=0.0753). At 7 DAP, non-treated control was lower than all other treatments but mefenoxam (p=0.0001). At 14 DAP, oxathiapiprolin was higher than ethaboxam (p=0.0533).

Experiment III (Table 3) had differences on germination only at 0 days after planting (DAP), where non-treated control had lower values than all other treatments (p=0.0002). Differences on root rot score were found on 3, 7 and 14 DAP. For the first, non-treated control had a higher score than all other treatments (p<0.0001), while at 7 DAP oxathiapiprolin was lower than all treatments, and pyraclostrobin was lower than non-treated control (p<0.0001). For root rot score at 14 DAP, oxathiapiprolin and compound A were lower than non-treated control, mefenoxam and ethaboxam (p=0.0108). Root fresh weight had differences between treatments only at 7 DAP, where oxathiapiprolin was higher than non-treated control and mefenoxam (p=0.0394) Shoot fresh weight had differences at 7 (p=0.0461) and 14 (p=0.0578) DAP, where oxathiapiprolin was higher than mefenoxam on both cases. The whole plant fresh weight had differences at 7 and 14 DAP, where oxathiapiprolin was higher than mefenoxam and ethaboxam on the first (p=0.0206) and compound A was higher than ethaboxam in the second (p=0.0446).

For experiment IV, differences between non-inoculated and inoculated pots happened in all variables (p<0.0001) but shoot fresh weight. No differences were found for root rot score, root fresh weight, shoot fresh weight and whole plant fresh weight for inoculations at 21 DAP only (Table 4).

Overall, germination was only impacted when soybean was inoculated at day of planting. Pyraclostrobin, ethaboxam and oxathiapiprolin was higher than control in two out of three experiments at 0 DAP, while mefenoxam and compound A resulted better than control only in one case.

Root rot score had differences at all times of inoculation, but only at 7 and 14 DAP differences happened in all three experiments. At 0 DAP, pyraclostrobin and ethaboxam were better than control. At 3 DAP, all fungicides were better than the non-treated control. At 7 DAP, oxathiapiprolin was superior to the control in all experiments. On the other hand, mefenoxam was no different than control in all three experiments. Moreover, pyraclostrobin and compound A were better than control in two out of three experiments, while ethaboxam was better than control only in one trial. At 14 DAP, similar to 7 DAP, oxathiapiprolin was superior to the control two out of three experiments, and ethaboxam, compound A and mefenoxam were closest to the non-treated control in all three cases. However, on experiment II (Table 2), compound A was better than mefenoxam. Pyraclostrobin was better than control only in one case. At 21 DAP, no fungicide was better than control and all differences in this time of inoculation happened between fungicides.

Root fresh weight had differences at all times of inoculation, and only at 0 and 7 DAP differences happened in two out of three experiments. At 0 DAP, no fungicide had root fresh weight higher than control, and the non-treated treatment was higher than ethaboxam, compound A, pyraclostrobin and oxathiapiprolin once. At 3 DAP, ethaboxam, mefenoxam and compound A was similiar to control in all three experiments. Pyraclostrobin and oxathiapiproblian were better than non-treated control in only one case. At 7 DAP, ethaboxam, mefenoxam and pyraclostrobin was similiar to control in all three experiments. compound A and oxathiapiproblian were better than non-treated control in only one case. At 14 and 21 DAP, all fungicides were similar to the non-treated control, and differences between fungicides happen only in one experiment where oxathiapiprolin was higher than mefenoxam.

Shoot fresh weight had differences at 3, 7 and 14 DAP, and only at 7 DAP differences happened in two out of three experiments. At 3 DAP, all fungicides were different from control in one case, and similar to it in the other two cases. At 7 DAP, compound A, pyraclostrobin, ethaboxam and oxathiapiprolin were different from control in one case, and similar to it in the other two cases. Moreover, mefenoxam was similar to non-treated control in all cases. At 14 DAP, all fungicides were similar to control in all experiments. Only in one of then, oxathiapiprolin was better than mefenoxam.

Whole plant fresh weight had differences at 0, 3, 7 and 14 DAP, and only at 7 and 14 DAP differences happened in two out of three experiments. At 0 DAP, all fungicides were similar to non-treated control, with the exception of oxathiapiprolin in one case. At 3 DAP, fungicides were similar to non-treated control in two cases, and better than it in one case. At 7 DAP, mefenoxam was similar to control in all cases, and only in one case compound A, pyraclostrobin, ethaboxam and oxathiapiprolin were better than it. At 14 DAP, all fungicides were similar to control in all cases, and differences between fungicides happened in two experiments.

Table 8. Germination (# of ten plants), root rot score (per cup), root fresh weight (g plant^{-1}), shoot fresh weight (g plant^{-1}), and whole plant fresh weight (g plant^{-1}) of plant inoculated with *Phytophthora sojae* zoospores at 0, 3(VC), 7 (VE), 14 (V_1), and 21 (V_2) days after planting (DAP), for experiment I.

treatment	germination		root score		root weight		shoot weight		plant weight	
					0 DAP					
non-treated	7.2	b*	2.0	a	0.64	a	1.11	a	1.74	a
mefenoxam	7.6	ab	1.6	ab	0.46	ab	1.05	a	1.51	a
compound A	8.4	ab	1.5	ab	0.48	ab	1.11	a	1.58	a
pyraclostrobin	9.2	a	1.3	b	0.46	ab	1.16	a	1.62	a
ethaboxam	8.4	ab	1.4	ab	0.44	b	1.13	a	1.57	a
oxathiapiprolin	8.4	ab	1.7	ab	0.52	ab	1.21	a	1.73	a
					3 DAP					
non-treated	8.2	a	3.4	a	0.41	b	0.88	c	1.29	b
mefenoxam	9.0	a	2.2	b	0.59	ab	1.13	ab	1.73	a
compound A	7.4	a	1.6	b	0.60	ab	1.32	a	1.92	a
pyraclostrobin	9.0	a	1.9	b	0.68	a	1.18	ab	1.85	a
ethaboxam	8.8	a	2.0	b	0.64	ab	1.19	ab	1.82	a
oxathiapiprolin	9.0	a	1.7	b	0.68	a	1.12	b	1.79	a
					7 DAP					
non-treated	9.0	a	3.0	a	0.68	bc	1.24	a	1.92	a
mefenoxam	8.2	a	2.7	ab	0.76	abc	1.25	a	2.02	a
compound A	9.4	a	2.4	b	1.04	a	1.34	a	2.37	a
pyraclostrobin	8.6	a	3.0	a	0.74	abc	1.26	a	2.00	a
ethaboxam	8.6	a	2.9	a	0.59	c	1.27	a	1.86	a
oxathiapiprolin	8.8	a	2.3	b	0.95	ab	1.40	a	2.34	a
					14 DAP					
non-treated	7.4	a	3.0	a	1.39	a	1.76	a	3.15	a
mefenoxam	7.8	a	2.7	abc	1.34	a	1.43	a	2.76	a
compound A	8.4	a	2.7	abc	1.52	a	1.70	a	3.22	a
pyraclostrobin	9.2	a	2.5	c	1.25	a	1.54	a	2.79	a
ethaboxam	8.2	a	3.0	ab	1.46	a	1.65	a	3.11	a
oxathiapiprolin	7.8	a	2.5	c	1.52	a	1.72	a	3.23	a
					21 DAP					
non-treated	8.6	a	3.0	ab	2.34	ab	2.21	a	4.56	a
mefenoxam	7.6	a	2.9	ab	2.27	b	2.14	a	4.40	a
compound A	8.2	a	3.1	a	2.49	ab	2.35	a	4.83	a
pyraclostrobin	9.0	a	3.1	a	2.63	ab	2.22	a	4.85	a
ethaboxam	7.8	a	2.8	ab	2.54	ab	2.27	a	4.80	a
oxathiapiprolin	8.0	a	2.5	b	2.79	a	2.26	a	5.05	a

*Means followed by the same letter do not differ by the Tukey method (α=0.1)

Table 9. Germination (# of ten plants), root rot score (per cup), root fresh weight (g plant^{-1}), shoot fresh weight (g plant^{-1}), and whole plant fresh weight (g plant^{-1}) of plant inoculated with *Phytophthora sojae* zoospores at 0, 3 (VC), 7 (VE), 14 (V_1), and 21 (V_2) days after planting (DAP), for experiment II.

treatment	germination		root score		root weight		shoot weight		plant weight	
0 DAP										
non treated	5.0	b*	2.0	a	1.26	a	0.84	a	2.10	a
mefenoxam	8.2	ab	1.5	ab	1.10	ab	0.68	a	1.78	ab
compound A	7.6	ab	1.5	ab	1.00	b	0.73	a	1.73	ab
pyraclostrobin	8.0	ab	1.7	ab	1.04	b	0.67	a	1.71	ab
ethaboxam	8.8	a	1.4	b	1.08	ab	0.76	a	1.84	ab
oxathiapiprolin	8.4	a	1.7	ab	1.02	b	0.67	a	1.69	b
3 DAP										
non treated	6.2	a	2.2	a	1.15	a	0.95	a	2.10	a
mefenoxam	7.0	a	1.8	a	1.27	a	1.16	a	2.44	a
compound A	8.0	a	2.0	a	1.21	a	0.99	a	2.19	a
pyraclostrobin	7.6	a	1.7	a	1.31	a	1.13	a	2.44	a
ethaboxam	9.0	a	2.0	a	1.27	a	1.06	a	2.33	a
oxathiapiprolin	7.8	a	1.8	a	1.35	a	1.00	a	2.35	a
7 DAP										
non treated	9.2	a	3.9	a	1.25	a	0.79	c	2.04	b
mefenoxam	7.4	a	3.1	ab	1.42	a	1.12	bc	2.54	ab
compound A	8.6	a	2.2	cd	1.47	a	1.55	ab	3.02	a
pyraclostrobin	8.8	a	2.7	bc	1.47	a	1.50	ab	2.97	a
ethaboxam	8.8	a	2.9	b	1.41	a	1.41	ab	2.82	a
oxathiapiprolin	8.8	a	2.0	d	1.48	a	1.61	a	3.09	a
14 DAP										
non treated	9.4	a	3.0	a	1.66	ab	1.63	a	3.29	ab
mefenoxam	8.2	a	2.9	a	1.53	b	1.68	a	3.21	ab
compound A	8.2	a	2.7	ab	1.55	ab	1.75	a	3.31	ab
pyraclostrobin	8.0	a	2.7	ab	1.63	ab	1.86	a	3.49	ab
ethaboxam	8.2	a	2.9	a	1.57	ab	1.54	a	3.11	b
oxathiapiprolin	8.2	a	2.2	b	1.80	a	2.06	a	3.86	a
21 DAP										
non treated	9.0	a	3.3	ab	2.19	a	3.05	a	5.24	a
mefenoxam	8.0	a	3.4	a	2.18	a	3.07	a	5.25	a
compound A	8.4	a	3.1	ab	2.15	a	3.44	a	5.58	a
pyraclostrobin	8.4	a	2.8	b	2.28	a	3.64	a	5.91	a
ethaboxam	8.0	a	3.2	ab	2.21	a	3.34	a	5.54	a
oxathiapiprolin	8.4	a	3.1	ab	2.27	a	3.79	a	6.07	a

*Means followed by the same letter do not differ by the Tukey method (α=0.1)

Table 10. Germination (# of plants), root rot score (per cup), root fresh weight (g plant^{-1}), shoot fresh weight (g plant^{-1}), and whole plant fresh weight (g plant^{-1}) of plant inoculated with *Phytophthora sojae* zoospores at 0, 3 (VC), 7 (VE), 14 (V_1), and 21 (V_2) days after planting (DAP), for experiment III.

treatment	germination		root score		root weight		shoot weight		plant weight	
					0 DAP					
non treated	1.7	b*	2.3	a	1.22	a	1.10	a	2.32	a
mefenoxam	7.7	a	2.2	a	1.16	a	1.31	a	2.46	a
compound A	9.3	a	2.0	a	1.25	a	1.29	a	2.54	a
pyraclostrobin	7.0	a	1.8	a	1.17	a	1.24	a	2.41	a
ethaboxam	9.0	a	2.2	a	1.00	a	1.22	a	2.23	a
oxathiapiprolin	8.0	a	2.1	a	1.17	a	1.29	a	2.46	a
					3 DAP					
non treated	7.3	a	2.9	a	1.21	a	1.44	a	2.65	a
mefenoxam	7.0	a	2.2	c	1.39	a	1.41	a	2.81	a
compound A	8.0	a	1.9	cd	1.43	a	1.35	a	2.77	a
pyraclostrobin	8.3	a	1.7	d	1.42	a	1.43	a	2.86	a
ethaboxam	8.7	a	2.6	b	1.07	a	1.37	a	2.44	a
oxathiapiprolin	8.3	a	2.0	cd	1.37	a	1.55	a	2.92	a
					7 DAP					
non treated	8.3	a	3.7	a	1.57	b	1.71	ab	3.28	ab
mefenoxam	8.7	a	3.4	ab	1.47	b	1.43	b	2.90	b
compound A	8.0	a	3.1	ab	1.95	ab	1.81	ab	3.76	ab
pyraclostrobin	9.0	a	3.0	b	1.72	ab	1.53	ab	3.25	ab
ethaboxam	7.7	a	3.4	ab	1.60	ab	1.52	ab	3.12	b
oxathiapiprolin	7.7	a	2.3	c	2.24	a	1.83	a	4.07	a
					14 DAP					
non treated	9.3	a	3.0	ab	2.24	a	1.65	ab	3.89	ab
mefenoxam	8.7	a	3.3	a	2.08	a	1.56	b	3.65	b
compound A	7.7	a	2.6	b	2.66	a	1.86	ab	4.52	a
pyraclostrobin	9.7	a	2.8	ab	2.36	a	1.76	ab	4.12	ab
ethaboxam	8.7	a	3.0	ab	2.17	a	1.67	ab	3.84	ab
oxathiapiprolin	8.0	a	2.5	b	2.43	a	1.99	a	4.41	ab
					21 DAP					
non treated	7.3	a	3.0	a	4.24	a	2.65	a	6.90	a
mefenoxam	8.3	a	2.8	a	3.71	a	2.27	a	5.98	a
compound A	7.0	a	3.3	a	3.82	a	2.23	a	6.04	a
pyraclostrobin	9.0	a	2.8	a	3.44	a	2.14	a	5.58	a
ethaboxam	7.7	a	2.8	a	3.61	a	2.34	a	5.95	a
oxathiapiprolin	8.7	a	2.8	a	3.57	a	2.42	a	5.99	a

*Means followed by the same letter do not differ by the Tukey method (α=0.1)

Table 11. Root rot score, root fresh weight (g plant^{-1}), shoot fresh weight (g plant^{-1}), and whole plant fresh weight (g plant^{-1}) of plant inoculated with *Phytophthora sojae* zoospores at 21 days after planting (V_2) (DAP), for experiment IV.

treatment	root score		root weight		shoot weight		plant weight	
				non inoculated				
non treated	1.5	a*	5.26	ab	2.34	a	7.60	ab
mefenoxam	1.8	a	5.60	ab	2.48	a	8.08	ab
compound A	1.5	a	5.50	ab	2.22	a	7.72	ab
pyraclostrobin	1.5	a	4.92	b	2.21	a	7.13	b
ethaboxam	1.5	a	5.81	ab	2.66	a	8.47	a
oxathiapiprolin	1.5	a	6.14	a	2.53	a	8.68	a
				inoculated				
non treated	3.5	a	3.60	a	1.98	a	5.58	a
mefenoxam	3.5	a	4.32	a	2.39	a	6.71	a
compound A	3.5	a	4.59	a	2.46	a	7.04	a
pyraclostrobin	3.5	a	4.58	a	2.28	a	6.86	a
ethaboxam	3.5	a	4.34	a	2.07	a	6.41	a
oxathiapiprolin	3.5	a	3.98	a	2.41	a	6.39	a

*Means followed by the same letter do not differ by the Tukey method (α=0.1)

The effects of seed treatment over time of inoculation are summarized in Figure 1. For experiment I (Figura 1A), non-treated control had a lower value of root rot score for 0 DAP than all others times of inoculation ($p<0.0001$). Mefenoxam at 0 DAP was lower than 7, 14 and 21 DAP, and 3 DAP was lower than 21 DAP ($p=0.0084$). compound A at 0 DAP was lower than 7, 14 and 21 DAP, while 3 DAP was lower than 14 and 21 DAP, and 7 DAP was lower than 21 DAP ($p<0.0001$). Pyraclostrobin at 0 DAP was lower than 7, 14 and 21 DAP, while 3 DAP was lower than 7 and 21 DAP, and 14 DAP was lower than 21 DAP ($p=<0.0001$). Ethaboxam at 0 and 3 DAP was lower than 7, 14 and 21 DAP ($p<0.0001$). Oxathiapiprolin at 0 and 3 DAP was lower than 14 and 21 DAP ($p=0.0045$). For experiment II (Figura 1B), non-treated control had a lower value of root rot score for 0 and 3 DAP than all others moments of inoculation ($p<0.0001$), similar to mefenoxam ($p<0.0001$) and pyraclostrobin ($p<0.0001$). Compound A at 0 DAP was lower than 14 and 21 DAP, but similar to 3 and 7 DAP ($p<0.0001$). Oxathiapiprolin at 0, 3, 7 and 14 DAP was lower than 21 DAP ($p<0.0001$). For experiment III (Figura 1C), non-treated control had a lower value of root rot score for 0 DAP than 7, 14 and 21 DAP, and 3 DAP was different from 7 DAP ($p=0.0066$). similar to mefenoxam ($p<0.0001$), pyraclostrobin ($p<0.0001$), and ethaboxam ($p<0.0001$). Compound A at 0 and 3 DAP was lower than 14 and 21 DAP, while 7 DAP was lower than 21 DAP ($p<0.0001$). Ethaboxam at 0 DAP was lower than 7 and 14 DAP only ($p=0.0021$). Oxathiapiprolin at 0 and 3 DAP was lower than 21 DAP ($p=0.0239$).

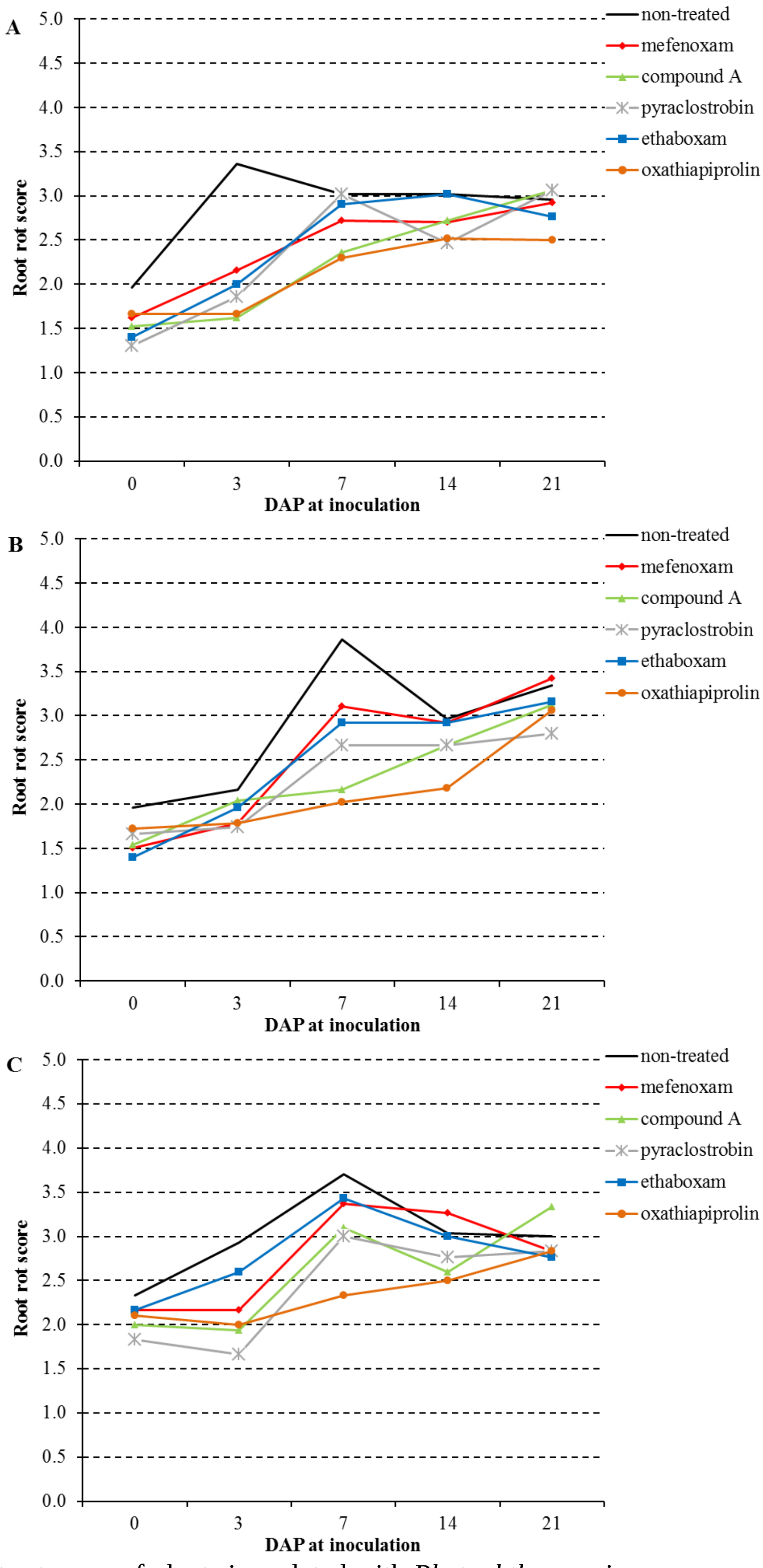

Figure 5. Root rot score of plants inoculated with *Phytophthora sojae* zoospores at 0, 3, 7, 14, and 21 days after planting (DAP), comparing five fungicides and one non-treated control on experiments I (A), II (B), and III (C).

The effect of inoculation at later growth stages could be measured by high root rot scores and lower root weights. Based on these measurements, seed treatments protected seedlings at 14 DAP in all 3 experiments. Although differences were found at 21 DAP, in none of the four experiments any fungicide was better than the non-treated control.

Overall, all fungicides performed better than non-treated control. Mefenoxam had lower root score than the control only in two cases (3 DAP, experiments I and III), while ethaboxam had this result in four cases (0 DAP on experiment II, 3 DAP on experiments I and III, and 7 DAP on experiment II). On the same way, compound A had a lower root rot score than the non-treated control in four cases (3 DAP on experiments I and III, 7 DAP on experiments I and II, and 14 DAP on experiment III). Pyraclostrobin had satisfactory results for root rot score in 6 cases (0 DAP on experiment I, 3 DAP on experiments I and III, 7 DAP on experiments II and III, and 14 DAP on experiment I). Finally, oxathiapiprolin had the best sets of results between all fungicides, overcoming the non-treated control for root rot score on 7 of the cases (3 DAP on experiments I and III, 7 DAP on all 3 experiments, and 14 DAP on experiments I and II). The inoculation at 7 DAP was the one with most frequent differences between treatments. In this case, oxathiapiprolin was better than compound A for root rot control only in one case, on experiment III (Table 3), where the former outcompeted all other fungicides. This is especially important because it shows that compound A has a similar performance to oxathiapiprolin with less than half the concentration per seed of the later, which confers to the former a better efficiency status than all other fungicides tested in this study.

Base on the afore mentioned, it may be claimed that 14 DAP seems to be the limit of efficacy of fungicide seed treatment for controlling Phytophthora root and stem rot. Although it is not possible to assure whether this happens mostly because the fungicide degradation or because root tissue growth, results from Sartori et al. (2020) might give a clue on this issue. According to the authors, root absorption on fungicide seed treatment on seedlings (14 to 18 DAP) is limited and it is concentrated on primary roots (tap root) mainly. Therefore, as the root systems develop, most of the new tissue becomes unprotected and susceptible to infection.

3.4. Conclusion

In conclusion, this study has shown that the maximum satisfactory activity of fungicide seed treatment for Phytophthora root rot control goes until 14 days after planting

(V_1), and that compound A was more efficient to manage *P. sojae* than others fungicides available in the market.

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