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RELATION BETWEEN FUNGICIDES AND FLOWER ABORTION IN DISEASE-FREE SOYBEAN PLANTS: PHYSIOLOGICAL, REPRODUCTIVE AND PRODUCTION ASPECTS

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ASPECTOS FISIOLÓGICOS, REPRODUTIVOS E COMPONENTES DE PRODUÇÃO EM PLANTAS DE SOJA, LIVRES DE DOENÇAS, TRATADAS COM DIFERENTES FUNGICIDAS

Autora: Verônica Barbosa Junqueira
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RESUMO

JUNQUEIRA, VERÔNICA BARBOSA. Instituto Federal Goiano – Campus Rio Verde – GO, Outubro de 2021. **Relação entre fungicidas e abortamento floral em plantas de soja livres de doença: aspectos fisiológicos, reprodutivos e de produção.** Orientador: DSc. Alan Carlos da Costa. Coorientadores: DSc. Caroline Müller, DSc. Adinan Alves da Silva.

Os fungicidas são insumos essenciais para a lucratividade das lavouras de soja, e a pulverização foliar destes pesticidas expandiu significativamente nas últimas décadas. No entanto, para além do controle e eliminação de doenças, esses pesticidas podem interferir na fisiologia, reprodução e produtividade de plantas livres de doenças. Dessa forma, objetivou-se com esta pesquisa avaliar os efeitos de três fungicidas comerciais sobre o processo fotossintético, a viabilidade do grão de pólen e os componentes da produção de plantas de soja livres de doenças, aplicados nas fases vegetativa e/ou reprodutiva da cultura. No primeiro experimento, plantas de soja livres de doença, cultivar SYN 1378C, foram tratadas nas fases de desenvolvimento de pré-florescimento (V8) e florescimento (R1), com a aplicação de três fungicidas comerciais: ciproconazol 150 g L^{-1} + difenoconazol 250 g L^{-1} (CPZ + DFZ; 250 mL ha^{-1}; sem adjuvante); azoxistrobina 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1} (AZB + BZP; 200 g ha^{-1}; adjuvante Nimbus® (Syngenta)); propiconazol 250 g L^{-1} + difenoconazol 250 g L^{-1} (PPZ + DFZ; 150 mL ha^{-1}; sem adjuvante). Em paralelo foi conduzido um tratamento controle, sem a aplicação de fungicida. O estudo foi realizado em duplicatas independentes, e cada experimento foi conduzido em blocos casualizados, com quatro repetições. Foram avaliadas as características de fitotoxicidade, trocas gasosas e fluorescência da clorofila *a*, viabilidade e germinação dos grãos de pólen, abortamento de flores e componentes da produção. Um segundo experimento foi realizado, utilizando a mesma cultivar de soja, tratada com a aplicação de outros três fungicidas comerciais: epoxiconazol 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + piraclostrobina 81 g L^{-1} (EPZ + FLX + PYR; 800 mL ha^{-1}; adjuvante Assist$^{®}$ 500 mL ha^{-1} (BASF Ltda.)); trifloxistrobina 150 g L^{-1} + protioconazol 175 g L^{-1} (TFX + PRZ; 400 mL ha^{-1}; adjuvante Aureo$^{®}$ (Bayer Ltda.)); e trifloxistrobina 150 g L^{-1} + protioconazol 175 g L^{-1} + bixafen 125 g L^{-1} (TFX + PRZ + BXF; 500 mL ha^{-1}; adjuvante Aureo$^{®}$ (Bayer Ltda.)) e um tratamento controle sem aplicação de fungicida. As plantas receberam uma (no estágio R1) duas (nos estágios R1 e R2) ou três (nos estágios R1, R2 e R3) aplicações dos fungicidas. O delineamento experimental foi em esquema fatorial (4 tratamentos x 3 aplicações), em blocos ao acaso, com oito repetições. Em ambos os experimentos, os seis fungicidas avaliados não afetaram as trocas gasosas, germinação do grão de pólen e produtividade da cultura. No entanto, o fungicida EPZ + FLX + PYR afetou negativamente as características fotoquímicas de plantas de soja, enquanto que o TFX + PRZ reduziu o número de vagens de plantas de soja após uma aplicação, o que pode ter refletido em um maior peso de mil grãos, como forma de compensação. Os fungicidas TFX + PRZ e TFX + PRZ + BXF prejudicaram a germinação de pólen da soja neste estudo, porém sem alterar os componentes de produção das plantas de soja. A aplicação dos fungicidas em diferentes estádios de desenvolvimento e diferentes números de aplicações não interferiram no metabolismo e a produção das plantas de soja livres de doença, em condições controladas.

PALAVRAS-CHAVES: germinação de pólen, trocas gasosas, fluorescência da clorofila *a*, produtividade, triazol, estrobilurinas, carboxamida, *Glycine max*

ABSTRACT

JUNQUEIRA, VERÔNICA BARBOSA. Goiano Federal Institute - Campus Rio Verde – GO, October, 2021. **Relation between fungicides and flower abortion in soybean disease-free plants: physiological, reproductive and production aspects.** Advisor: DSc. Alan Carlos da Costa. Coadvisor: DSc. Caroline Müller, DSc. Adinan Alves da Silva.

Fungicides are essential inputs to the profitability of soybean crops, and foliar spraying of these pesticides has significantly expanded in recent decades. However, in addition to controlling and eliminating diseases, these pesticides can interfere with the physiology, reproduction and productivity of disease-free plants. Thus, we aimed with this research to evaluate the effects of three commercial fungicides on the photosynthetic process, the viability of the pollen grain and the yield components of disease-free soybean plants, applied in the vegetative and/or reproductive phases of the crop. In the first experiment, disease-free soybean plants of the cultivar SYN 1378C, were treated in the pre-bloom (V8) and bloom (R1) stages of development, with the application of three commercial fungicides: cyproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1} (CPZ + DFZ; 250 ml ha^{-1}; no adjuvant); azoxystrobin 300 g Kg^{-1} + benzovindiflupyr 150 g Kg^{-1} (AZB + BZP; 200 g ha^{-1}; Nimbus® adjuvant (Syngenta)); propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1} (PPZ + DFZ; 150 mL ha^{-1}; no adjuvant). In parallel, a control treatment was carried out, without the application of fungicide. The study was carried out in independent duplicates, and each experiment was carried out in randomized blocks, with four replications. The characteristics of phytotoxicity, gas exchange and chlorophyll a fluorescence, pollen grain viability and germination, flower abortion and yield components were evaluated. A second experiment was carried out, using the same soybean cultivar, treated with the application of three other commercial fungicides: epoxiconazole 50 g L-1 + fluxapyroxade 50 g L-1 + pyraclostrobin 81 g L-1 (EPZ + FLX + PYR; 800 mL ha-1; Assist® adjuvant 500 mL ha-1 (BASF Ltda.)); trifloxystrobin 150 g L-1 + prothioconazole 175 g L-1 (TFX + PRZ; 400 mL ha-1; Aureo® adjuvant (Bayer Ltda.)); and trifloxystrobin 150 g L-1 + prothioconazole 175 g L-1 + bixafen 125 g L-1 (TFX + PRZ + BXF; 500 mL ha-1; Aureo® adjuvant (Bayer Ltda.)) and a control treatment without application of fungicide. The plants received one (in the R1 stage) two (in the R1 and R2 stages) or three (in the R1, R2 and R3 stages) fungicide applications.

The experimental design was in a factorial scheme (4 treatments x 3 applications), in randomized blocks, with eight replications. In both experiments, the six fungicides evaluated did not affect gas exchange, pollen grain germination and crop yield. However, the fungicide EPZ + FLX + PYR negatively affected the photochemical characteristics of soybean plants, while TFX + PRZ reduced the number of soybean plant pods after one application, which may have reflected in a higher weight of one thousand grains as a form of compensation. The fungicides TFX + PRZ and TFX + PRZ + BXF affected the germination of soybean pollen in this study, but without changing the yield components of the soybean plants. The application of fungicides at different stages of development and different numbers of applications did not alter the metabolism and production of disease-free soybean plants under controlled conditions.

KEYWORDS: pollen germination, gas exchange, chlorophyll *a* fluorescence, crop yield, triazole, strobilurins, carboxamide, *Glycine max*

1. GENERAL INTRODUCTION

Soybean (*Glycine max* (L.) Merr.), which belongs to the Fabaceae (Leguminosae) family, is one of the most economically important legumes in the world. Its grains are rich in proteins, unsaturated fatty acids, such as oleic and linoleic acids (omega 3, 6 and 9) and dietary fiber (Yao et al. 2020), being used in human food (Kim et al., 2015) and animal feed (Hassan et al., 2020). Soy oil, besides being an important food ingredient, also has wide industrial use, mainly in the production of biofuels, lubricants and plastics. Also, soybean cultivation in crop rotation improves soil fertility by providing nitrogen from the symbiosis with nitrogen-fixing bacteria, in addition to facilitating the management of diseases, insects and weeds (Cordeiro and Echer, 2019) .

Originated from China, soybeans were domesticated over 3,000 years ago (Anderson et al., 2019), however, only in the first decades of the 20th century there was a significant expansion in its cultivation, mainly in the United States and Brazil. In the United States, with the creation of the intensive cooperative soybean breeding program in 1936 (Johnson and Bernard, 1962). In Brazil, from the inclusion of soybean breeding in experimental work with the "Campanha da Cultura da Soja" in 1951 (Miyasaka e Silva, 1958), and with strong government incentives after the 60s (Cattelan and Dall'Agnol, 2018), we started to select more productive soybean genotypes adapted to different edaphoclimatic regions. Initially, genetic breeding aimed at greater productivity, but still in the 1960s, breeders started to backcross and launched the first varieties with greater resistance to disease (Johnson and Bernard, 1962).

In 1961, the United States and China stood out as the world's largest soybean producers, with 18.5 and 6.3 million tons (Mt), respectively; and, in the same period, Brazil produced approximately 0.3 Mt (CONAB, 2019), in the South and Southeast regions of the country. However, with the continuous and growing advances in the area of plant breeding, the selection of soybean genotypes allowed to obtain varieties well adapted to low latitude, less susceptible to changes in the photoperiod and tolerant to other abiotic stresses (Carvalho et al., 2017), thus expanding the crop to the Center-West region of Brazil in the 70s, and to the Northeast and North regions in the following decades (CONAB, 2020). In 1974, Brazil became the second largest producer of soybeans in the world, and it currently ranks first with a production of 128.50 Mt, followed by the

United States with 96.67 Mt, which together are responsible for approximately 70% of the world production of this crop (USDA, 2021).

The occurrence of fungal diseases in soybeans has been responsible for a reduction in profitability. The main significant loss in soybean production occurred with the discovery of soybean rust (*Phakopsora pachyrhizi*), which caused losses of up to 4.6 million tons of soybeans in Brazil (Godoy et al., 2016) and total economic losses 51% larger in the United States (Bandara et al., 2020a). Although soybean rust is the best-known soybean disease, several other diseases can also negatively impact yield, such as soybean cyst nematode, charcoal rot, seedling diseases, bacterial blight and *Sclerotium* blight (Bandara et al. , 2020b).

Fungicides are fundamental to the economic profitability of agricultural crops and foliar spraying has expanded significantly in recent decades. The most common classes of fungicides marketed for use in soybeans include demethylation inhibitors (DMI; also known as triazoles), quinone outside inhibitors (QoI; also known as strobilurins) and succinate dehydrogenase inhibitors (SDHI) (also known as carboxamides) (Faske & Emerson, 2020), where the strobilurin and triazole fungicide families are the most commonly used for the management of soybean diseases (Bayer, 2021). Triazoles were introduced on the market in the 1970s, when the introduction of more active and less phytotoxic fungicides began (Morton and Staub, 2008). Triazoles belong to the group of sterol biosynthesis inhibitors, which constitutes the largest and most important group of compounds ever developed for the control of fungal diseases in plants and animals, accounting for more than 30% of global sales of fungicides (Krämer et al., 2007).

Strobilurins and carboxamides are mitochondrial electron transport chain inhibitors. Strobilurins block electron transfer from cytochrome *b* to cytochrome *c1* (Complex IV); while carboxamides inhibit the enzyme succinate dehydrogenase (Complex II), which stops the electron transport to oxygen. The actions of strobilurin and carboxamide interfere with the formation of ATP, which is the vital energy molecule for growth of the fungus (Amaro et al., 2018). The first strobilurins released entered the market in 1996 (Barlett et al., 2002; Morton and Staub, 2008) and accounted for up to 25% of global fungicide sales (Leadbeater et al., 2012). Carboxamides were developed in the 1960s, but only in the 2000s did farmers start using them to control plant diseases (Morton and Staub, 2008).

Farmers around the world use fungicides to control and prevent soy diseases (Xia et al., 2006). However, the excessive use of foliar fungicides can be a problem for the crop, since in addition to controlling pathogens, they can negatively affect plant metabolism. Harmful effects of some fungicides have already been reported as phytotoxicity soon after application (Wu and von Tiedemann, 2002), affecting leaf anatomy (Shahid et al., 2018), photosynthetic structures (Petit et al., 2012; Singh and Sahota, 2018) and the viability of the pollen grain (Junqueira et al., 2017), which in turn can compromise the quality of the grains.

There are reports of different plant responses to triazoles, strobilurin and carboxamide fungicides. Triazole fungicides affected the NPQ of *Cucumis sativus* L. (Xia et al., 2006), while another type of triazole protected *Hordeum vulgare* against exposure to ozone and salt stress (Wu and von Tiedemann, 2002). Strobilurins reduced the photosynthetic efficiency of soybean plants (Nason et al., 2007), while pyraclostrobin (a type of strobilurin) increased the net photosynthesis and grain yield of that same crop (Fagan et al., 2010). The combined application of triazole, strobilurin and carboxamide resulted in positive effects on wheat physiology and yield in the absence of disease pressure (Ajigboye et al., 2014). Many physiological effects of carboxamide and strobilurins have already been reported (reviewed by Amaro et al., 2020) and because of that, companies often indicate the use of these fungicides to farmers even in the absence of diseases, in order to improve the physiological traits and, consequently, the yield. However, published studies on the effects of fungicides on disease-free soybean plants are scarce (Swoboda and Pedersen, 2009; Grichar, 2013; Mahoney and Gillard, 2014).

The purpose of this study is to show how fungicides act on plant metabolism without considering disease control. There is some research showing toxic effects of these products in some species, but doses higher than recommended for the field are often used (Petit et al., 2012; Dias, 2012), or the authors suggest “physiological effect” of the fungicide in comparison to plants infected by pathogens (Fagan et al., 2010; Amaro et al., 2020). Furthermore, there are no studies on the effects of fungicides on flowering of disease-free soybean plants. Thus, this study focused on replicating field conditions, with recommended doses, used at the recommended stages, in order to obtain applicable results for farmers.

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2. OBJECTIVES

2. 1 General

To characterize the effects of fungicides from the chemical groups of triazole, strobilurins and carboxamides on aspects of physiology, reproduction and production components of disease-free soybean plants

2.2 Specific

To analyze visual symptoms of leaf phytotoxicity resulting from the application of fungicides on soybean plants at different stages of development;

Evaluate the effects of fungicides with different number of applications in the photosynthetic process of soybean plants;

Determine if fungicides affect flower abortion and other reproductive structures of soybean plants;

To study the effect of fungicides on the viability and germination of pollen grains from disease-free soybean plants;

Check whether different product applications and/or at different stages of development affect the production components of disease-free soybean plants.

CHAPTER I

Do fungicides affect the physiology, reproductive development and productivity of healthy soybean plants?

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ABSTRACT

Fungicides are widely used to control diseases in soybean crops. We hypothesized that fungicides applied to healthy soybean plants compromise the plant's physiology, affect the reproductive process and reduce crop productivity. We aimed to evaluate the photosynthetic process, pollen grain viability and yield components of soybean plants exposed to three commercial fungicides. The experiment was repeated twice using soybean cultivar SYN 1378C, disease-free plants, which were treated with the application of three commercial fungicides: i) control treatment (without any fungicide application); ii) cyproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1} (CPZ+DFZ; 250 mL ha^{-1}; without adjuvant); iii) azoxystrobin 300 g Kg^{-1} + benzovindiflupyr 150 g Kg^{-1} (AZB+BZP; 200 g ha^{-1}; Nimbus® adjuvant (Syngenta)); and iv) propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1} (PPZ+DFZ; 150 mL ha^{-1}; without adjuvant) in both soybean pre-bloom (V8) and bloom (R1) developmental stages. The experimental design was randomized blocks with four replicates. Phytotoxicity, gas exchange and chlorophyll *a* fluorescence traits, pollen grain viability, pollen grain germination, flower abortion and soybean yield components were evaluated. The fungicides did not affect the physiological traits, pollen grain germination and crop yield.

Keywords: *Glycine max*, strobilurin, triazole, carboxamide, pollen.

1 INTRODUCTION

Growing demand for food has driven the expansion of agricultural areas and the pursuit of higher productivity. Soybean stands out as one of the main economically important crops for being a source of protein and vegetable oil, both for human and animal food (Xia et al., 2006), and as a feedstock for biodiesel production (Xia et al., 2006). The maintenance of high soybean productivity depends on disease control in these crops, especially fungal ones. Fungal diseases are a major concern for farmers who grow soybeans. Among the fungal diseases, soybean rust (*Phakopsora pachyrhizi*) has affected soybean production worldwide and may cause losses up to 80 % in yield (Xia et al., 2006). Thus, the use of foliar fungicides is almost imperative for both disease control and prevention. It is noteworthy to mention that although soybean resistance *loci* have been mapped, no resistance genes have been cloned, thus there are no commercial soybean cultivars with durable resistance to *P. pachyrhizi* (Wu and von Tiedemann, 2002).

Farmers all around the world use fungicides to control soybean diseases (Xia et al., 2006). However, overuse of foliar fungicides can be a problem for the crop since besides controlling the pathogens, it may negatively affect plants metabolism. Harmful effects of some fungicides were reported as phytotoxicity right after application (Wu and von Tiedemann, 2002), affecting leaf anatomy (Shahid et al., 2018), photosynthetic structures (Petit et al., 2012; Singh and Sahota, 2018) and pollen grain viability (Junqueira et al., 2017), which in turn may compromise grain quality.

Fungicides belonging to the triazole and strobilurin chemical groups are the most used in soybean cultivation. In Brazil, there are 303 registered commercial products for fungal disease control in soybean, of which 50% belong to the triazole groups and 25% belong to the strobilurins group, which can be formulated alone or in combination (AGROFIT, 2020). Triazoles act by inhibiting the synthesis of ergosterol, a lipid present in the fungal membrane (Villani et al., 2016), which performs a function similar to that of the phytosterols (Yang et al., 2015). Strobilurins, on the other hand, have as their mode of action the electrons block in Complex III of the mitochondrial electron transport chain (Feng et al., 2020).

Another widely used fungicide chemical group in soybeans is carboxamides, which comprises 5% of registered fungicides in Brazil. Like strobilurins, carboxamides also act by interfering with the pathogen's mitochondrial respiration, more specifically in complex II (Amaro et al., 2018). Thus, respiratory chain inhibitor fungicides are believed to block not only electron transport in fungi but also in plants due to the similarity of target proteins. Fungicides are mainly used from the beginning of the reproductive period, when symptoms become more visible. This period is critical for productivity. Several producers and companies in the region have raised questions about the possible effect of applying fungicides during soybean flowering stage. They believe. that when the fungicides are applied during soybean flowering, a reduction in pod establishment may occur due to increased flower abortion. This may be a consequence of the fungicide effects on the pollen tube germination, a fundamental process to ensure fertilization. Pollen germination depends on favorable interactions between pollen grains and stigma, and if it does not occur it will affect grain formation (Pacini and Dolferus, 2019). Thus, pollen grain germination in soybean plants can directly reflect the crop grain yield. This makes this trait important for determining the most efficient and least crop-damaging fungicides, and avoids soybean yield losses after application.

There are different responses of plants to triazole, strobilurin and carboxamide fungicides described. Triazole fungicides affected the NPQ of *Cucumis sativus* L (Xia et al., 2006), while another kind of triazole protected *Hordeum vulgare* against ozone exposure and salt stress (Wu and von Tiedemann, 2002). Strobilurin fungicides reduced the photosynthetic efficiency of soybean plants (Nason et al., 2007), while pyraclostrobin (a kind of strobilurin) increased net photosynthesis and grain yield also in soybeans (Fagan et al., 2010). Triazole, strobilurin and carboxamide combined application resulted in positive effects on wheat physiology and yield in the absence of disease pressure (Ajigboye et al., 2014). Many physiological effects of carboxamide and strobilurins are reported (Amaro et al., 2020) and because of that, companies often indicate farmers to use fungicides even in the absence of diseases in order to improve physiological traits and, consequently, yield.

However, published studies concerning fungicide effects on disease-free soybean plants are scarce (Swoboda and Pedersen, 2009). Our goal here was to show how these products act in plant metabolism without considering disease control. There are some researches showing toxic effects of fungicides in some species, but frequently the authors use doses beyond the recommended for the fields (Petit et al., 2012), or suggest "physiological effect" in relation to infected control plants (Fagan et al., 2010). Also, there are no studies concerning fungicide effects on soybean flowering. So, in this study we focused on replicating field conditions, with recommended doses, used in recommended stages in order to get applicable results for the farmers.

We hypothesized that fungicides applied to healthy soybean plants compromise the physiology of the plant, affect the reproductive process and reduce crop productivity. To test the hypothesis, we aimed to evaluate the photosynthetic process, pollen grain viability and yield components of soybean plants exposed to three commercial fungicides applied at the vegetative and reproductive phases, following technical recommendations according to the genotype and region conditions.

2 MATERIAL AND METHODS

2.1 Plant Material and Experimental Conditions

Two experiments were conducted sequentially, in a greenhouse at the Laboratory of Ecophysiology and Plant Productivity at the Instituto Federal Goiano, Campus Rio Verde, Goiás, Brazil, with controlled temperature (~26 °C) and relative humidity (62 – 86 %). The experiments were carried out independently, the first from December to March, and the second one from January to April. For both experiments, soybean seeds (SYN 1378 C, Syngenta, Brazil) were sown in plastic pots containing 12 L of substrate. The substrate was prepared from a mixture of Red Latosol (LVdf) soil and sand in the ratio 2:1, , which had the following physicochemical characteristics: pH H_2O – 5.8; P – 0.9 mg dm^{-3}; K – 9.0 mg dm^{-3}; Ca – 0.59 $cmol_c$ dm^{-3}; Mg – 0.17 $cmol_c$ dm^{-3}; Al – 0.05 $cmol_c$ dm^{-3}; H^+Al – 1.8 $cmol_c$ dm^{-3}; S – 0.8 mg dm^{-3}; B – 0.1 mg dm^{-3}; Cu – 0.5 mg dm^{-3}; Fe – 118.0 mg dm^{-3}; Mn – 16.7 mg dm^{-3}; Zn – 0.2 mg dm^{-3}; Na – 1.8 mg dm^{-3}; SB – 30 %; CTC – 2.6 $cmol_c$ dm^{-3}; OM – 6.2 %; clay – 38.5%; silt – 7.5 % and sand – 54 %. The substrate was fertilized according to the soil chemical analysis and the recommendation for soybean crop (Sfredo, 2008). Liming was performed using dolomitic limestone, increasing the base saturation to 60% and the plants were fertilized with 1.5 g dm^{-3} of mono-ammonium phosphate (MAP), 1.8 g dm^{-3} of potassium chloride (KCl); 0.8 g dm^{-3} of magnesium sulfate ($MgSO_4$); 0.5 g dm^{-3} of urea (CH_4N_2O); 0.05 g dm^{-3} of sulfate copper ($CuSO_4$); 0.008 g dm^{-3} of boric acid (H_3BO_3); and 0.003 g dm^{-3} of zinc sulfate ($ZnSO_4$).

The treatments consisted of applying the following commercial fungicides: i) control treatment (without any fungicide application); ii) cyproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1} (CPZ+DFZ; 250 mL ha^{-1}; without adjuvant); iii) azoxystrobin 300 g Kg^{-1} + benzovindiflupyr 150 g Kg^{-1} (AZB+BZP; 200 g ha^{-1}; Nimbus® adjuvant (Syngenta Ltda.)); and iv) propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1} (PPZ+DFZ; 150 mL ha^{-1}; without adjuvant). These fungicides were chosen because these combinations are common in field applications and most of the registered fungicides for disease control in soybean plants are from the triazole, strobilurin and carboxamide chemical groups. The fungicides were applied at pre-bloom (V8) and bloom (R1) stages of the soybean plants, with the addition of the adjuvant when recommended by the manufacturer.

The fungicide application was carried out using a CO_2-charged hand boom sprayer equipped with 4 Tee Jet nozzles, which delivered 150 L ha^{-1}, as recommended by the manufacturer for disease control on soybeans. Fungicide application was performed directly over the plants by keeping the bar at 0.4 m high from the top of the plants. The doses were sequentially applied on a windless day, thus ensuring uniform exposures of plants. The plants of each treatment were at a safe distance to avoid mixing doses. All plants were kept at 90% of field capacity of the substrate and absent of pests and diseases throughout the cycle.

The experimental design was randomized blocks with four treatments (control and 3 fungicides) and four replicates for both experiments.

2.2 Phytotoxicity

The visual symptoms on soybean leaves were photographed in R1 stage, 7 days (Zuntini et al., 2019) after the second application of fungicide. The images were obtained with a digital camera (Fujifilm FinePix, SL 300, Brazil) in optical zoom 30x, 14 megapixels and high LCD resolution.

2.3 Physiological traits

2.3.1 Gas exchange

Gas exchange were measured in soybean plants at stage R1, five days after the second application of fungicide to determine the maximum net photosynthetic rate (*A*, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_S, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and the dark respiration (R_D, µmol CO_2 m^{-2} s^{-1}). The parameters were measured using an infrared gas analyzer (IRGA, model LI-6400XTR, LI-COR, Lincoln, NE, USA).

The evaluations were performed in fully expanded leaves. The A, g_S, and E measurements were performed between 9:00 and 11:00 am under constant photosynthetic photon flux density (PPFD, 1000 µmol photons m^{-2} s^{-1}), atmospheric CO_2 concentration (C_a) (400 ± 25 µmol mol^{-1}), and at environmental temperature (26 ± 1 ºC) and relative humidity (74 ± 12%). For R_D measurement, the leaves were dark-adapted for at least 5 h, and the evaluation was performed after 10:00 pm. The respiration was assessed the night before photosynthetic measurements.

2.3.2 Chlorophyll *a* fluorescence

Variables of chlorophyll fluorescence were performed in the same leaf of the photosynthesis measurements using a modulated portable fluorometer coupled to IRGA. First, evaluations were conducted on dark-adapted leaves, so that the reaction centers were fully opened (all oxidized primary electron acceptors) with minimum heat loss. Under this condition, it was possible to estimate the initial fluorescence (F_0), maximum fluorescence (F_m), and potential quantum yield of photosystem II [$F_v/F_m = (F_0-F_m)/F_m)$] (Genty et al., 1989). After the light adaptation of leaves, the chlorophyll fluorescence before saturation pulse (F) and the maximum fluorescence in light-adapted leaves (F_m’) were evaluated, and used to estimate the effective quantum yield of PSII [$Y_{II} = (F_m’-F)/F_m’$] (Genty et al., 1989). The quantum yield of regulated energy dissipation [$Y_{NPQ} = (F/F_m’) - (F/F_m)$] was calculated according to (Genty et al., 1989) and (Hendrickson et al., 2004). The Y_{II} was also used to estimate the apparent electron transport rate (ETR = Y_{II}.PPFD.A_{leaf}.0,5) (Bilger et al., 1995), where PPFD is the photons flow (µmol m^{-2} s^{-1}) on the leaves; A_{leaf} the amount corresponding to the fraction of incident light that is absorbed by the leaves; and 0.5 the excitation energy fraction directed to the PSII (Laisk and Loreto, 1996).

2.4 Pollen grain viability

Flower buds were collected at the R1 stage for all treatments, 24 h after fungicide application. For each plant, about 5 flower buds were collected, fixed in Carnoy solution (ethanol 75% and glacial acetic acid 25%), and stored at -20 °C. The evaluation of the possible effects of fungicides on pollen viability was estimated by counting the mature pollen for each plant. Slides were prepared by macerating anthers from three floral buds in aceto carmine (1%). The material was covered with cover glass and images were obtained under an Olympus microscope (BX61, Tokyo, Japan), coupled to a DP-72 camera, using the clear field option. Stained pollen grains were considered viable, whereas unstained pollen grains were considered inviable (Gupta et al., 2018). At least 300 pollen grains were counted per experimental unit.

2.5 Pollen grain germination

In vitro grain pollen germination was measured 24 h after fungicide application. The culture medium consisted of sucrose (15%), calcium nitrate (0.03%), boric acid (0.01%), agar (0.6%) and Milli-Q water. The solution was slowly heated for complete agar dissolution (Koti et al., 2005). Five freshly open flowers were randomly collected from each experimental unit and pollen from each flower was homogenously spread on a on a glass slide containing 100 µL of culture medium. The glass slides were kept in a Petri dish with water saturated atmosphere, and incubated at 25 ºC for 2 h. After the germination, the glass slide was covered with a cover slip and analysed under an Olympus microscope (BX61, Tokyo, Japan), coupled to a DP-72 camera, using the clear field option. The pollen grains were considered germinated when the pollen tube exceeded the diameter of the own pollen grain (Benkő et al., 2020). At least 100 pollen grains were counted per experimental unit.

2.6 Flower abortion

The number of aborted reproductive structures was evaluated from the moment the plants reached the R1 developmental stage. Flower abortion was recorded every three days until the end of the cycle. Flower abortion percentage per plant was calculated in relation to the total number of reproductive structures produced by the plants.

2.7 Soybean yield components

The pods were harvested manually when the grains have reached physiological maturity in order to determine the soybean yield components. We evaluated pod number per plant, total grains per plant, aborted grains per plant (%), hundred-grain weight (g) and total production (g.plant^{-1}).

2.8 Statistical analysis

The obtained data were submitted to factorial analysis of variance and the means were compared by Tukey test ($p < 0.05$), using the statistical software SISVAR (Analysis of Variance System, version 5.4, Lavras, Brazil).

3 RESULTS

Both in experiment I and II fungicides CPZ+DFZ and PPZ+DFZ did not cause visual phytotoxicity effects on soybean plants seven days after treatment application (Figure 1-A, B, C, D and G). Fungicide AZB+BZP caused visual symptoms of mild phytotoxicity, characterized as small necrotic dots over the leaf blade, which did not increase with time (Figure 1-E and F).

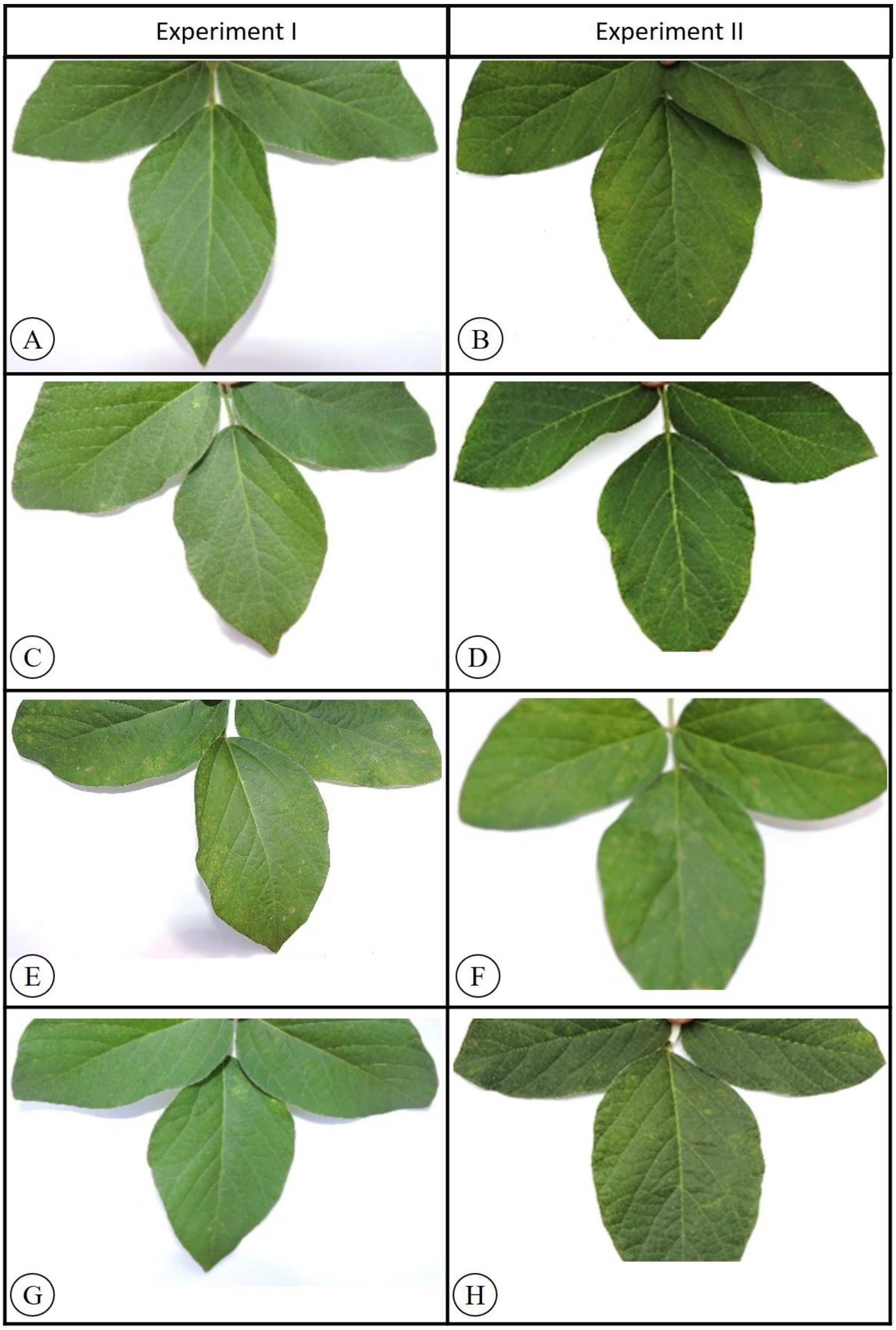

Figure 1. Leaves of soybean plants exposed to control (A and B) and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages: (C and D) CPZ+DFZ (ciproconazole 150 g L-1 + difenoconazole 250 g L-1), (E and F) AZB+BZP (azoxystrobin 300 g Kg-1 + benzovindiflupir 150 g Kg-1) and (G and H) PPZ+DFZ (propiconazole 250 g L-1 + difenoconazole 250 g L-1), in the experiments I (A, C, E and G) and II (B, D, F, H), 7 days after the treatment applications, of experiments I and II.

In general, fungicide application did not cause changes in the photosynthetic rate (*A*), transpiratory rate, stomatal conductance (g_S) (*E*) and dark respiration (R_D) of soybean plants (Table 1). The fungicide AZB+BZP reduced R_D only in experiment II.

Table 1. Net photosynthetic rate (*A*), stomatal conductance (g_S), transpiration rate (*E*) and the dark respiration (R_D) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages (assessments were performed five days after R1 application): CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) e PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}).

Treatment	*A* (μmol CO_2 m^{-2} s^{-1})	g_S (mol H_2O m^{-2} s^{-1})	*E* (mmol H_2O m^{-2} s^{-1})	R_D (μmol CO_2 m^{-2} s^{-1})
Experiment I				
Control	21.89 ± 0.81a	0.45 ± 0.16a	7.78 ± 0.33a	2.41 ± 0.17a
CPZ+DFZ	21.95 ± 1.56a	0.52 ± 0.09a	7.66 ± 0.24a	2.08 ± 0.12a
AZB+BZP	22.10 ± 1.63a	0.32 ± 0.09a	7.24 ± 0.29a	2.23 ± 0.35a
PPZ+DFZ	20.15 ± 1.01a	0.55 ± 0.15a	7.92 ± 0.47a	2.70 ± 0.78a
Experiment II				
Control	22.59 ± 2.22a	0.62 ± 0.08a	5.41 ± 0.53a	2.37 ± 0.38ab
CPZ+DFZ	22.24 ± 1.49a	0.78 ± 0.04a	6.30 ± 0.18a	2.49 ± 0.18a
AZB+BZP	23.83 ± 1.40a	0.76 ± 0.05a	6.20 ± 0.22a	1.32 ± 0.10b
PPZ+DFZ	25.27 ± 1.81a	0.80 ± 0.05a	6.44 ± 0.26a	2.01 ± 0.29ab

Mean ± SEM ($n = 4$), followed by the same letter in the column, do not differ significantly from each other, in each experiment, as determined by Tukey's test ($p > 0.05$).

We did not observe significant effects of the fungicides on potential quantum yield of PSII (F_v/F_m), electron transport rate (ETR), effective quantum yield of PSII (Y_{II}) and quantum yield of regulated energy dissipation (Y_{NPQ}), in both experiments (Table 2).

Table 2. Potential quantum yield of PSII (F_v/F_m), electron transport rate (ETR), effective quantum yield of PSII (Y_{II}) and quantum yield of regulated energy dissipation (Y_{NPQ}) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages (assessments were performed five days after R1 application): CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) e PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}).

Treatment	F_v/F_m	ETR	Y_{II}	Y_{NPQ}

Experiment I				
Control	0.83 ± 0.001a	145.15 ± 3.19a	0.33 ± 0.01a	0.36 ± 0.03a
CPZ+DFZ	0.83 ± 0.004a	137.31 ± 7.73a	0.32 ± 0.02a	0.32 ± 0.03a
AZB+BZP	0.83 ± 0.002a	142.27 ± 6.56a	0.33 ± 0.02a	0.33 ± 0.03a
PPZ+DFZ	0.82 ± 0.005a	135.89 ± 6.39a	0.31 ± 0.01a	0.32 ± 0.03a
Experiment II				
Control	0.82 ± 0.006a	151.04 ± 7.86a	0.35 ± 0.02a	0.33 ± 0.04a
CPZ+DFZ	0.81 ±0.006a	137.90 ± 7.33a	0.32 ± 0.02a	0.36 ± 0.02a
AZB+BZP	0.83 ± 0.002a	149.02 ± 6.82a	0.34 ± 0.02a	0.33 ± 0.02a
PPZ+DFZ	0.82 ± 0.006a	153.11 ± 8.62a	0.35 ± 0.02a	0.32 ± 0.03a

Mean ± SEM (*n* = 4), followed by the same letter, in the column, do not differ significantly from each other, in each experiment, as determined by Tukey's test (p > 0.05)

Pollen grain germination was reduced by fungicide AZB+BZP only in experiment I (Table3, Figure 2), despite pollen viability was not affected by any of the fungicides in both experiments (Table 3, Figure 3).

Table 3. Pollen grain viability and pollen grain germination of soybean plants exposed to control and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages: CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) e PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}).

Treatment	Pollen grain viability (%)	Pollen grain germination (%)
Experiment I		
Control	95.29 ± 1.31a	80.29 ± 2.86ab
CPZ+DFZ	93.57 ± 1.66a	85.39 ± 1.55a
AZB+BZP	92.33 ± 1.62a	76.72 ± 1.63b
PPZ+DFZ	97.14 ± 0.77a	81.11 ± 1.75ab
Experiment II		
Control	98.31 ± 0.65a	97.13 ± 0.42a
CPZ+DFZ	91.65 ± 0.11a	81.14 ± 6.24a
AZB+BZP	93.38 ± 2.10a	92.34 ± 2.15a
PPZ+DFZ	88.61 ± 4.68a	89.44 ± 5.68a

Mean ± SEM (*n* = 4) followed by the same letter in the column, do not differ significantly from each, in each experiment, other as determined by Tukey's test (p > 0.05).

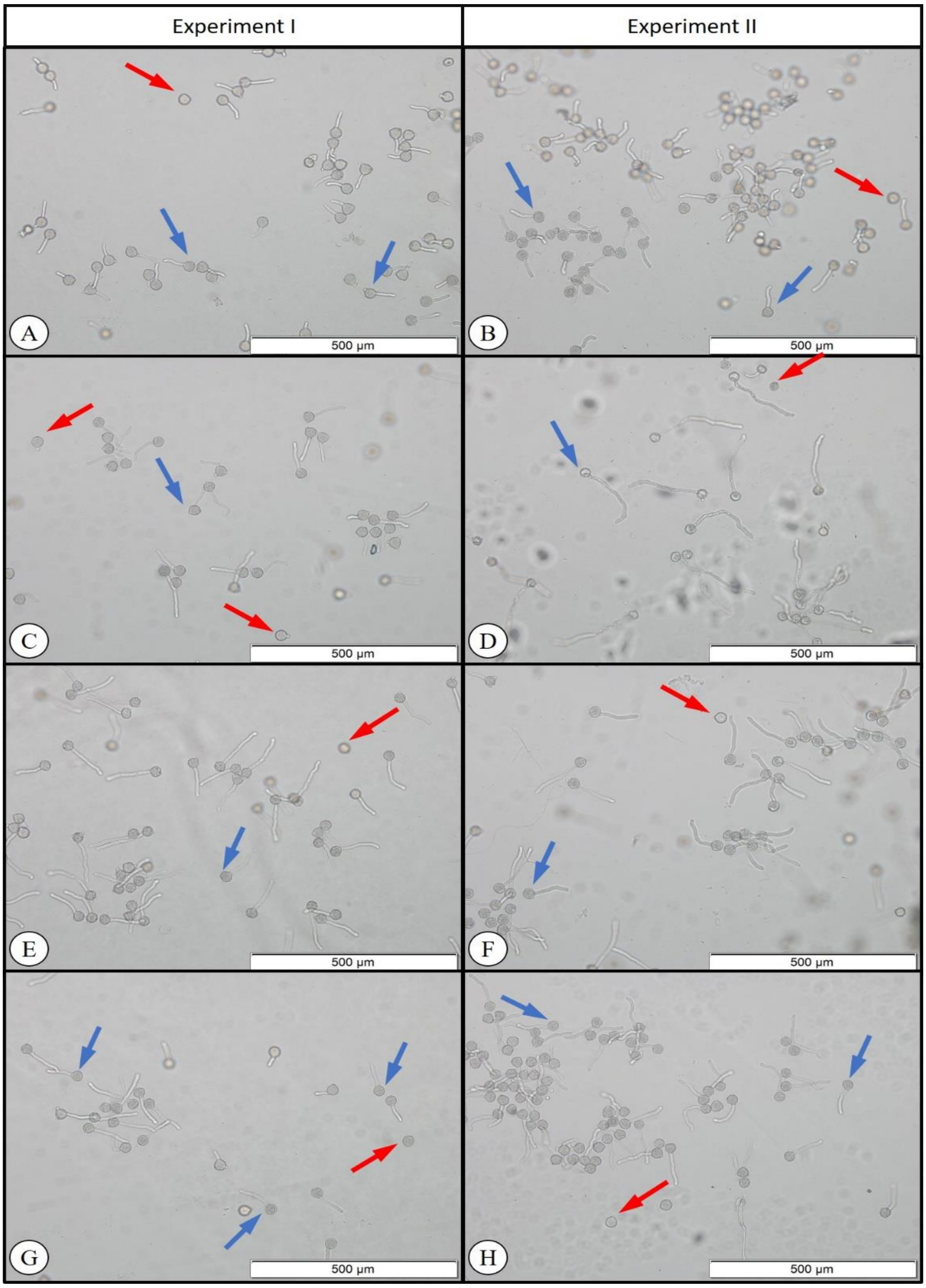
Experiment I
Experiment II
A
500 µm
B
500 µm
C
500 µm
D
500 µm
E
500 µm
F
500 µm
G
500 µm
H
500 µm

Figure 2. Pollen grain germination of soybean plants exposed to control (A and B) and fungicide in pre-bloom (V8) and bloom (R1) growth stages: (C and D) CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), (E and F) AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) and (G and H) PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}), of experiments I (A, C, E and G) and II (B, D, F, H). Blue arrows indicate germinated pollen grains and red arrows indicate not germinated pollen grains, in each experiment. Bar = 500 μm and 200 μm.

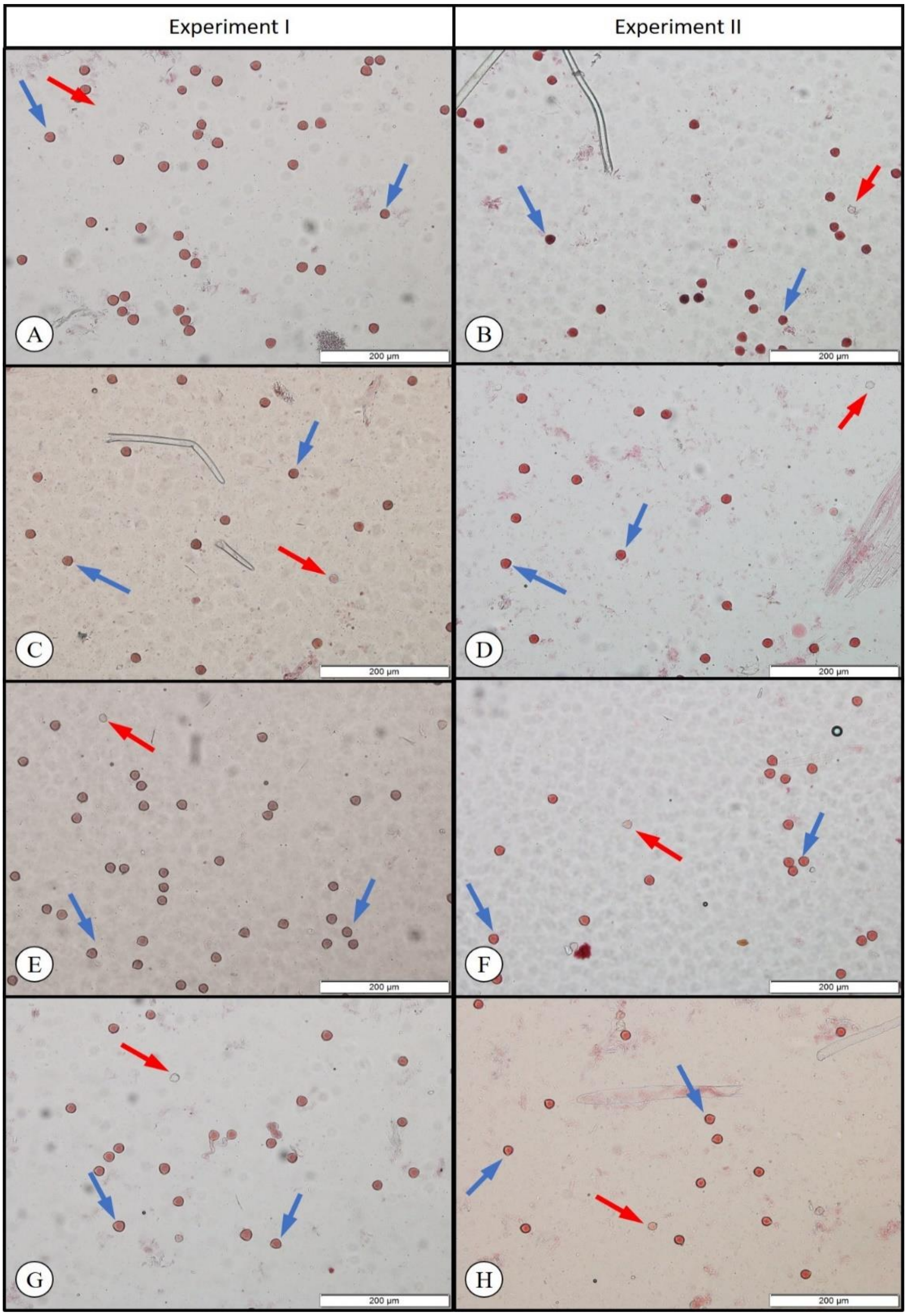

Figure 3. Pollen grain viability of soybean plants exposed to control (A and B) and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages: (C and D) CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), (E and F) AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) and (G and H) PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}), in the experiments I (A, C, E and G) and II (B, D, F, H). Blue arrows indicate viable pollen grains and red arrows indicate inviable pollen grains, in each experiment. Bar = 500 µm and 200 µm.

The yield components were not affected by any of the fungicides in experiment I (Table 4 and 5). In experiment II there was a reduction in pod number and total grains of control treatment when compared to AZB+BZP (Table 4). Also, control plants showed reduced hundred-grain weight values when compared to CPZ+DFZ and PPZ+DFZ (Table 5). These changes did not affect total production among the treatments in experiments I and II (Table 5).

Table 4. Flower abortion, pod number, total grains and aborted grains of soybean plants exposed to control and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages: CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), AZB+BZP (azoxystrobin 300 g Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) e PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}).

Treatment	Flower abortion (%)	Pod number	Total grains	Aborted grains (%)
Experiment I				
Control	72.30 ±2.75a	108.75 ± 12.51a	239.00 ± 25.5a	8.25 ± 2.17a
CPZ+DFZ	69.98 ± 4.09a	111.75 ± 22.68a	239.25 ± 55.0a	16.25 ± 4.03a
AZB+BZP	73.39 ± 2.02a	111.75 ± 8.42a	239.00 ± 17.1a	12.50 ± 3.12a
PPZ+DFZ	69.90 ± 3.78a	109.00 ± 8.15a	236.50 ± 18.9a	18.00 ± 4.71a
Experiment II				
Control	75.66 ± 3.59a	81.50 ± 8.67b	182.00 ± 21.78b	13.75 ± 4.53a
CPZ+DFZ	66.72 ± 2.75a	85.50 ± 8.86ab	193.50 ± 21.88b	13.75 ± 0.85a
AZB+BZP	63.58 ± 3.69a	114.75 ± 8.88a	280.25 ± 18.82a	13.25 ± 1.97a
PPZ+DFZ	69.02 ± 1.23a	93.25 ± 3.47ab	210.75 ± 2.17ab	13.00 ± 4.42a

Mean ± SEM (*n* = 4), followed by the same letter, in the column, do not differ significantly from each other, in each experiment, as determined by Tukey's test ($p > 0.05$)

Table 5. Hundred-grain weight and total production of soybean plants exposed to control and fungicide treatments in pre-bloom (V8) and bloom (R1) growth stages: CPZ+DFZ (ciproconazole 150 g L^{-1} + difenoconazole 250 g L^{-1}), AZB+BZP (azoxystrobin 300 g

Kg^{-1} + benzovindiflupir 150 g Kg^{-1}) e PPZ+DFZ (propiconazole 250 g L^{-1} + difenoconazole 250 g L^{-1}).

Treatment	Hundred-grain weight (g)	Total production (g.plant^{-1})
Experiment I		
Control	12.03 ± 0.88a	393.08 ± 20.87a
CPZ+DFZ	12.78 ± 1.01a	372.75 ± 60.47a
AZB+BZP	11.04 ± 1.20a	432.25 ± 58.24a
PPZ+DFZ	12.75 ± 0.78a	376.50 ± 47.10a
Experiment II		
Control	10.00 ± 0.46c	344.50 ± 28.00a
CPZ+DFZ	12.99 ± 0.37a	316,25 ± 55.61a
AZB+BZP	10.98 ± 0.42bc	317.50 ± 12.36a
PPZ+DFZ	12.11 ± 0.61ab	303.25 ± 21.61a

Mean ± SEM (*n* = 4) followed by the same letter in the column, do not differ significantly from each, in each experiment, other as determined by Tukey's test ($p > 0.05$).

4 DISCUSSION

Fungicide application, according to technical recommendation, does not affect photosynthetic metabolism, pollen grain viability and yield components of healthy soybean plants, under controlled conditions. Fungicides are expected to control disease and maintain maximum crop productivity (Shahid and Khan, 2018).

Specific symptoms associated with fungicide phytotoxicity can appear as interveinal chlorosis and they are reported following the application of several commercial products, as fluopyram, a succinate dehydrogenase inhibitor fungicide (Kandel et al., 2018) and mixtures of triazoles and strobilurins (Zuntini et al., 2019). In this study, we observed that the plants treated with the fungicide azoxystrobin + benzovindiflupyr (AZB+BZP) showed symptoms of mild phytotoxicity, evidenced by small necrotic dots over the leaf blade in both experiments. Phytotoxicity is commonly observed in plants treated with pesticides combined with mineral oil, as the adjuvant Nimbus® used in AZB+BZP-treatment. Although mineral oil is required to improve the biological efficiency of foliar sprays by increasing the penetration of the active ingredients into the leaf tissues (Räsch et al., 2018), the interaction between the adjuvant and the fungicide may increase cuticular permeability and intensify phytotoxicity potential (Appah et al., 2020). In addition, as mineral oil is derived from petroleum, it removes the leaf waxy layer, which makes the leaves exposed and sensitive to external agents and the epidermis may be injured, reducing growth and interfering in plant development (Assunção et al., 2019). Therefore, careful assessment of adjuvants under field conditions should be performed when it is necessary to mix adjuvant with the fungicide for the application.

Photosynthetic rates of soybean plants were not affected by either of the three fungicides tested. However, fungicide AZB+BZP reduced R_D in soybean plants of experiment II, which did not reflect in reduction in any other gas exchange traits. This response is expected from a fungicide as the AZB+BZP, which contains two chemical compounds that impairs cell respiration. AZB + BZP fungicide is formulated with a strobilurin (azoxystrobin) and a carboxamide (benzovindiflupyr). Strobilurins are compounds that inhibit cytochrome bc1 (mitochondrial complex III) of the fungal cell respiration chain, reducing ATP synthesis (Amaro et al., 2020). Complex III is the same for all eukaryotes, so a partial reduction in respiratory rate and ATP synthesis is expected in plants treated with strobilurins. Carboxamides are also mitochondrial electron transport chain (ETC) inhibitor fungicides, which lead fungi to death by interfering with succinate dehydrogenase (complex II). In the present study, AZB+BZP fungicide affected the R_D in the experiment II but not in experiment I. Mitochondrial ETC in plants has the alternative oxidase enzyme (AOX), which provides an alternative pathway for the oxidation of NADH in the presence of inhibitors of other complexes. AOX is also found in some fungi, many of which are pathogenic. It has been reported that the resistance of some fungi to mitochondrial inhibitor fungicides is due to the activation of alternative oxidase (Thomazella et al., 2012; Tian et al., 2020). (Nason et al., 2007) exposed wheat plants to five different strobilurins and observed that one of them reduced dark respiration in the leaves. The reduction in ATP production of respiration occurs when AOX is activated (Selinski et al., 2018). According to the authors, it happens because AOX is not proton pumping and as the electrons that flow to AOX bypass the proton pumping complexes III and IV, less ATP will be produced.

The fungicides did not affect the photochemical parameters of soybean disease-free plants. A fungicide containing a mixture of isopyrazam + epoxiconazole (carboxamide + triazole) applied in wheat plants in the absence of disease pressure resulted in higher effective quantum yield of PSII (Y_{II}), which positively correlated with increased biomass and yield (Ajigboye et al., 2014). The authors did not observe similar responses in isolated treatment with epoxiconazole or azoxystrobin; therefore, they attributed the increase in Y_{II} to isopyrazam.

Fagan et al. (2010) tested the effect of a strobilurin (pyraclostrobin) and a triazole (tebuconazole) on soybean plants and observed that pyraclostrobin increased photosynthetic rate, which reflected in higher thousand-grain weight and productivity of soybean plants in comparison to triazole-treated and control plants. The authors did not describe in the study whether the plants were totally disease-free or not. Marek et al. (2018) tested strobilurins and carboxamides on tomato plants inoculated with *Alternaria solani* and concluded that all fungicides improved photosynthetic efficiency and fruit production. Also, Zambiazzi et al. (2018) observed positive effects of triazole, strobilurin and carboxamide-based fungicides on soybeans, with improvement on physiological and production characteristics of the plants. Nevertheless, most of these authors tested the fungicide in disease control, thus, the higher photosynthetic efficiency and productivity is probably due to the fungal control, instead of improving the plant physiology itself. In the present study, disease-free soybean plants did not show increased physiological or production parameters as a result of fungicide applications.

Our study showed that fungicides AZB+BZP reduced pollen grain germination in the soybean cultivar SYN 1378C in experiment I. Despite of this reduction in pollen germination, which could lead to less grain production, the total grains of soybean plants treated by this fungicide was not smaller than the other treatments. Pollen viability was not reduced by any of the fungicides. Several researchers reported reduction in pollen viability and/or germination after fungicide application with a range of species. Diniconazole-M (triazole) and carboxin thiram (carboxamide-dimethyl dithiocarbamate) fungicides reduced, on average, 14% and 85%, respectively, the grain pollen germination of *Prunus persica* (Kargar and Imani, 2011) and *P. dulcis* (Zarrabi and Imani, 2011), with changes in their morphology. The authors recommend fungicide application in pre-bloom or to wait as long as possible during full bloom in order to prevent yield losses. Padilla et al. (2017) investigated the effect of pyraclostrobin + epoxiconazole on *Solanum betaceum* and *Rubus glaucus* pollen grains *in vitro* and observed *S. betaceum* was more sensitive. According to the authors, the fungicide reduced the germination of *S. betaceum* pollen grains by 63% and of *R. glaucus* by 18%, after 6 h, with pollen tube deformation in both species. There is a wide variety of responses depending on the applied fungicide and the species evaluated. However, the previously cited studies tested the fungicide directly on the culture media, which will hardly happen in the field. On the other hand, we observed in a previous study that the fungicide containing pyraclostrobin + epoxiconazole (strobilurin + triazole) applied in commercial dosage in *Zea mays* plants caused a reduction in pollen viability, but not in germination (Junqueira et al., 2017). To be more realistic, both in our previous study and in this one, the fungicide was applied to the plants via spraying, to simulate the procedure performed in the field. Even though it is an easy and quick phytotoxicity assessment, we believe that the effect of the fungicide is exacerbated when applied directly to the pollen grains in the culture medium.

In the present study, none of the fungicides tested affected any of the yield components. The absence of fungicide benefits in yield components of disease-free soybean plants was also observed by (Swoboda and Pedersen, 2009), which highlight that the use of fungicides in soybean plants should occur only for the management of diseases. Similar results were found by Mahoney and Gillard (2014), who investigated the effect of fungicides containing triazoles and strobilurins on disease-free *Phaseolus vulgaris* plants, and concluded that when there is low disease pressure, fungicide application does not contribute to higher production.

5. CONCLUSIONS

The application of three different commercial triazole, strobilurin or carboxamide–based fungicides during pre-bloom and bloom stage on soybean disease-free plants, did not affect the physiological traits, pollen grain germination and crop yield.

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CHAPTER II

Flower abortion, physiology, and production of soybean healthy plants treated with triazole, strobilurin and carboxamide fungicides

(Format according to the journal Pesticide Biochemistry and Physiology)

ABSTRACT

Fungicides are a key for agricultural crops' economic profitability, and foliar spraying has expanded dramatically in recent decades. However, these pesticides may interfere with plant’s metabolism causing reduction in physiological, reproductive and production traits. We proposed the hypothesis that fungicides from the chemical groups of triazoles, strobilurins and carboxamides may interfere in physiology and reproductive processes of disease-free soybean plants leading to a reduction in productivity. We aimed to evaluate the effect of the number of applications of three fungicides containing triazole, strobilurin and carboxamide compounds on gas exchange, chlorophyll *a* fluorescence, viability and germination of pollen grain, floral abortion and production component of disease-free soybean plants. The experiment was carried out using soybean cultivar SYN 1378C, disease-free plants, which were treated with the application of three commercial fungicides: epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1} (EPZ+FLX+PYR, ; 800 mL ha^{-1}; Assist® adjuvant 500 mL ha^{-1}(BASF Ltda.)); trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} (TFX+PRZ; 400 mL ha^{-1}; Aureo® adjuvant (Bayer Ltda.)); and trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1} (TFX+PRZ+BXF; 500 mL ha^{-1}; Aureo® adjuvant (Bayer Ltda.)); and a control treatment without any fungicide application. The plants received one (in R1 stage) two (in R1 and R2 stages) or three (in R1, R2 and R3 stages) fungicide applications. The experimental design was factorial with randomized blocks with four treatments (control and 3 fungicides), three applications and eight replicates. EPZ+FLX+PYR fungicide caused general reduction of photochemical traits of soybean plants. TFX+PRZ reduced the pod number of soybean plants after one application, which may have been reflected in higher HGW as compensation. TFX+PRZ and TFX+PRZ+BXF reduced soybean pollen germination in this study, but it did not reflect in lower yield components.

Key-words: *Glycine max*, photosynthesis, strobilurin, triazole, carboxamide, pollen.

1 INTRODUCTION

Fungicides are a key for agricultural crops' economic profitability, and foliar spraying has expanded dramatically in recent decades, mainly after losses of up to 4.6 million tons of soybeans in Brazil (Godoy et al., 2016) and total economic losses 51% higher in the United States (Bandara et al., 2020a) with the discovery of soybean rust (*Phakopsora pachyrhizi*), for example. The most common fungicide classes marketed for use in soybeans include demethylation inhibitors (DMI; also known as triazole), quinone outside inhibitors (QoI; also known as strobilurin), and succinate dehydrogenase inhibitor (SDHI) (also known as carboxamide) (Faske & Emerson, 2020). The fungicide families strobilurin and triazole are the most commonly employed for in-season soybean disease management (Bayer, 2021).

Triazoles were introduced on the market in the 1970s, when the introduction of more active and less phytotoxic fungicides started (Morton and Staub, 2008). Triazoles belong to the group of sterol biosynthesis inhibitors, which constitutes the largest and most important group of compounds ever developed for the control of fungal diseases in plants and animals, accounting for more than 30 % of global fungicide sales (Krämer et al., 2007). Strobilurin and carboxamides are mitochondrial electron transfer chain inhibitors. Strobilurins block electron transfer from cytochrome b to cytochrome c1 (Complex IV); while carboxamides inhibit the enzyme succinate dehydrogenase (Complex II), which impairs the electron transport to oxygen. The actions of both strobilurin and carboxamide interfere with the formation of ATP, which is the vital energy for fungal growth (Amaro et al., 2018). The first released strobilurin entered the market in 1996 (Barlett et al., 2002; Morton and Staub, 2008), and today they account for up to 25% of the global fungicide sales (Leadbeater et al., 2012). Carboxamides were developed in the 1960s, but only in the 2000s farmers began to use them to control plant diseases (Morton and Staub, 2008).

Soybean (*Glycine max* (L.) Merr.), which belongs to the Fabaceae (Leguminosae) family, is one of the most economically important legumes in the world. Grains are rich in proteins, unsaturated fatty acids, such as oleic and linoleic acids (omega 3, 6, and 9), and dietary fiber (Yao et al. 2020), being used for human food (Kim et al., 2015) and animal feed (Hassan et al., 2020). Soy oil, in addition to being an important food ingredient, also has wide industrial use, mainly in the production of biofuels, lubricants, and plastics. In addition, soybean cultivation in crop rotation improves soil fertility by providing nitrogen from the symbiosis with nitrogen-fixing bacteria, in addition to facilitating the management of diseases, insects, and weeds (Cordeiro and Echer, 2019). In the 2019/20 crop, world soybean production reached 339.70 MT, with Brazil (128.50 MT) and the United States (96.67 MT) accounting for 66.3% of global production (USDA, 2021).

Soybean plants are commonly treated with fungicides in the field at four main growth stages: early vegetative development stage (V4 or 25 days after emergence), bloom (R1 – before closing of rows in the field), R1 + 14 days (14 days after R1 stage) and R1+28 days (28 days after R1 stage). The use of fungicides on soybeans has increased dramatically since the mid-2000, mainly due to increased disease development and greater fungicide availability, but also because of reports of physiological benefits that contribute to a grain yield increase (Wise and Mueller, 2011; Tedford et al., 2017). Most of farmers have used fungicides preventively, even in the absence of diseases or with low disease pressure. This has gradually increased, and several studies have suggested the fungicide application even in disease-free plants to increase photosynthetic rates, which, consequently, could be favorable to productivity, in a phenomenon called “physiological effect” (Lopes et al., 2018; Pobezhimova et al., 2019; Amaro et al., 2020). However, there are few studies that actually use completely healthy plants as a control treatment; besides, some of the “physiological effects” occur after some kind of induced abiotic stress as drought (Ali et al., 2019). As a consequence, the number of available fungicides, as well as field application have increased, but little is known about their benefits in the absence of disease (Weisz et al., 2011; Wise and Mueller, 2011; Mallowa et al., 2015; Friskop et al., 2018).

On the other hand, a few researches have showed toxic effects of fungicides. According to some studies, fungicide spraying has negative effects on plant physiology, such as reduced growth, disruption of reproductive organ development, nitrogen and carbon metabolism changes (reviewed by Dias 2012, Petit et al. 2012, Sharma et al., 2019), without considering the environmental (Zubrod et al., 2019; Belsky and Joshi, 2020) and human (Preeti et al., 2015) risks caused by the excessive use of pesticides. Triazoles, strobilurins and carboxamides have mode of actions that may interfere in similar rotes that are found in plants. Sterol biosynthesis pathway – the target of triazoles on fungi – is similar to phytosterols biosynthesis pathway. Phytosterols are plant sterols found in plant cell membranes and they are precursors of the brassinosteroids, a group of plant hormones that control plant growth and development. Sterols also create lipid microdomains, which are involved in transmembrane signal transmission (Valitova et al., 2016). As both sterols in fungi and phytosterols on plants have similar biosynthesis pathways, it is expected that phytosterol biosynthesis may be compromised after a triazole spray (Darnet et al., 2020). Strobilurin and carboxamides targets also exist on plants, since mitochondrial complex II and complex III are present in all eukaryotes. Reductions in plant's respiration are commonly found after using these fungicides, however not in the extent to cause death, since plants have a strobilurin and carboxamide-insensitive alternative oxidase (Amaro et al., 2018).

In this study, we propose the hypothesis that fungicides from the chemical groups of triazoles, strobilurins and carboxamides may interfere in physiology and reproductive processes of disease-free soybean plants leading to a reduction in productivity. We aimed to evaluate the effect of the number of applications of three fungicides containing triazole, strobilurin and carboxamide compounds on gas exchange, chlorophyll *a* fluorescence, viability and germination of pollen grain, floral abortion and production component of disease-free soybean plants.

2 MATERIAL AND METHODS

2.1 Plant material and experimental conditions

The experiment was carried out in a greenhouse at the Laboratory of Ecophysiology and Plant Productivity at the Instituto Federal Goiano, Campus Rio Verde, Goiás, Brazil, with controlled temperature (~26 °C) and relative humidity (62 – 86 %). Soybean seeds (SYN 1378 C, Syngenta, Brazil) were sown in 12 dm^3 plastic pots containing substrate prepared from a mixture of Red Latosol (LVdf) soil and sand (2:1), with the following physicochemical characteristics: pH H_2O – 5.8; P – 0.9 mg dm^{-3}; K – 9.0 mg dm^{-3}; Ca – 0.59 $cmol_c$ dm^{-3}; Mg – 0.17 $cmol_c$ dm^{-3}; Al – 0.05 $cmol_c$ dm^{-3}; H^+Al – 1.8 $cmol_c$ dm^{-3}; S – 0.8 mg dm^{-3}; B – 0.1 mg dm^{-3}; Cu – 0.5 mg dm^{-3}; Fe – 118.0 mg dm^{-3}; Mn – 16.7 mg dm^{-3}; Zn – 0.2 mg dm^{-3}; Na – 1.8 mg dm^{-3}; SB – 30 %; CTC – 2.6 $cmol_c$ dm^{-3}; OM – 6.2 %; clay – 38.5%; silt – 7.5 % and sand – 54 %. Liming was performed using dolomitic limestone, increasing the base saturation to 60% and the plants were fertilized with urea (CH_4N_2O, 0.5 g dm^{-3}), potassium chloride (KCl, 1.8 g dm^{-3}), mono-ammonium phosphate (MAP, 1.5 g dm^{-3}), magnesium sulfate ($MgSO_4$, 0.8 g dm^{-3}), sulfate copper ($CuSO_4$, 0.05 g dm^{-3}), boric acid (H_3BO_3, 0.008 g dm^{-3}), and zinc sulfate ($ZnSO_4$, 0.003 g dm^{-3}), according to the recommendation for soybean crop (Sfredo, 2008).

2.2 Treatments and experimental design

Fungicides containing mixtures of triazole (epoxiconazole or prothioconazole), strobilurin (pyraclostrobin or trifloxystrobin) and/or carboxamide (fluxapiroxade or bixafen) were selected from commercially products commonly used to prevent and control diseases on soybean plants. The fungicides used were: epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1} (EPZ+FLX+PYR ; 800 mL ha^{-1}; Assist® adjuvant 500 mL ha^{-1} (BASF Ltda.)); trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} (TFX+PRZ; 400 mL ha^{-1}; Aureo® adjuvant (Bayer Ltda.)); and trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1} (TFX+PRZ+BXF; 500 mL ha^{-1}; Aureo® adjuvant (Bayer Ltda.)). The doses correspond to the manufacturer recommendation for soybean plants.

Fungicides were applied at three different soybean development stages at R1 (beginning of flowering); R2 (full flowering) and R3 (beginning of pods, ten days after the R2 application). The treatments consisted of a control treatment (without any fungicide application); and one (R1), two (R1 and R2), or three (R1, R2 and R3) applications of the commercial fungicides on soybean disease-free plants.

The fungicide applications were carried out using a CO_2-charged hand boom sprayer equipped with 4 Tee Jet nozzles, which delivered 150 L ha^{-1}, as recommended by the manufacturer for disease control on soybeans. Fungicide application was performed directly over the plants by keeping the bar at 0.4 m high from the top of the plants. The doses were sequentially applied on a windless day, thus ensuring uniform exposures of plants. The plants of each treatment were at a safe distance to avoid mixing doses. All plants were kept at 90% of field capacity of the substrate and absent of pests and diseases throughout the cycle.

The experimental design was factorial with randomized blocks with four treatments (control and 3 fungicides), three applications and eight replicates.

2.3 Gas Exchange

Gas exchange and chlorophyll *a* fluorescence traits were measured five days after each fungicide spray using an infrared gas analyzer coupled with a modulated fluorometer chamber (IRGA - LI-6400XTR, Licor®, Lincoln, Nebraska, USA). Photosynthetic rate (*A*, µmol CO2 m^{-2} s^{-1}), stomatal conductance (*g*s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H2O m^{-2} s^{-1}), the ratio between the internal and external concentration of CO_2 (C_i/C_a) and the dark respiration (R_D, µmol CO_2 m^{-2} s^{-1}) were measured on the last fully expanded leaf. From these data, the efficiency of carboxylation (A/C_i) was estimated. These measures were obtained under constant photosynthetically active radiation (PAR) (1000 µmol photons m^{-2} s^{-1}), atmospheric concentration of CO_2 (C_a) (~400,30 µmol mol^{-1}), temperature (~25,55 ºC) and humidity (~60,02%). R_D measurements were performed the night before the gas exchange assessments after 10:00 pm, with the leaves dark-adapted for at least 5 hours.

2.4 Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence assessments were performed on the same leaf of the photosynthesis measurements using IRGA. The initial fluorescence (F_0), maximum fluorescence (F_m), and potential quantum yield of photosystem II (PSII) [$F_v/F_m = (F_0-F_{m)}/F_{m)}$] were obtained on 30 min dark-adapted leaves. In light-adapted leaves, the steady-state fluorescence (F), the maximum fluorescence in light-adapted leaves (F_m’), the photochemical coefficient, which indicates the fraction of PSII centers that are open (qL = (F_m’–F)/(F_m’–F_0’) x (F_0’/F)) (Kramer et al., 2004)), the photochemical quenching (qP = (F_m’–F)/(F_m’–F_0’), the electron transport rate (ETR = Y_{II} x PPFD x A_{leaf} x 0,5) (Bilger et al., 1995), and the non-photochemical quenching (NPQ) [(Fm−Fm')/Fm'] was determined.

2.5 Pollen grain viability

In order to assess pollen grain viability, five flower buds were collected per plant 24 h after each fungicide application, immediately fixed in Carnoy solution containing ethanol (75%) and glacial acetic acid (25%), and stored at -20 °C until the analysis. For the analysis, slides were prepared by macerating anthers from floral buds in aceto carmine (1%) and the material was covered with cover glass. Images were obtained using an Olympus microscope (BX61, Tokyo, Japan), coupled to a DP-72 camera, with the clear field option. Pollen viability was estimated by counting the mature pollen for each plant, considering the stained pollen grains as viable, and unstained pollen grains as non-viable (Gupta et al., 2018). At least 300 pollen grains were counted per replicate. Pollen grain viability was not performed in R3 stage because there were no more appropriate flowers to collect in this stage of development.

2.6 Pollen grain germination

The pollen grain germination was determined in fresh material randomly collected from each experimental unit, 24 h after treatment application. fresh collected material. Pollen grains from five open flowers were homogenously spread on a glass slide containing 100 µL of the culture medium containing sucrose (15%), calcium nitrate (0.03%), boric acid (0.01%), agar (0.6%) and Milli-Q water prepared according to Koti et al. (2005). The glass slides were placed in Petri dishes with water saturated atmosphere, and incubated at 25 ºC. After 2 h, the glass slide was covered with a cover slip and the germination was analysed using an Olympus microscope (BX61, Tokyo, Japan), coupled to a DP-72 camera, with the clear field option. The pollen grains were considered germinated when the pollen tube exceeded the diameter of the pollen grain itself according to Benkő et al. (2020). At least 100 pollen grains were counted per replicate. Pollen grain germination was not performed in R3 stage because there were no more appropriate flowers to collect in this stage of development.

2.7 Flower abortion

The number of aborted flowers (flower buds and flowers) was counted from the first flower issued, and performed every three days, throughout the plant cycle. To ensure the most accurate counting of flower abortion, a structure was set up around and above the soil on each plant, where the fallen flowers were collected. The percentage of flower abortion was calculated in relation to the total number of reproductive structures, included young pods and pods, produced by the plant.

2.8 Soybean yield components

At physiological maturity stage, the pods were manually harvested to determine the pod number per plant (PN plant^{-1}), number of grains per pod (NG pod^{-1}), total grains per plant (TG plant^{-1}), hundred-grain weight (HGW, g) and grain yield (GY, g plant^{-1}) were assessed.

2.9 Statistical analysis

The data obtained were subjected to factorial analysis of variance by the F test ($p \leq 0.05$) and treatment means were compared by Tukey test ($p \leq 0.05$) using the Analysis of Variance System (SISVAR®, Analysis of Variance System, version 5.4, Lavras, Brazil). For multivariate analysis, missing values were estimated using Bayesian Principal Component Analysis (bPCA) (Schmitt; Mandel; Guedj, 2015) by initially modelling 10 Principal Components (nPCs), employing Q2 for cross-validation using the package pcaMethods (Stacklies et al., 2007). Data were scaled by means of mean-centering and scaled by the root-mean-square sqrt(sum(x^2)/(n-1)) using scale function in R (R CORE TEAM, 2021). PCAs (Principal Component Analysis) were modelled and graphs were generated using FactoMineR and factoextra packages, respectively (Kassambara; Mundt, 2020; Lê; Josse; Husson, 2008) using nPCs found while imputing data with bPCA.

3 RESULTS

Net photosynthetic rate (*A*) and stomatal conductance (g_S) had no significant interaction between treatments and number of fungicide applications. However, the *A* of EPZ+FLX+PYR-treated plants was around 17,8% lower than TFX+PRZ and TFX+PRZ+BXF treatments, after three fungicide applications (Table 1). The transpiration rate (*E*) was significantly higher in control and TFX+PRZ-treated plants after one fungicide application, when compared to three fungicide applications (Table 1).

The instantaneous efficiency of carboxylation (A/C_i) was higher for plants that received two fungicide applications than three sprays (Table 2). The ratio between internal and external CO_2 concentration (C_i/C_a) and the dark respiration (R_D) and were not affected by the fungicides, regardless of the number of applications (Table 2). The R_D was higher for EPZ+FLX+PYR-treated plants after two applications, when compared to three fungicides applications; and also, higher when compared with the TFX+PRZ+BXF-treated plants after the second fungicide spray (Table 2).

The initial fluorescence (F_0) had no significant effect for both treatments and number of applications (Table 3). The potential quantum yield of PSII (F_v/F_m) was significantly different for number of fungicide applications, and the effective quantum yield of PSII (Y_{II}) for the treatment. The F_v/F_m had lower values in control plants, after three fungicide applications when compared to one or two applications. For the EPZ+FLX+PYR-treated plants, F_v/F_m was lower after one application, when compared to two fungicide applications (Table 3). Higher F_v/F_m was observed after two fungicide applications when compared to one or three applications, regardless the treatments. Plants treated with EPZ+FLX+PYR had the lowest rates of Y_{II}, when compared to the control plants, after two fungicides applications (Table 3).

Photochemical quenching coefficient (q_L) of EPZ+FLX+PYR-treated plants was lower after three fungicide applications, when compared to the control plants. We observed higher q_L values for three fungicide applications when compared to one or two sprays for all treatments (Table 4). Electron transport rate (ETR) did not differ between treatments within each application period, but a lower ETR was observed for EPZ+FLX+PYR-treated plants after two and three fungicides applications, when compared to Control and TFX+PRZ+BXF, respectively. Non-photochemical quenching

(NPQ) was higher for plants treated with three fungicide applications than two sprays (Table 4).

The fungicides did not affect pollen viability among treatments, regardless the number of fungicide applications (Table 5, Figure 1). However, at the second fungicide application, TFX+PRZ promoted a lower pollen viability when compared to the Control (Table 5). Pollen germination was lower for TPX+PRZ+BXF-treated plants after one fungicide application, when compared to two applications. Also, at the first application, TFX+PRZ and TFX+PRZ+BXF reduced the pollen germination of soybean plants when compared to the Control treatment (Table 5, Figure 2). Higher flower abortion was observed after the first application for the fungicides TFX+PRZ and TFX+PRZ+BXF, compared to the second and third applications, respectively. Also, these treatments showed a higher flower abortion when compared to the EPZ+FLZ+PYR. On the other hand, at the third application the control plants had a higher flower abortion compared to the fungicide treatments (Table 5).

The rate of grain abortion (%) and the number of grains per pod were not affected by any fungicide treatment or the number of applications (Table 6). Whereas for pod number, the treatment TFX+PRZ showed lower values after the first fungicide application, compared to the third one and also comparing to EPZ+FLX+PYR (Table 6).

The total number of grains, hundred-grain weight (HGW) and grain yield did not differ significantly among treatments within each number of applications (Table 7). The TFZ+PRZ showed a lower value for total grain after the first fungicide application, when compared to the second and third ones. HGW was lower in EPZ+FLZ+PYR and TF+PRZ+BXF, compared to the second and third applications, respectively, also after one fungicide application. The grain yield was reduced by TFX+PRZ and TFX+PRZ+BXF after one fungicide application, when compared to three applications (Table 7).

Multivariate analysis of data obtained from soybean plants exposed to different numbers of application and fungicides showed that the three principal components (PCs), based on relative values, explained 53.7% of the total variation. The photochemical traits contributed mainly for the PC1 (26.1%), and FA and yield components, as PN, TG and GY, contributed to PC2 (17.0%) (Figure 3A). However, the high degree of overlap on the score plot indicated that there is no clear separation between treatments and control plants, regardless of the number of applications performed (Figure 3B).

Table 1. Net photosynthetic rate (*A*), stomatal conductance (g_S) and transpiration rate (*E*) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatments	*A* (µmol CO_2 m^{-2} s^{-1})			g_S (mol H_2O m^{-2} s^{-1})			*E* (mmol H_2O m^{-2} s^{-1})		
Number of applications	1	2	3	1	2	3	1	2	3
Control	21.67 ± 1.22Aa	21.74 ± 1.55Aa	19.66 ± 1.14Aab	0.61 ± 0.06Aa	0.64 ± 0.06Aa	0.65 ± 0.03Aa	7.47 ± 0.40Aa	6.59 ± 0.67ABa	6.17 ± 0.31Ba
EPZ+FLX+PYR	20.33 ± 1.62Aa	19.75 ± 2.66Aa	17.18 ± 1.46Ab	0.56 ± 0.03Aa	0.67 ± 0.05Aa	0.56 ± 0.04Aa	6.99 ± 0.41Aa	6.47 ± 0.24Aa	5.92 ± 0.56Aa
TFX+PRZ	20.53 ± 1.73Aa	21.51 ± 1.13Aa	20.83 ± 1.27Aa	0.56 ± 0.10Aa	0.57 ± 0.09Aa	0.65 ± 0.03Aa	6.85 ± 0.61Aa	6.53 ± 0.39ABa	6.35 ± 0.41Ba
TFX+PRZ+BXF	20.46 ± 0.92Aa	21.78 ± 0.99Aa	20.97 ± 1.01Aa	0.73 ± 0.16Aa	0.60 ± 0.09Aa	0.61 ± 0.02Aa	7.75 ± 0.61Aa	6.64 ± 0.42Aa	6.12 ± 0.33Aa
Control		21.02 ± 1.33 a			0.635 ± 0.049 a			6.74 ± 0.53 a	
EPZ+FLX+PYR		19.09 ± 2.00 a			0.594 ± 0.047 a			6.46 ± 0.45 a	
TFX+PRZ		20.96 ± 1.35 a			0.592 ± 0.078 a			6.58 ± 0.47 a	
TFX+PRZ+BXF		21.07 ± 0.96 a			0.647 ± 0.105 a			6.84 ± 0.54 a	
1 application		20.75 ± 1.36 A			0.615 ± 0.101 A			7.26 ± 0.52 A	
2 applications		21.19 ± 1.67 A			0.618 ± 0.071 A			6.56 ± 0.44 B	
3 applications		19.66 ± 1.36 A			0.618 ± 0.033 A			6.14 ± 0.40 B	
TREAT		0.0282			0.5895			0.6150	
NUMB		0.0635			0.9963			0.0002	
TREAT × NUMB		0.4886			0.3757			0.8408	
Block		0.0000			0.9491			0.6404	

Mean ± SEM (*n* = 8) followed by the same letter do not differ significantly according to Tukey’s test; capital letters in the line and lower-case letters in the column.
TREAT = treatment; NUMB = number of applications.

Table 2. Ratio between internal and external CO_2 concentration (C_i/C_a), instantaneous efficiency of carboxylation (A/C_i), and dark respiration (R_D) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatments	C_i/C_a			A/C_i			R_D (µmol CO_2 m^{-2} s^{-1})		
Number of applications	1	2	3	1	2	3	1	2	3
Control	0.82 ± 0.03Aa	0.83 ± 0.01Aa	0.85 ± 0.01Aa	0.07 ± 0.01Aa	0.07 ± 0.01Aa	0.06 ± 0.00Aa	1.79 ± 0.12Aa	2.39 ± 0.25Aab	2.18 ± 0.16Aa
EPZ+FLX+PYR	0.82 ± 0.02Aa	0.85 ± 0.01Aa	0.85 ± 0.00Aa	0.06 ± 0.01Aa	0.06 ± 0.01Aa	0.05 ± 0.00Aa	2.34 ± 0.19ABa	2.67 ± 0.67Aa	1.66 ± 0.12Ba
TFX+PRZ	0.81 ± 0.03Aa	0.78 ± 0.06Aa	0.84 ± 0.01Aa	0.07 ± 0.01Aa	0.08 ± 0.01Aa	0.07 ± 0.00Aa	2.13 ± 0.23Aa	1.94 ± 0.24Aab	1.58 ± 0.32Aa
TFX+PRZ+BXF	0.84 ± 0.03Aa	0.78 ± 0.07Aa	0.83 ± 0.01Aa	0.06 ± 0.00Aa	0.08 ± 0.01Aa	0.07 ± 0.00Aa	1.91 ± 0.16Aa	1.79 ± 0.20Ab	2.11 ± 0.40Aa
Control	0.832 ± 0.018 a			0.066 ± 0.005 a			2.119 ± 0.208 a		
EPZ+FLX+PYR	0.840 ± 0.015 a			0.060 ± 0.007 a			2.222 ± 0.432 a		
TFX+PRZ	0.807 ± 0.039 a			0.069 ± 0.008 a			1.882 ± 0.275 a		
TFX+PRZ+BXF	0.818 ± 0.041 a			0.069 ± 0.009 a			1.935 ± 0.271 a		
1 application	0.820 ± 0.025 A			0.065 ± 0.006 AB			2.041 ± 0.194 A		
2 applications	0.810 ± 0.045 A			0.072 ± 0.010 A			2.196 ± 0.403 A		
3 applications	0.843 ± 0.009 A			0.061 ± 0.005 B			1.882 ± 0.288 A		
TREAT	0.3808			0.1502			0.2581		
NUMB	0.1090			0.0301			0.1737		
TREAT × NUMB	0.5850			0.7109			0.0517		
Block	0.6721			0.0534			0.6667		

Mean ± SEM (n = 8) followed by the same letter do not differ significantly according to Tukey's test; lower case letters in the column and capital letters in the line.
TREAT = treatment; NUMB = number of applications.

Table 3. Initial fluorescence (F_0); potential quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII (Y_{II}) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatments	F_0			F_v/F_m			Y_{II}		
Number of applications	1	2	3	1	2	3	1	2	3
Control	632.7 ± 20.7Aa	617.0 ± 12.7Aa	614.6 ± 10.3Aa	0.818 ± 0.005ABa	0.827 ± 0.004 Aa	0.812 ± 0.006 Ba	0.31 ± 0.01Aa	0.34 ± 0.01Aa	0.32 ± 0.02Aab
EPZ+FLX+PYR	635.3 ± 16.8Aa	635.0 ± 9.9Aa	624.6 ± 35.2Aa	0.812 ± 0.005 Ba	0.822 ± 0.002 Aa	0.817 ± 0.004 ABa	0.31 ± 0.02 Aa	0.29 ± 0.04Ab	0.28 ± 0.02 Ab
TFX+PRZ	623.0 ± 13.1Aa	611.9 ± 8.0Aa	619.1 ± 15.4Aa	0.819 ± 0.004 Aa	0.826 ± 0.002 Aa	0.820 ± 0.004 Aa	0.30 ± 0.02 Aa	0.32 ± 0.02Aab	0.33 ± 0.02 Aab
TFX+PRZ+BXF	635.1 ± 19.7Aa	646.7 ± 11.1Aa	615.1 ± 10.3Aa	0.818 ± 0.006 Aa	0.824 ± 0.003 Aa	0.815 ± 0.005 Aa	0.31 ± 0.01Aa	0.31 ± 0.01Aab	0.34 ± 0.02 Aa
Control	621.46 ± 15.01 a			0.819 ± 0.006 a			0.324 ± 0.014 a		
EPZ+FLX+PYR	631.63 ± 22.29 a			0.817 ± 0.004 a			0.292 ± 0.025 b		
TFX+PRZ	618.01 ± 12.18 a			0.822 ± 0.004 a			0.315 ± 0.019 ab		
TFX+PRZ+BXF	632.32 ± 14.97 a			0.819 ± 0.005 a			0.317 ± 0.014 ab		
1 application	631.55 ± 17.10 A			0.817 ± 0.005 B			0.306 ± 0.015 A		
2 applications	627.66 ± 11.87 A			0.825 ± 0.003 A			0.314 ± 0.023 A		
3 applications	618.35 ± 19.60 A			0.816 ± 0.005 B			0.316 ± 0.019 A		
TREAT	0.3387			0.5352			0.0269		
NUMB	0.2674			0.0003			0.5865		
TREAT × NUMB	0.7443			0.6242			0.1920		
Block	0.0003			0.0012			0.0040		

Mean ± SEM (*n* = 8) followed by the same letter do not differ significantly according to Tukey's test; lower case letters in the column and capital letters in the line.
TREAT = treatment; NUMB = number of applications.

Table 4. Photochemical quenching coefficient (q_L), electron transport rate (ETR) and non-photochemical quenching (NPQ) of the youngest fully expanded leaf of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatment	q_L			ETR			NPQ		
Number of applications	1	2	3	1	2	3	1	2	3
Control	0.22 ± 0.02ABa	0.23 ± 0.01Ba	0.31 ± 0.01Aa	136.4 ± 4.7Aa	148.1 ± 5.1Aa	138.9 ± 7.4Aab	1.20 ± 0.18Aa	1.12 ± 0.15Aa	1.42 ± 0.10Aa
EPZ+FLX+PYR	0.22 ± 0.02Ba	0.18 ± 0.02Ba	0.24 ± 0.03Ab	133.0 ± 6.5Aa	124.9 ± 15.7Ab	123.5 ± 9.3Ab	1.19 ± 0.26Aa	1.15 ± 0.21Aa	1.30 ± 0.21Aa
TFX+PRZ	0.21 ± 0.02Ba	0.20 ± 0.02Ba	0.28 ± 0.02Aab	129.9 ± 9.8Aa	138.9 ± 7.6Aab	143.2 ± 7.1Aab	1.22 ± 0.18Aa	1.00 ± 0.09Aa	1.32 ± 0.11Aa
TFX+PRZ+BXF	0.21 ± 0.03Aa	0.20 ± 0.01Aa	0.28 ± 0.02Aab	133.2 ± 3.5Aa	134.3 ± 5.9Aab	147.3 ± 7.1Aa	1.15 ± 0.21Aa	1.12 ± 0.11Aa	1.18 ± 0.10Aa
Control	0.254 ± 0.024 a			141.10 ± 6.06 a			1.251 ± 0.154 a		
EPZ+FLX+PYR	0.214 ± 0.026 b			127.14 ± 10.87 b			1.214 ± 0.218 a		
TFX+PRZ	0.230 ± 0.025 ab			137.33 ± 8.29 ab			1.179 ± 0.140 a		
TFX+PRZ+BXF	0.233 ± 0.026 ab			138.26 ± 6.17 ab			1.150 ± 0.142 a		
1 application	0.216 ± 0.022 B			133.12 ± 6.34 A			1.190 ± 0.200 AB		
2 applications	0.204 ± 0.017 B			136.56 ± 9.86 A			1.101 ± 0.142 B		
3 applications	0.278 ± 0.024 A			138.20 ± 8.43 A			1.305 ± 0.136 A		
TREAT	0.0172			0.0258			0.6905		
NUMB	0.0000			0.4580			0.0296		
TREAT × NUMB	0.4489			0.2179			0.8638		
Block	0.0022			0.0039			0.0000		

Mean ± SEM (*n* = 8) followed by the same letter do not differ significantly according to Tukey's test; lower case letters in the column and capital letters in the line.
TREAT = treatment; NUMB = number of applications.

Table 5. Pollen viability (%), pollen germination (%) and flower abortion (%) of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Pollen viability and germination assessments were performed 24 h after each application and Flower abortion was counted every three days after R1 stage until plant senescence: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatment	Pollen viability (%)			Pollen germination (%)			Flower abortion (%)		
Number of applications	1	2	3	1	2	3	1	2	3
Control	96.80 ± 1.12Aa	96.80 ± 1.12Aa	ND	88.71 ± 4.37Aa	88.71 ± 4.37Aa	ND	73.98 ± 2.77Aab	73.98 ± 2.77Aa	73.98 ± 2.77Aa
EPZ+FLX+PYR	93.23 ± 3.25Aa	94.95 ± 1.01Aab	ND	86.24 ± 3.39Aab	88.78 ± 3.06Aa	ND	67.58 ± 3.82Ab	68.12 ± 2.83Aa	65.95 ± 2.58Aab
TFX+PRZ	94.65 ± 1.88Aa	90.73 ± 3.16Ab	ND	71.21 ± 7.84Ac	83.96 ± 3.74Aa	ND	77.20 ± 3.35Aa	68.71 ± 2.29Ba	69.78 ± 1.71ABab
TFX+PRZ+BXF	96.62 ± 2.17Aa	93.10 ± 2.60Aab	ND	78.20 ± 4.35Bbc	84.38 ± 4.04Aa	ND	77.95 ± 2.65Aa	72.16 ± 3.51ABa	65.19 ± 3.97Bb
Control	96.80 ± 1.07 a			88.71 ± 4.17 a			73.98 ± 2.64 a		
EPZ+FLX+PYR	94.09 ± 2.36 a			87.51 ± 3.17 ab			67.21 ± 3.01 b		
TFX+PRZ	92.69 ± 2.67 a			77.59 ± 6.63 c			71.90 ± 2.98 ab		
TFX+PRZ+BXF	94.86 ± 2.45 a			81.29 ± 4.30 bc			71.77 ± 4.05 ab		
1 application	95.33 ± 2.23 A			81.09 ± 5.91 B			74.18 ± 3.55 A		
2 applications	93.90 ± 2.31 A			86.46 ± 3.79 A			70.74 ± 2.95 AB		
3 applications	ND			ND			68.72 ± 3.16 B		
TREAT	0.0842			0.0000			0.0049		
NUMB	0.2072			0.0029			0.0046		
TREAT × NUMB	0.2252			0.0610			0.0634		
Block	0.0173			0.0000			0.1290		

Mean ± SEM (*n* = 8) followed by the same letter do not differ significantly according to Tukey's test; lower case letters in the column and capital letters in the line.
ND – Not Done – There were no appropriate flowers to pollen viability and germination assessments at R1+10 stage.
TREAT = treatment; NUMB = number of applications..

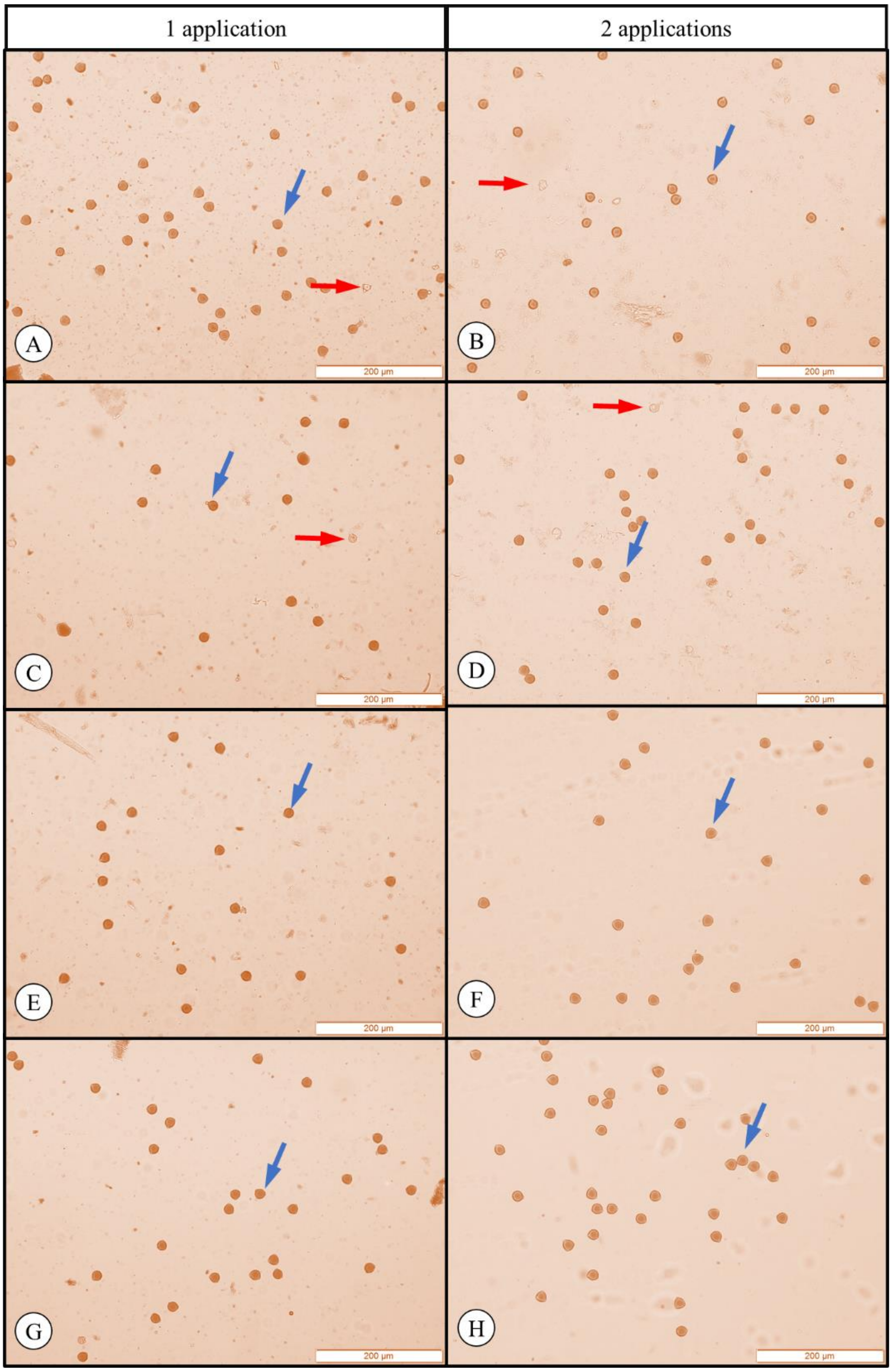

Figure 1. Pollen viability of soybean plants exposed to control and fungicide treatments with one application (in R1 stage) or two applications (in R1 and R2 stages). Assessments were performed 24 h after each application: control (without any fungicide application) (A and B); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) (C and D); TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) (E and F) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1}) (G and H). Blue arrows indicate viable pollen grains and red arrows indicate unviable pollen grains. Bar = 200 µm.

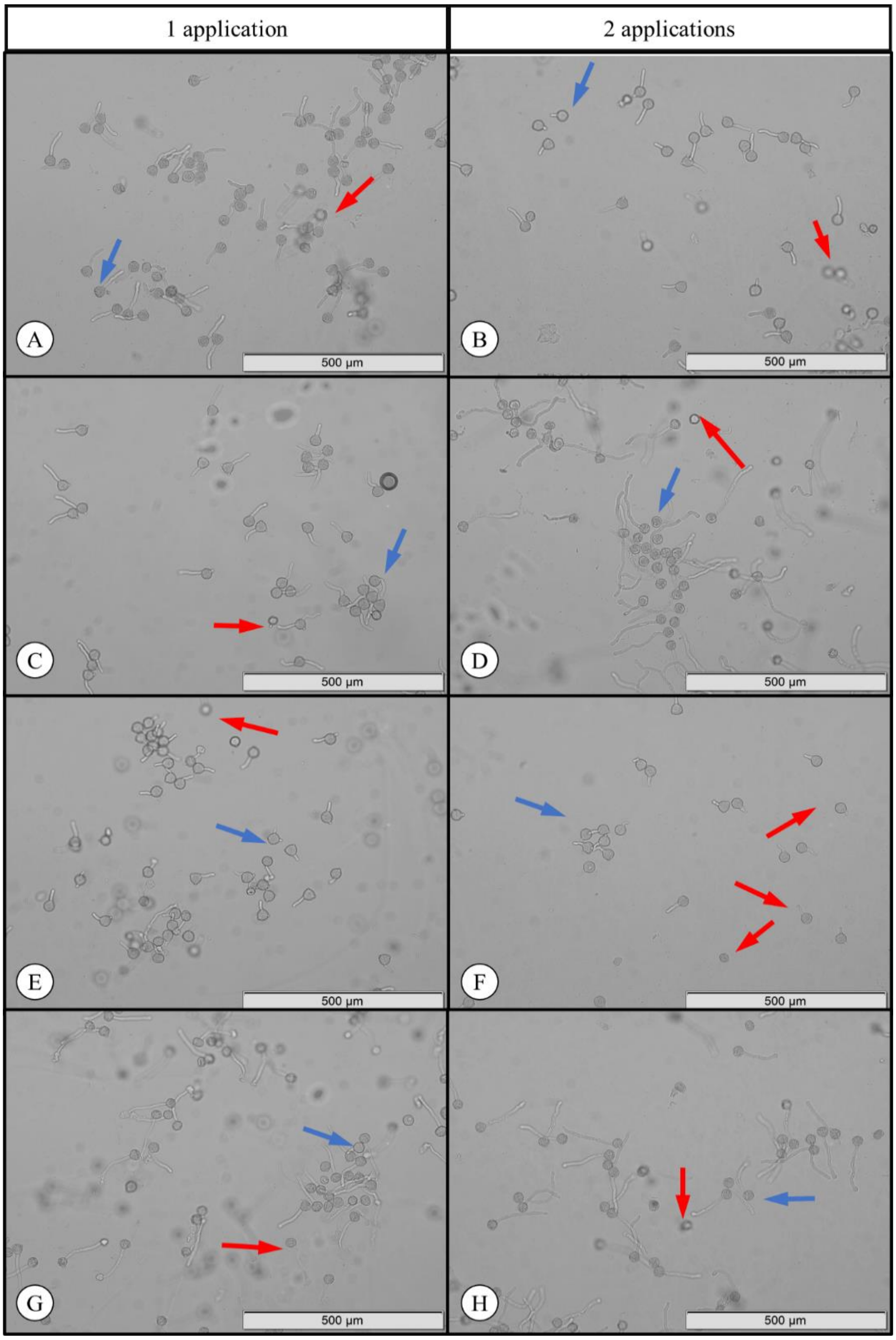

Figure 2. Pollen germination of soybean plants exposed to control and fungicide treatments with one application (in R1 stage) or two applications (in R1 and R2 stages). Assessments were performed 24 h after each application: control (without any fungicide application) (A and B); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) (C and D); TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) (E and F) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1}) (G and H). Blue arrows indicate germinated pollen grains and red arrows indicate not germinated pollen grains. Bar = 500 µm.

Table 6. Grain abortion (GA, %), pod number (PN, plant $^{-1}$) and grains per pod (NG, pod $^{-1}$) of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed at physiological maturity. Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatment	GA (%)			PN (plant^{-1})			NG (pod^{-1})		
Number of applications	1	2	3	1	2	3	1	2	3
Control	5.59 ± 1.76Aa	5.59 ± 1.76Aa	5.59 ± 1.76Aa	95.13 ± 11.04Aab	95.13 ± 11.04Aa	95.13 ± 11.04Aa	2.21 ± 0.03Aa	2.21 ± 0.03Aa	2.21 ± 0.03Aa
EPZ+FLX+PYR	6.90 ± 1.39Aa	6.29 ± 1.22Aa	4.48 ± 0.97Aa	107.25 ± 8.96Aa	97.75 ± 4.46Aa	117.00 ± 9.76Aa	2.23 ± 0.07Aa	2.17 ± 0.08Aa	2.29 ± 0.08Aa
TFX+PRZ	6.77 ± 1.97Aa	6.02 ± 1.60Aa	6.56 ± 1.11Aa	77.38 ± 15.19Bb	102.25 ± 6.18ABa	115.50 ± 9.17Aa	2.30 ± 0.09Aa	2.30 ± 0.08Aa	2.25 ± 0.04Aa
TFX+PRZ+BXF	6.10 ± 1.24Aa	6.11 ± 3.69Aa	5.84 ± 1.39Aa	96.50 ± 10.94Aab	103.75 ± 16.27Aa	112.25 ± 8.81Aa	2.17 ± 0.05Aa	2.22 ± 0.06Aa	2.20 ± 0.03Aa
Control	5.59 ± 1.69 a			95.13 ± 10.55 a			2.21 ± 0.03 a		
EPZ+FLX+PYR	5.89 ± 1.24 a			107.33 ± 8.51 a			2.23 ± 0.08 a		
TFX+PRZ	6.45 ± 1.54 a			98.38 ± 12.63 a			2.28 ± 0.07 a		
TFX+PRZ+BXF	6.01 ± 2.28 a			104.17 ± 12.22 a			2.20 ± 0.05 a		
1 application	6.34 ± 1.56 A			94.06 ± 12.19 B			2.23 ± 0.06 A		
2 applications	6.00 ± 2.17 A			99.72 ± 10.14 AB			2.23 ± 0.07 A		
3 applications	5.62 ± 1.32 A			109.97 ± 10.07 A			2.24 ± 0.05 A		
TREAT	0.8874			0.1934			0.0990		
NUMB	0.7449			0.0131			0.9326		
TREAT × NUMB	0.9652			0.1626			0.5506		
Block	0.0171			0.0006			0.0208		

Mean ± SEM ($n = 8$) followed by the same letter do not differ significantly according to Tukey’s test; lower case letters in the column and capital letters in the line.
TREAT = treatment; NUMB = number of applications.

Table 7. Total number of grains (TG, plant $^{-1}$), hundred-grain weight (HGW, g) and grain yield (GY, g plant^{-1}) of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed at physiological maturity. Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1})

Treatment	TG (plant $^{-1}$)			HGW (g)			GY (g plant^{-1})		
Number of applications	1	2	3	1	2	3	1	2	3
Control	210.5 ± 23.9Aa	210.5 ± 23.9Aa	210.5 ± 23.9Aa	11.01 ± 0.76Aa	11.01 ± 0.76Aa	11.01 ± 0.76Aa	23.62 ± 3.68Aa	23.62 ± 3.68Aa	23.62 ± 3.68Aa
EPZ+FLX+PYR	237.6 ± 16.3Aa	212.0 ± 12.2Aa	268.1 ± 24.6Aa	10.68 ± 0.43Ba	12.81 ± 0.93Aa	11.26 ± 0.35ABa	25.24 ± 1.57Aa	26.78 ± 1.31Aa	30.23 ± 2.96Aa
TFX+PRZ	175.1 ± 32.1Ba	236.4 ± 18.2Aa	258.5 ± 17.1Aa	12.34 ± 1.48Aa	11.52 ± 0.58Aa	11.96 ± 0.54Aa	20.14 ± 3.67Ba	27.26 ± 2.40ABa	30.74 ± 1.91Aa
TFX+PRZ+BXF	208.1 ± 23.6Aa	231.3 ± 36.9Aa	247.3 ± 20.6Aa	10.26 ± 1.36Ba	12.09 ± 0.52ABa	12.61 ± 0.48Aa	22.28 ± 4.21Ba	27.43 ± 3.52ABa	31.12 ± 2.74Aa
Control	210.50 ± 22.83 a			11.01 ± 0.72 a			23.62 ± 3.52 a		
EPZ+FLX+PYR	239.25 ± 20.49 a			11.58 ± 0.73 a			27.42 ± 2.20 a		
TFX+PRZ	223.33 ± 27.61 a			11.94 ± 0.94 a			26.05 ± 3.32 a		
TFX+PRZ+BXF	228.88 ± 27.67 a			11.65 ± 0.96 a			26.94 ± 3.77 a		
1 application	207.84 ± 25.47 B			11.07 ± 1.10 A			22.82 ± 3.37 B		
2 applications	222.53 ± 23.89 AB			11.86 ± 0.75 A			26.27 ± 2.83 AB		
3 applications	246.09 ± 22.92 A			11.71 ± 0.60 A			28.93 ± 3.09 A		
TREAT	0.2413			0.2490			0.1402		
NUMB	0.0096			0.1238			0.0006		
TREAT × NUMB	0.1283			0.0473			0.2641		
Block	0.0088			0.0003			0.0002		

Mean ± SEM (*n* = 8) followed by the same letter do not differ significantly according to Tukey's test; lower case letters in the column and capital letters in the line.
TREAT = treatment; NUMB = number of applications.

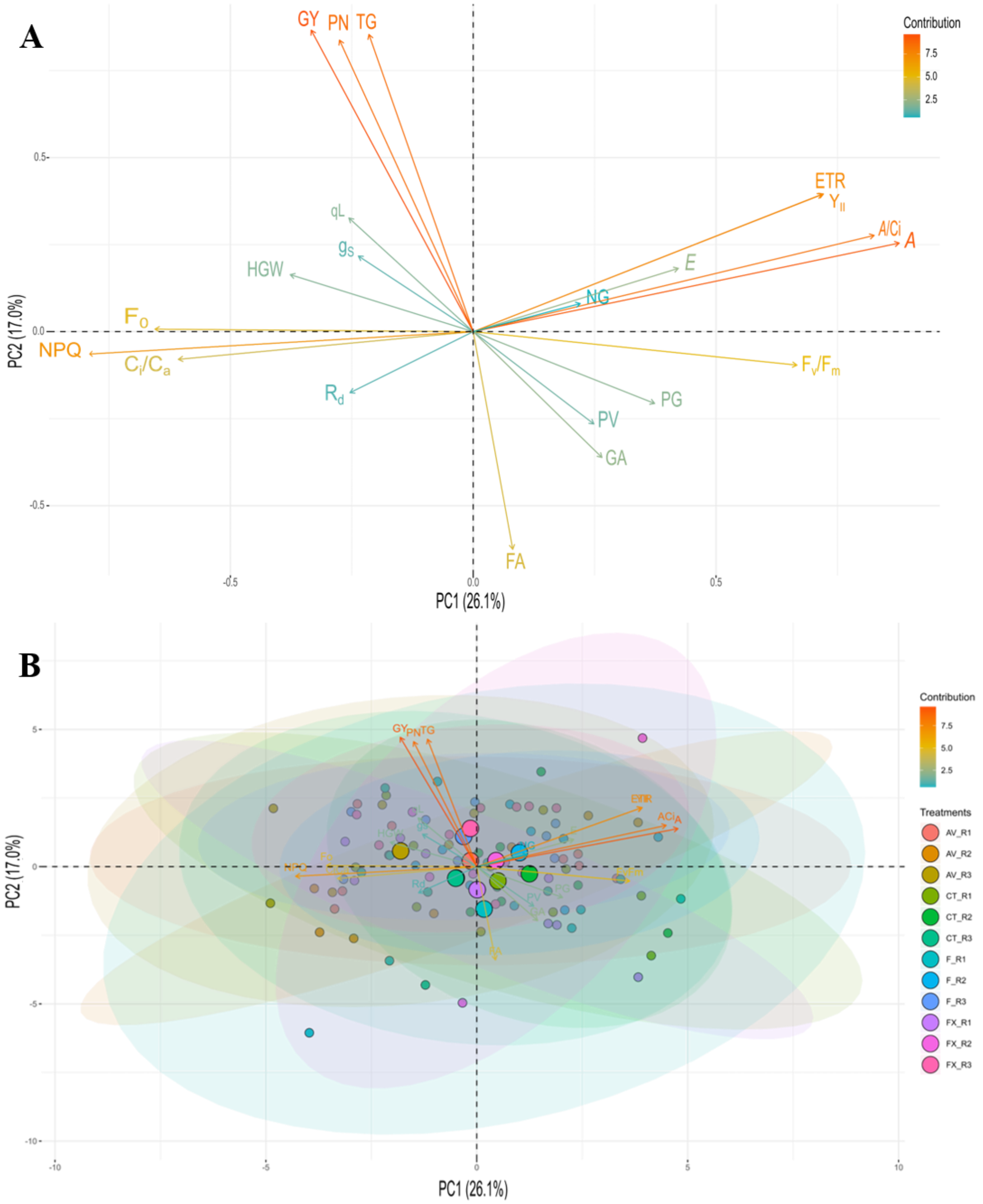

Figure 3. Principal component analysis (A) and score plot (B) for physiological and pollen grain traits, flower abortion and soybean yield components phytotoxicity, physiological, and morphological traits of soybean plants of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L-1 + fluxapiroxade 50 g L-1 + pyraclostrobin 81 g L-1) TFX+PRZ trifloxystrobin 150 g L-1 + prothioconazole 175 g L-1) and TFX+PRZ+BXF (trifloxystrobin 150 g L-1 + prothioconazole 175 g L-1 + bixafen 125 g L-1).

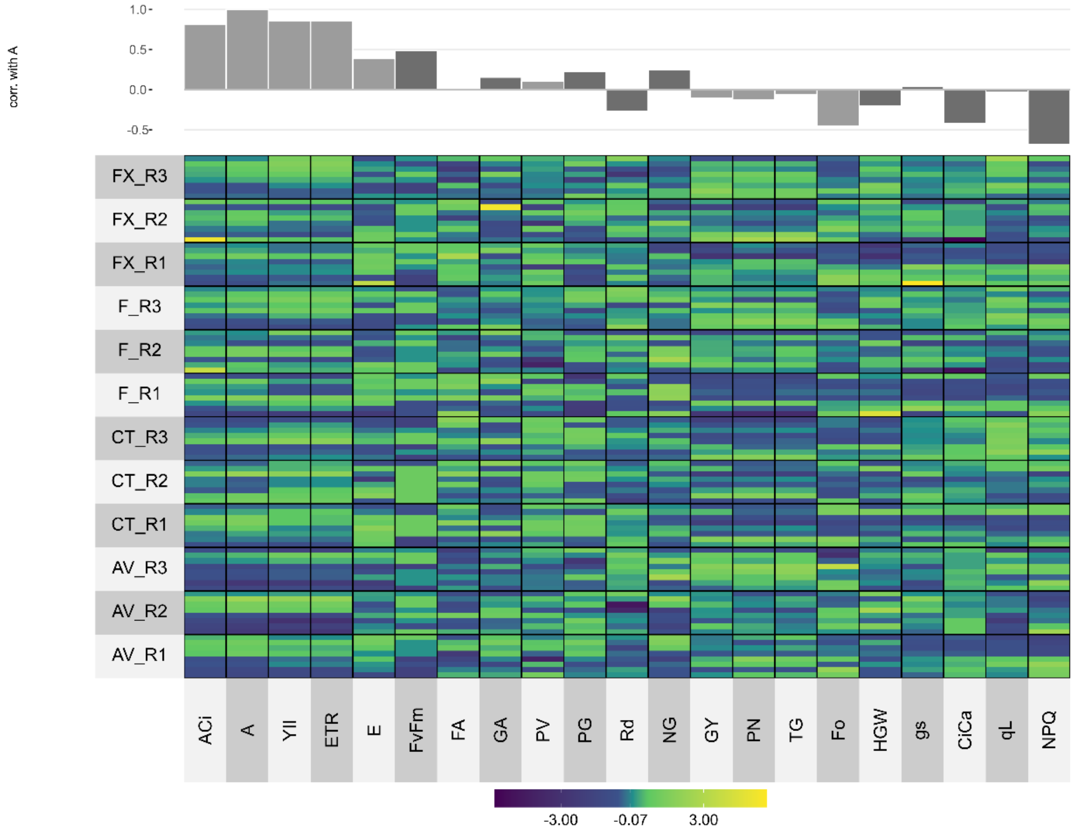

Figure 4. Heatmap for physiological and pollen grain traits, flower abortion and soybean yield components phytotoxicity, physiological, and morphological traits of soybean plants of soybean plants exposed to control and fungicide treatments with one application (in R1 stage), two applications (in R1 and R2 stages) and three applications (in R1, R2 and R3). Assessments were performed five days after each application: Control (without any fungicide application); EPZ+FLX+PYR (epoxiconazole 50 g L^{-1} + fluxapiroxade 50 g L^{-1} + pyraclostrobin 81 g L^{-1}) TFX+PRZ trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1}) and TFX+PRZ+BXF (trifloxystrobin 150 g L^{-1} + prothioconazole 175 g L^{-1} + bixafen 125 g L^{-1}).

4 DISCUSSION

Triazole, strobilurin and carboxamide fungicides are the most common chemical groups of fungicides used for disease control and prevention in soybean plants (Zuntini et al., 2019). In the present study, it was verified that, in general, physiological traits were not affected by any fungicide evaluated. Significant differences were found for the factor number of applications, which are probably more related to the development stage when the assessments were performed than to the exposure to the fungicides.

The assessments performed after one application in the R1 stage resulted in a higher transpiration rate, probably due to the higher plant metabolism expected in the reproductive stage in plants with adequate water supply and controlled temperature and humidity conditions, and not as a response to the fungicides applied. It was also observed by the highest efficiency of carboxylation found in R3 stage, when plants require a high photosynthetic efficiency to supply the development of pods, that appear with great intensity during this stage (Mundstock & Thomas, 2005). However, the pod number/*A*/Ci was not affected by any of the fungicides applied.

An increase in photosynthetic rate and carboxylation efficiency of Japanese cucumber plants treated with fungicides was observed by Amaro et al. (2020). The authors attributed this increase to a reduction in the respiratory rate expected in plants subjected to fungicides that contain strobilurins or carboxamides, chemicals that target complexes of the mitochondrial electron transport chain (Wood & Hollomon, 2003). However, although the authors concluded that the fungicides caused physiological effects that culminated in higher productivity, this fact was only observed in grafted cucumber plants (Amaro et al., 2020), a technique described to improve the productivity of cucumber plants, regardless of fungicide application (Bayoumi et al., 2021). Furthermore, our recent researches have shown that even in crops as corn and soybean, no improvement in physiological and production traits were observed when the plants were treated with strobilurin + triazole (Junqueira et al., 2017), or combinations of different triazoles (Junqueira et al., 2021), respectively.

Other studies report negative effects on physiological parameters such as photosynthetic rate, transpiration rate, stomatal conductance and internal CO_2 concentration after the application of strobilurins (Nason et al, 2007; Debona et al., 2016). Fluzilazole, a triazole molecule, was found to cause a little reduction in photosynthesis, stomata conductance and internal concentration of CO_2 of cucumber plants (Xia et al., 2006). Nonetheless, positive effects of fungicides on net photosynthesis have been found for corn (Blandino et al. 2012), beans (Jadoski et al. 2015) and soybeans (Fagan et al., 2010) exposed to strobilurins. The authors frequently attribute the increase in net photosynthetic rate to a reduction in respiration, which, directly interferes with the net assimilation rate (Bryson et al., 2000). Reduced respiration is commonly found after strobilurin application, due to its mode of action, that consists in the blockage of the electron transport chain in complex III (cytochrome bc_1) (Wood and Hollomon, 2003). However, in this study, only TFX+PRZ+BXF, which contains a triazole, a strobilurin and a carboxamide reduced soybean plant respiration after two applications, without affecting the photosynthetic rate. The combination of a strobilurin and a carboxamide intensifies the electron blockage, since carboxamides are also mitochondrial electron transport chain inhibitors, at complex II (succinate dehydrogenase). The mode of action of the fungicides targets the fungi, but, as the complexes II and III are also present in plants' mitochondria, a temporary reduction in plant respiration may occur.

Slight changes in potential quantum yield of PSII (F_v/F_m) were found for number of applications, but all values ranged from 0.81 to 0.83, which do not represent plant stress. In wheat plants treated with azoxystrobin, F_v/F_m was an extremely sensitive parameter, where values up to 0.22 were found for plants treated with fungicide concentration of 120 mg L^{-1} in hydroponic conditions (Lan et al., 2019). This dose is lower than the highest dose recommended by the manufacturer for wheat, however, the hydroponic growing medium allows for rapid absorption and high availability of the product for plants, which is often reduced in spraying due to the barriers that the product needs to overcome to be absorbed by plants. It is also important to emphasize that in this work we used the doses recommended by the manufacturer for all the fungicides tested, aiming to verify possible effects commonly seen by farmers. The EPZ+FLX+PYR reduced effective quantum yield of PSII (Y_{II}) and electron transport rate (ETR) with two and three applications, and decreased photochemical quenching coefficient (q_L) after three applications, resulting in 12.6% lower A. Fungicide EPZ+FLX+PYR has pyraclostrobin, a strobilurin fungicide that is believed to block the electron transfer between PSI and PSII, which may have been responsible for the impairments observed in the soybean plants. Strobilurins are reported to bind to the Qi site in the cytochrome *bf* complex in chloroplasts (Nason et al., 2007). Despite of the reduction in photochemical processes caused by EPZ+FLX+PYR fungicide, it did not reflect in decreased yield components of the soybean plants.

The pollen grain germination values were lower in plants that received only one fungicide spray in R1 than two applications (both in R1 and R2 stages). It is possible that the flowers at R1 stage are more sensitive than those at stage R2, as they are at the beginning of flowering, and therefore the exposure of the fungicide resulted in a reduction in pollen germination. In addition, given the stage of the fungicide spray and evaluation – which happened 24 h after each application –, full-bloom (R2) flowers are better established and probably less sensitive to chemical application. Concerning the treatments, control plants had a higher pollen germination rate when compared to TFX+PRZ and TFX+PRZ+BXF treatments, which suggests a phytotoxic effect of those fungicides. Both fungicides contain the same strobilurin molecule (trifloxystrobin) and triazole (prothioconazole), and they only differ by the carboxamide bixafen. The combination of trifloxystrobin and prothioconazole was found to be more damaging to pollen grain germination than the combination of epoxiconazole, pyraclostrobin and fluxapyroxade, which are also a triazole, a strobilurin and a carboxamide, respectively. Although the applied fungicides caused a significant reduction in the *in vitro* pollen grain germination, this was not reflected in a higher flower abortion rate, which was also observed in soybean plants treated with cyproconazole + difenoconazole, azoxystrobin + benzovindiflupyr and propiconazole + difenoconazole fungicides (Junqueira et al., 2021). Considering that the number of pollen grains produced is extremely superior to the number of ovules, the relation between these two traits will probably only exist under extreme stress conditions, such as high temperature stress, as described by Djanaguiraman et al. (2012).

A soybean plants is known to abort a large number of its flowers and pods, in order to keep on the plant only the flowers that can be supported with photoassimilates throughout the cycle (Passos et al., 2011). There is a concern among farmers regarding the no application of fungicides during flowering, due to the likely impacts on floral abortion. We, however, we have observed that the possible floral abortion occurring in plants treated with fungicide does not compromise yield components, such as the total number of grains, hundred-grain weight and grain yield in soybean plants exposed to six different commercial fungicides as observed by Junqueira et al. (2021) and in the present study.

Slight and isolated changes were observed in the yield components of soybean plants after different fungicide application. The TFX+PRZ caused a lower number of pods only for one application; however, no significant difference was found for grain yield among fungicide treatments. We also observed a lower pod number for plants treated once with TFX+PRZ. These plants showed higher values of HGW, which is probably to compensate the reduced number of pods produced. Also investigating soybean plants, Swoboda and Pedersen (2009) did not find differences in yield between fungicide-treated and untreated plants, but the authors mention a few differences in yield components, such as node production, total biomass and stem weight. Other results suggest that foliar fungicides confer yield benefits in mitigating soybean yield losses, however the absence of disease is not mentioned (Bandara et al., 2020).

Researchers around the world have been looking for molecules that are able to stimulate the metabolism of crop plants and improve their productivity. The great marketing that exists concerning fungicides and their protective role, more recently associated with physiological benefits on plants, places these products at the top of the list of many farmers when it comes to managing high crop yields. However, several published studies demonstrate that a positive effect of the application of fungicides on disease-free plants, or with low disease severity, is rarely found (Swoboda and Pedersen, 2009; Faske & Emerson, 2020). So, in the absence of diseases, using a fungicide to boost grain production is unlikely to exceed the break-even cost (Faske & Emerson, 2020). Also, the overuse of fungicides raises production costs and may contribute to the emergence of fungicide-resistant diseases. Nonetheless, there are a number of non-fungicide products on the market that are designed to help plants enhance their physiology, metabolism, and productivity. Thus, the use of fungicides on disease-free plants just to have some gains would be counterproductive. In this way, and considering the data obtained, the use of fungicides is recommended only when the disease can impact yield, in order to assure the break-even cost.

5 CONCLUSIONS

Fungicides have no physiological benefits on soybean disease-free plants, since no physiological trait was improved by any of the products or the number of fungicide applications.

EPZ+FLX+PYR fungicide caused general reduction of photochemical traits of soybean plants.

TFX+PRZ reduced the pod number of soybean plants after one application, which may have been reflected in higher HGW as compensation.

TFX+PRZ and TFX+PRZ+BXF reduced soybean pollen germination in this study, but it did not affect the yield components.

Fungicides do not improve soybean metabolism and production in the absence of a yield-limiting disease, in controlled conditions.

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5. GENERAL CONCLUSION

The application of three different commercial fungicides based on triazole, strobilurin or carboxamide (CPZ+DFZ; AZB+BZP and PPZ+DFZ) during the pre-flowering and flowering stages in disease-free soybean plants did not affect the physiological characteristics, pollen grain germination and crop yield.

The fungicides EPZ + FLX + PYR, TFX + PRZ and TFX + PRZ + BXF do not present physiological benefits in plants free from soybean diseases, since no physiological attributes were improved by any of the products or by the number of applications. The fungicide EPZ + FLX + PYR caused a general reduction in the photochemical characteristics of soybean plants. TFX + PRZ reduced the number of soybean plant pods after one application, which may have been reflected in a higher HGW as a compensation. TFX + PRZ and TFX + PRZ + BXF reduced soybean pollen germination in this study, which did not reflect changes in production components. The fungicides EPZ + FLX + PYR, TFX + PRZ and TFX + PRZ + BXF do not improve the metabolism and production of soybeans in the absence of a disease that limits production, according to the results of this study.

Based on the data from the present study, the use of fungicides is not recommended when the disease does not affect the productivity of soybean plants, since excessive applications can compromise the economic viability of the crop.