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Simone Gripa Minuzzi

APLICAÇÃO FOLIAR DE TIOSSULFATO DE AMÔNIO E METILENO UREIA ASSOCIADO A FUNGICIDA EM SOJA E SUA INTERFERÊNCIA NO PATOSSISTEMA *Glycine max - Phakopsora pachyrhizi*

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Agronomia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do grau de **Doutora em Agronomia.**

Orientador: Prof. Dr. Alessandro Dal'Col Lúcio

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RESUMO

APLICAÇÃO FOLIAR DE TIOSSULFATO DE AMÔNIO E METILENO UREIA ASSOCIADO A FUNGICIDA EM SOJA E SUA INTERFERÊNCIA NO PATOSSISTEMA *Glycine max - Phakopsora pachyrhizi*

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A ferrugem asiática da soja (FAS), causada pelo patógeno *Phakopsora pachyrhizi*, é a principal doença ocorrente na cultura soja e é causadora de dano significativo na produtividade. A medida de controle preferencialmente utilizada é a química. Porém, em virtude da redução da eficiência dos mesmos, a adoção de medidas integradas visando o controle da doença, tem sido amplamente discutida. Dentre essas medidas, destaca-se a utilização de fertilizantes foliares. Nossos estudos objetivaram elucidar o efeito de fertilizantes foliares à base de nitrogênio e/ou enxofre, isolados ou associados ao fungicida picoxistrobina + ciproconazol, visando o controle de *P. pachyrhizi*. Também foi avaliado as respostas bioquímicas das plantas expostas a estes tratamentos, bem como o efeito na produtividade sob diferentes ambientes de cultivo. Foram conduzidos ensaios em casa de vegetação e campo. No primeiro estudo o efeito da utilização de tiossulfato de amônio e metileno ureias nas cultivares NS 5445, BMX Tornado e TMG 7062 foi investigado com base em parâmetros bioquímicos, de ativação de enzimas, peroxidação lipídica e concentração de compostos fenólicos, e de controle da doença. A partir disso foi verificado que os fertilizantes foliares reduziram o estresse oxidativo das plantas sob infecção de *P. pachyrhizi*, além de proporcionar controle da doença. Foram observadas as primeiras evidências bioquímicas relacionadas a ativação da fenilalanina amônia liase e alta peroxidação lipídica na cultivar TMG 7062, como parte da maquinaria de defesa dessa cultivar. No segundo estudo foi investigado o efeito da associação dos fertilizantes foliares com o fungicida pré-formulado a base de picoxistrobina + ciproconazol também com base nos parâmetros bioquímicos citados, e seu efeito sobre o controle da doença. O fungicida pré-formulado induziu grande estresse oxidativo nas plantas das três cultivares e as associações com os fertilizantes no geral não amenizaram o dano nas membranas lipídicas, nem levaram a ativação de rotas de defesa avaliados. Entretanto, proporcionaram incremento de controle da FAS, presumindo-se, portanto, efeito tóxico ao fungo via deposição de elementos químicos na superfície foliar, impedindo a germinação e colonização do fungo nos tecidos foliares. Nesse mesmo sentido, foi analisado o efeito de controle dos fertilizantes foliares isolados e associados ao fungicida pré-formulado sob diferentes ambientes de cultivo (Itaara/RS e Planaltina/DF) dentro do programa de controle estabelecido. Para tanto, foram avaliadas a severidade da FAS, produtividade e peso de mil sementes nos diferentes tratamentos e sob duas cultivares, NS 5445 e BMX Tornado. Pôde-se verificar que, em condições de baixa pressão da doença (Planaltina), as associações de picoxistrobina + ciproconazol com os fertilizantes foliares incrementaram o controle da FAS. Porém sob alta pressão da doença (Itaara), as mesmas associações, não tiveram incrementos de controle dentro do programa de controle. Em relação ao recente uso de tiossulfato de amônio e metileno ureias na agricultura, os dados desta tese trazem informações novas, que podem contribuir na definição de estratégias no manejo da ferrugem asiática.

Palavras-chave: Soja. Ferrugem asiática. Tiossulfato de amônio. Metileno ureia. Fertilizante foliar.

ABSTRACT

FOLIAR APPLICATION OF AMMONIUM TIOSULPHATE AND METHYLENE UREA ASSOCIATED TO FUNGICIDE IN SOYBEAN AND ITS INTERFERENCE IN THE PATHOSYSTEM *Glycine max - Phakopsora pachyrhizi*

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Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is the main disease on soybean crop and can cause important yield reduction. The chermical control is the most suitable for a disease like ASR. However, due to the constant reduction of their efficacy, the adoption of integrated management control of ASR has been widely discussed for the soybean sustainability. These measures include the use of foliar fertilizers to increase disease control. The studies aimed to elucidate the effects of nitrogen and / or sulfur leaf fertilizers isolated and associated with a commercial fungicide composed by picoxystrobin + cyproconazole on the of ASR control and, as well as the biochemical responses of the plants exposed to these treatments, as well as the yield effect under different growing environments. Field trials were conducted in greenhouse and field, divided in three chapters. The first study considered the effect of the use of ammonium thiosulfate and methylene ureas in the cultivars NS 5445, BMX Tornado and TMG 7062 was investigated based on biochemical parameters, enzyme activation, lipid peroxidation and phenolic compound concentration, and control. From this it was verified that the foliar fertilizers reduced the oxidative stress of the plants under infection of *P. pachyrhizi*, besides providing control of the disease. The first biochemical evidence of the great activation of phenylalanine ammonia lyase and high lipid peroxidation in cultivar TMG 7062 was identified. The second study considered the effect of the association of foliar fertilizers with the pre-mixture fungicide based on picoxystrobin + cyproconazole was also investigated, based on the biochemical parameters mentioned above, and its effect of disease control. The pre-mixture fungicide induced great oxidative stress in the plants of the three cultivars and the associations with the fertilizers overall did not alleviate the damage in the lipid membranes nor led to the activation of evaluated routes of defense. However, they increased the FAS control, thus presuming a toxic effect on the fungus through the deposition of chemical elements on the leaf surface, preventing the germination and colonization of the fungus in the foliar tissues. The third study analized the effect of the isolated and associated foliar fertilizers to the pre-mixture fungicide on ASR under different cropping environments (Itaara/RS and Planaltina/DF) within the established control program. In order to determined such association it was evaluated the severity of ASR, yield and thousand seed mass in the different treatments and under two cultivars, NS 5445 and BMX Tornado. It was verified that, under conditions of low disease pressure (Planaltina), the associations of picoxystrobin + cyproconazole with foliar fertilizers increased ASR control, but under high disease pressure (Itaara) they did not have any control increments within the established control program. New information regarding the recent use of ammonium thiosulfate and methylene urea, which may contribute to the definition of strategies for the management of ASR is presented.

Keywords: Soybean. Asian soybean rust. ammonium thiosulphate. methylene urea. Foliar fertilizer.

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LISTA DE ABREVIATURAS

12 HAI – 12 hours after inoculation
4 HAA – 4 hours after application
AT – ammonium thiosulphate
AUARPC – Area under asian rust progress curve
FAS – Ferrugem Asiática da Soja
H_2O_2 - hydrogen peroxide
MDA – malondialdehyde
MEU – methylene ureas
Mz – Mancozeb
NDAFS – Number of days for the appearance of the first symptoms
N - Nitrogen
PAL – Phenylalanine ammonia-lyase
POX - peroxidase
ROS – reactive oxygen species
S – Sulfur
TSP - total soluble phenolics
TSM – thousand seed mass

SUMÁRIO

INTRODUÇÃO

A soja destaca-se como a principal cultura de grãos no Brasil, tendo um crescimento na área plantada de 2% na safra 16/17 em relação à anterior, atingindo uma produção de 114.041,9 milhões de toneladas, aproximadamente 16,31% superior a safra passada (CONAB, 2017). A Ferrugem Asiática da Soja (FAS), causada pelo fungo *Phakopsora pachyrhizi*, tem comprometido a sustentabilidade da cultura da soja. A partir de 2001, quando a doença foi encontrada no Paraguai e também no Brasil (YORINORI et al., 2005), o patógeno tornou-se altamente adaptado às condições do ambiente e vem causando danos expressivo ao longo dos anos (GODOY et al., 2016), constituindo um grande desafio à produção de soja no país.

As principais estratégias de redução de inóculo e manejo da doença se baseiam na adoção do vazio sanitário, utilização de cultivares resistentes que podem aumentar a eficiência do controle químico que se constitui na principal ferramenta de manejo utilizada atualmente (LANGENBACH et al., 2016; GODOY et al., 2016; BALARDIN et al., 2010; SCHERM et al., 2009; MILES et al., 2007).

Existem inúmeros fungicidas registrados para controle desta doença, que pertencem a quatro grupos químicos, são eles: estrobilurinas -inibidores da respiração celular do fungo, agem na quinona externa nas cristas mitocondriais (*QoIs – Quinone outside Inhibitors*); triazóis - os inibidores da desmetilação da cadeia carbônica na síntese de esteróis nas membranas celulares (*DMIs – DeMethylation Inhibitors*) (GODOY et al., 2016; LAUGENBACH et al., 2016); Carboxamidas - inibidores da respiração mitocondrial, os quais se ligam ao complexo II da cadeia de transporte de elétrons, tendo como alvo a enzima succinato desidrogenase (*SDHI - Succinate DeHydrogenase Inhibitors*) (KEON et al., 1991); e os multissítios - mecanismo de ação em múltiplos sítios do patógeno conferindo amplo espectro de controle.

Entretanto, todos os produtos pertencentes aos três primeiros grupos químicos citados agem em sítios específicos no metabolismo do patógeno e por isso são considerados de alto risco à perda de sensibilidade e resistência do patógeno (BRENT; HOLLOMON, 2007), e a menor sensibilidade do fungo aos fungicidas tem sido, frequentemente, relatada no Brasil. Nas safras 2007/08 a 2009/10, começou a ser notado problemas de eficácia a fungicidas do grupo DMIs no controle de FAS (GODOY, 2011) e mais tarde para QoIs (GODOY et al., 2014) e, mais recentemente, na safra (2016/17), foi verificado perda de eficiência do fungicida registrado contendo SDHI, atribuída a menor sensibilidade do fungo a fungicidas SDHI, em razão da mutação I86F na subunidade C do gene “sdh” do fungo *P. pachyrhizi* (GODOY et al. 2017).

O uso intensivo e inadequado de fungicidas, como o uso repetido de moléculas com o mesmo mecanismo de ação, a utilização de produtos de forma isolada com mecanismo sítio-específico, alterações de doses recomendadas, cobertura insuficiente do dossel depositando um número de gotas inferior ao necessário para conter a dose letal do fungicida, o uso de produtos sistêmicos de forma erradicativa, contribuem definitivamente para a seleção de isolados fúngicos menos sensíveis, e por consequência, pode diminuir a eficiência dos fungicidas no controle da FAS ao longo do tempo (BRENT; HOLLOMON, 2007).

Em virtude disso, a adoção de medidas integradas para o controle da doença tem sido amplamente discutida. Dentre essas medidas, incluem-se a utilização de fertilizantes foliares a fim de incrementar o controle da doença. O efeito da nutrição mineral e seus benefícios no controle de doenças em plantas foi compilado por Datnoff et al. (2007). Todos os nutrientes das plantas têm um impacto direto nos patógenos, e no crescimento microbiano, sendo todos eles, bem como suas proporções, são importantes no controle da doença e afetarão a incidência ou severidade da mesma (HUBER; HANEKLAUS, 2007).

Neste sentido, a introdução de enxofre e/ou nitrogênio pode ser uma ferramenta eficiente dentro do manejo integrado de doenças, especialmente da FAS, na cultura da soja. O Enxofre (S) é componente estrutural de muitos metabólitos como cisteína, glutationa, fitoalexinas e glucosinolatos, os quais têm sido investigados por suas funções em toda a planta, como indução e aumento da resistência das culturas a infeções por patógenos fúngicos (BLOEM et al. 2007; HANEKLAUS et al., 2007; 2009). Recentemente, a maioria dos metabólitos contendo S apresenta um modo de ação antifúngico comprovado (BLOEM et al., 2015). A cisteína (AZARAKHSH et al., 2015), assim como a glutationa (DE KOK et al., 1981), têm importante função na ativação do sistema antioxidante e redução da peroxidação lipídica nas plantas durante a patogênese (interação soja-FAS). Haneklaus et al. (2006) concluíram que a fertilização com enxofre reduziu o índice de doença em várias relações patógeno/hospedeiro, de 5–50% e 17–35% em experimentos em casa de vegetação e à campo, respectivamente.

Assim como para o enxofre, o nitrogênio (N) também apresenta efeito positivo sobre a fisiologia e bioquímica da planta sob infecção por patógenos, visto que também é componente estrutural de todas as proteínas, as quais formarão enzimas que estão envolvidas no equilíbrio redox, e também na formação de aminoácidos como glicina, fenilalanina, cisteína, glutamato, prolina e outros, que também tem importante função antioxidante. Teixeira et al. (2017) verificaram que aminoácidos tem função direta no sistema antioxidante por aumentar a habilidade da planta em lidar com a produção de espécies reativas de oxigênio.

Sabe-se que um aumento na concentração de espécies reativas de oxigênio (EROs) como, superóxido (O^-_2), peróxido de hidrogênio (H_2O_2), e radical hidroxila (OH^-) é uma característica observada em plantas sob algum estresse, tanto infectadas por patógenos (MAGBANUA et al. 2007; DEBONA et al. 2012), como em virtude da aplicação de fungicidas para conter o patógeno (CHEN et al., 2010; DIAS, 2012; FAIZE et al., 2011). Embora o acúmulo de EROs possa inicialmente contribuir para resistência da planta as doenças (HAMMOND-KOSACK; JONES 1996), o imbalanço entre a produção e remoção de EROs pode resultar em dano oxidativo (MAGBANUA et al. 2007; DEBONA et al. 2012; FORTUNATO et al. 2015). O estresse oxidativo causa peroxidação lipídica nas membranas celulares e danos aos pigmentos, proteínas e ácidos nucléicos (APEL; HIRT, 2004).

Para remover o excesso das EROs produzidas nessas duas situações, as plantas têm desenvolvido mecanismos enzimáticos e não enzimáticos (APEL; HIRT, 2004), que tem mostrado importante função na resistência das plantas as doenças e também na redução do estresse causado pela aplicação de fungicidas. A enzima peroxidase (POX) esta comumente envolvida na defesa contra o dano oxidativo, assim como as enzimas superóxido dismutase, catalase, glutationa-S-transferase, ascorbato peroxidase, glutationa redutase, e glutationa peroxidase (MITTLER, 2002). Além da ativação das enzimas antioxidantes, aumento na atividade da fenilalanina amônia liase (PAL) bem como maiores concentrações de compostos fenólicos totais solúveis também estão relacionadas diretamente a maior resistência do hospedeiro à infecção.

Com base no exposto, foram desenvolvidos trabalhos com a finalidade de elucidar o efeito de fertilizantes foliares à base de nitrogênio e/ou enxofre, isolados ou associados ao fungicida picoxistrobina + ciproconazol utilizado no controle de *P. pachyrhizi* e quanto às respostas bioquímicas das plantas quanto ao estresse oxidativo e ativação da rota de produção de compostos fenólicos expostas a estes tratamentos, além do efeito em produtividade sob diferentes ambientes de cultivo.

Manuscript I (Will be Submitted to Tropical Plant Pathology)

Ammonium thiosulphate and Methylene urea relieve *Phakopsora pachyrhizi* – induced oxidative stress in soybean

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Abstract

Asian Soybean Rust (ASR), caused by *Phakopsora pachyrhizi*, is a major disease found throughout all soybean growing areas in Brazil. However, specific information regard possible impacts set forth by the usage of foliar fertilizers containing nitrogen and sulfur to the soybean-pathogen interaction is still limited. Therefore, at the present work our goal was to determine the biochemical parameters related to plant response to pathogens is affected by the application of foliar fertilizers, which is a good indicator of the overall response of a plant to pathogen atttack. To this end, the activities of the enzymes peroxidase (POX) and phenylalanine ammonia-lyase (PAL), as well as the concentration of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and total soluble phenolics (TSP) were determined 4 hours after application of distilled water, ammonium thiosulphate methylene urea and mancozeb, and 12 hour after inoculation on three soybean cultivars, i.e. ‘NS 5445’, ‘BMX Tornado’ and ‘TMG 7062’, which are widely regarded as partially susceptible, susceptible, and tolerant to ASR, respectively In order to control parameters, the variables assessed were number of days for the appearance of the first symptoms (NDAFS), and the area under asian rust progress curve (AUARPC). Results indicated that *Phakopsora pachyrhizi* infection, regardless of the actual treatments, induced oxidative stress in all soybean cultivars. All treatments reduced the *P. pachyrhizi*-induced oxidative stress by constraining the fungal infection rather than by activating the antioxidant enzyme POX. However, a sustained level of TSP and PAL activities appeared to contribute to the increase of control through of the activation of defense pathways observed in the foliar fertilizers and mancozeb plants. These results show that the ammonium thiosulphate and methylene urea application reduced the *P. pachyrhizi*-induced oxidative stress in all the cultivars, besides this activated the PAL pathway and higher TSP were observed, thus contributing to greater soybean resistance to *P. pachyrhizi*.

Keywords: soybean, Asian soybean rust, nitrogen, sulfur, mancozeb.

Introduction

Biotic stresses such as pathogen infections can cause negative impact on soybean (*Glycine max* (L) Merr.) yield. Asian Soybean Rust (ASR), caused by *Phakopsora pachyrhizi* (Sydow & Sydow), is the most destructive disease causing yield losses up to 90% (Bromfield, 1984; Hartman et al., 2015) as environmental conditions are conducive to disease development. Disease infection takes place when temperatures range from 10 ºC to 27.5 ºC (optimum 20-23 ºC) and a minimum dew period of six hours is observed (Melching et al., 1989; Alves et al., 2006). The typical symptoms of that disease are small sporulating lesions formed mainly on the upper surface of soybean leaflets, which are frequently associated with leaf chlorosis. High severity cause premature defoliation and early maturity, resulting in significant yield losses (Hartman et al., 2015).

An increase in the concentration of reactive oxygen species (ROS), such as superoxide (O^-_2), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-), is a remarkable feature observed in plants infected by pathogens (Debona et al., 2012; Magbanua et al., 2007). Although ROS accumulation can initially contribute to plant disease resistance because of their importance as antimicrobial, cell wall strengthening and signaling molecules (Hammond-Kosack & Jones 1996), the imbalance between production and removal of ROS can result in oxidative damage (Magbanua et al., 2007; Debona et al., 2012; Fortunato et al., 2015). Oxidative stress causes lipid peroxidation in the cell membrane and damage to pigments, proteins, and nucleic acids (Apel & Hirt, 2004).

Plants have developed nonenzymatic and enzymatic mechanisms (Apel & Hirt, 2004) to remove the excess of ROS generated during the host–pathogen interaction, which has been shown to play a key role in plant disease resistance. The peroxidase enzyme (POX) is commonly involved in the host defense mechanisms against oxidative stress. Similarly, the superoxide dismutase, catalase, glutathione-S-transferase, ascorbate peroxidase, glutathione reductase, and glutathione peroxidase enzymes worked in the host pathogen interaction (Mittler, 2002).

Besides the activation of antioxidative enzymes, the increase of phenylalanine ammonia lyase (PAL) activity as well as higher total soluble phenolics (TSP) concentration also related to greater host resistance to disease infection. According to Liang et al. (2005), PAL activity was dependent on the level of basal resistance of cucumber cultivars to *Podosphaera xanthii*. Cruz et al. (2013) concluded that the high level of PAL activity in the leaves of plants sprayed with acibenzolar-S-methyl reflects the importance of the phenylpropanoid pathway for soybean resistance against infection by *P. pachyrhizi*. Fortunato et al. (2012) reported that the severity of Fusarium wilt on banana plants supplied with Si decreased as a consequence of an increase in the activities of phenylalanine ammonia-lyases, polyphenoloxidases, chitinases, β-1,3-glucanases, and peroxidases as well as higher amounts of total soluble phenolics (TSP) and lignin in the cell.

Due to the limited availability of soybean cultivars displaying a desirable level of resistance to ASR, its management has been primarily done by spraying fungicides onto the crop, although some cultural practices may also lower disease infection (Hartman et al., 2005; Yorinori et al., 2005). However, nearly 100 fungicides have recently been deregistered for ASR control in Brazil. Reduction of *P. pachyrhizi* sensitivity to these fungicides as well as ASR-induced yield losses in treated plots were the primary drivers for this decision. Therefore, it is crucial to investigate new disease management strategies reassuring soybean production sustainability.

In this sense, the introduction of foliar fertilizers into a ASR-management program might be a promising tool. An overview of current knowledge on the effect of mineral nutrition on plant diseases was compiled by Datnoff et al. (2007). Sulfur (S) is a constituent of the amino acids cysteine and methionine and an essential component of proteins, as well as nitrogen (N), and both are present in metabolites with direct antifungal action.

The objectives of this work were to determine whether the reduction of ASR severity can be linked with an increase in the activities of defense enzyme phenylalanine ammonia-lyase as well as the antioxidative enzyme peroxidase. We also quantified total soluble phenolics on plants sprayed with a range of foliar fertilizers and fungicide and assessed oxidative damage as a treatment response.

Material and Methods

Plant growth

The experiment was conducted during the 2016-2017 growing season at Itaara, Rio Grande do Sul, Brazil, in partially controlled greenhouse conditions. Maximum and minimum temperatures recorded were 29 °C and 16 °C, respectively. Temperatures were regulated through hoods. Relative humidity was controlled by a computerized moisturizing system and ranged from 65 to 90%. Experiments included seeds from soybean cultivars “NS 5445”, “BMX Tornado”, and “TMG 7062”. Seeds were treated with fipronil + pyraclostrobin + thiophanate-methyl (50 + 5 + 45 g a.i. 100 kg^{-1} of seed, respectively), and inoculated with *Bradyrhizobium japonicum* prior to sowing. Seeds were hand-sewn in 5-L pots containing rice husk + soil (2:1), and cultural and fertilization practices performed according to technical recommendations for soybean crop production.

Treatment application

Soybean plants were either sprayed with distilled water (control; hereinafter referred to as treatment T1), ammonium thiosulphate at 510g S ha^{-1} (T2), methylene urea at 3300g N ha^{-1} (T3), or T4 – Mancozeb (1,125g a. i. ha^{-1}). Spraying took place as a preventive measure at early flowering (R1) (Fehr & Caviness, 1977), that is, prior to any ASR disease symptoms being noticeable. Treatments were applied using a knapsack sprayer, and the spray boom equipped with four XR 11002 flat-fan nozzles spaced 0.5 m apart, and calibrated to deliver 150 L ha^{-1} at 30 psi (206.8 kPa).

Inoculation of plants with *Phakopsora pachyrhizi*

On the same treatment spraying day, but six hours after spray, soybean leaves were artificially inoculated following the methodology presented by Lenz et al. (2011) using a uredospore suspension of *P. pachyrhizi* ($4x10^4$ spores mL^{-1}) with water, adhesive spreader Tween 80 ppm (for uredospores adhesion to the leaf), and uredospores of *P. pachyrhizi* mixture at R1 stage (early flowering) (Fehr & Caviness, 1977). Such uredospores were previously collected from ASR-infected plants. Immediately after inoculation, pots were transferred to a growth chamber with an average temperature of 25 ± 2 ºC and a relative humidity of 90 ± 5% and subjected to an initial 12 h dark period.

Assessment of number of days for the appearance of the first symptoms (NDAFS) and area under asian rust progress curve of ASR.

The last two fully expanded leaflets of each plant were marked with colored tape and repeatedly evaluated, and the actual number of days for the appearance of the first symptoms (NDAFS). Observations were done at daily basis and after the second day of inoculation using a 20X magnifier to check the initial symptoms. This methodology allowed the estimation of residual length under each treatment. Severity was determined by assigning visual scores of diseased leaflet areas as proposed by Godoy et al. (2006). Afterwards, the AUARPC parameter was calculated according to Campbell & Madden (1990).

Biochemical analysis

For all biochemical essays, the fourth and fifth trifoliate leaves, as counted from the plant bottom up, were collected from two plants within each replication either at four hours after application (4HAA) or 12 hours after inoculation (12HAI). Leaf samples were kept in liquid nitrogen during sampling, and subsequently stored at -80ºC until further analysis.

Determination of peroxidase (POX) enzyme activity

The POX enzyme activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra (1976). In total, 0.5 g of leaf tissue was ground into a fine powder using a mortar and pestle to which liquid nitrogen was added.

Upon grinding, the fine powder was homogenized in 2000 µl of a solution containing 50 mM of a potassium phosphate buffer (pH 6.8). The homogenate was centrifuged at 12,000 x g for 15 min at 4ºC, and the supernatant was used as a crude enzyme extract. The reaction was started after the addition of 15 µl of the crude enzyme extract to 985 µl of reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2.

Activity of the POX enzyme was determined through absorbance of colored purpurogallin at 420 nm for 1 min at 25°C. An extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance & Maehley, 1995) was used to calculate the POX enzyme activity, which was expressed as micromoles of purpurogallin produced per minute, per milligram of protein.

Determination of malondialdehyde (MDA) concentration

Oxidative damage in the leaf cells was estimated as the concentration of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA) following Cakmak and Horst (1991).

In total, 0.1 g of leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was homogenized in 2000 μl of 0.1% (wt vol^{-1}) trichloracetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. After centrifugation, 500 μl of the supernatant was added into 1500 μl of TBA solution (0.5% in 20% TCA), and the reaction proceeded for 30 min in a boiling water bath at 95°C.

After this period, the reaction was stopped by transferring eppendorf's to an ice bath. Samples were centrifuged at 9,000 × *g* for 10 min, and the specific absorbance was determined at 532 nm. The nonspecific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. An extinction coefficient of 155 mM^{-1} cm^{-1} (Heath & Packer, 1968) was used to calculate the MDA concentration, which was expressed as micromoles per kilogram of fresh weight.

Determination of hydrogen peroxide (H_2O_2) concentration

In total, 0.1 g of leaf tissue was ground into a fine powder following a similar procedure as described for malondialdehyde (MDA) determination. The fine powder was homogenized in a volume of 1,500 μl of TCA 0,1%.

The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C (Loreto & Velikova, 2001). In total, 500 μL of the supernatant were added to a reaction mixture containing 500 μL of potassium phosphate buffer 10 mM (pH 7.0) and 1000 μl of potassium iodide (1M). The absorbance of the samples was determined at 390 nm. The concentration of H_2O_2 in the samples was estimated based on a standard curve of H_2O_2 and expressed as millimoles per gram of fresh weight.

Determination of phenylalanine ammonia lyase (PAL) enzyme activity

For the determination of PAL enzyme activity, 0.3 g of leaf tissue was ground following the same procedures described previously. The fine powder obtained was homogenized in 2000 µL of a solution containing 50 mM potassium phosphate buffer (pH 6.8) and 1 mM phenyl-methylsulfonyl fluoride (PMSF) in an ice bath.

The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant used to determine the PAL enzyme activity. The latter was achieved by following the methodology proposed by Guo et al. (2007) with some modifications. The reaction was started by adding 100 µL of the crude enzyme extract to a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 20 mM *L*-phenylalanine; the final volume was 1000 µL.

The reaction mixture was incubated in a water bath at 30°C for 1 h, and 50 µl of HCl 6 N were added afterwards to stop the reaction. The absorbance of *trans*-cinnamic acid derivatives was measured at 290 nm. A similar procedure was used for the control samples, but the reaction was immediately stopped with 50 µl of HCl 6 N after the addition of the crude enzyme extract to the reaction mixture. The extinction coefficient of 100 M^{-1} cm^{-1} was used to calculate PAL activity (Zucker, 1965).

Determination of Total Soluble Phenolics (TSP)

A total of 0.1 g of leaf tissue was ground into a fine powder with liquid nitrogen in a mortar and pestle and homogenized in 1000 µl of a solution containing 80% (vol/vol) methanol in an ice bath.

The homogenate was centrifuged at 17,000 × *g* for 30 min, and the supernatant used to determine TSP concentration by following the methodology proposed by Zieslin and Ben-Zaken (1993), with modifications proposed by Rodrigues et al. (2005). The reaction was started after the addition of 0.2 M Folin-Ciocalteu phenol reagent to 150 µl of the methanolic extract and kept at 25°C for 5 min. Next, 0.1 M sodium carbonate was added to the solution, which was maintained at 25°C for 10 min. Afterwards, 1000 µL of deionized water was added to the mixture and it was incubated at 25°C for 1 h.

The absorbance was read at 725 nm, and TSP concentration calculated based on a calibration curve using pyrogallol (Sigma-Aldrich, São Paulo, Brazil) as a standard.

Experimental design and data analysis

A 2 factorial experiment, consisting of three soybean cultivars (NS 5445, BMX Tornado and TMG 7062) as well as four treatments (ammonium thiosulphate, methylene urea, mancozeb, or untreated control) was arranged in a completely randomized design with three replications.

Each experimental unit consisted of 5L pots containing two soybean plants. Data were subjected to analysis of variance (ANOVA) and the average compared when appropriate by performing the Scott-Knott test at 5% probability using the Assistat software (Silva & Azevedo, 2002).

Results

ANOVA results indicated that all the factors in this study were significant for the Asian soybean rust (ASR), for AUARPC and NDAFS parameters (Table 1) that varied according to each cultivar. The ASR AUARPC was significantly lower in TMG 7062 relative to soybean cultivars NS 5445 and BMX Tornado, regardless of the treatments, as well NDAFS (Table 2). Moreover, regardless the cultivar there was a significant decrease in the ASR AUARPC when those treated with foliar fertilizers and mancozeb were compared to untreated plants. The mancozeb treatment achieved the greatest control effectiveness of all treatments and genotypes 80.74% for NS 5445, 71.65% for BMX Tornado, and 58.10% for TMG 7062 (Table 2).

Similarly, to control parameters, all the isolated factors as well as their interactions were statistically significant for POX and PAL enzyme activity, and H_2O_2, MDA and TSP concentration (Table 3). H_2O_2 concentration was the only variable at which the cultivar–treatment interaction was found not to be significant.

POX enzyme activity significantly increased in ammonium thiosulphate-treated plants for NS 5445 (moderate susceptibility) in the sampling time 4haa compared methylene urea (57.45%) and mancozeb (reduction of 64.15%) treatments (Fig. 1A). However, at 12 hai, ammonium thiosulphate and mancozeb treatments significantly reduced POX activity 42.21% and 58.41%, respectively, compared to no treated plants. Nevertheless, an opposite response was observed in soybean cultivars BMX Tornado and TMG 7062. The actual, POX activity recorded in these cultivars was significantly reduced at 4 haa in ammonium thiosulphate-treated plants by 67.92% and 54.60% in BMX Tornado and TMG 7062, respectively (Figures 2A and 3A), and continued being reduced at 12 hai for TMG 7062 (49.18%) (Fig. 3B); however, it should be noticed that all treatments led to a significantly reduced POX activity in BMX Tornado at 12 hai (Fig. 2B).

Regarding H_2O_2 concentration, there was significant difference in both sampling periods for mancozeb-treated plants for NS 5445 (Figures 1C and 1D), which translated into an increase of 68.02% (4haa) and 62.88% (12hai). However, there were no significant differences between each treatment compared to no treated plants at 4 haa for either BMX Tornado (Fig. 2C) and TMG 7062 (Fig. 3C). All treatments reduced significantly the H_2O_2 concentration 12hai for BMX Tornado (Fig. 2D), although at 12 hai, only the methylene urea treatment significantly increased POX activity (44.08%) quantified in the TMG 7062 cultivar (Fig. 3D).

Regarding the MDA concentration, significant differences among the treatments occurred 4haa for NS 5445 and BMX Tornado cultivars, at which all treatments significantly increased MDA concentrations compared to no treated plants (Figures 1E and 2E). Exception was found to ammonium thiosulphate and methylene urea treatments for TMG 7062 4haa, which significantly reduced the MDA concentration (11.58% and 19.25%, respectively) (Fig. 3E). On sampling time 12 hai, the ammonium thiosulphate treatment significantly reduced MDA concentration for NS 5445 (74.92%) (Fig. 1F), while methylene urea and mancozeb treatments significantly reduced the MDA concentrations for BMX Tornado, 13.49% and 28.76% respectively, and TMG 7062, 35% and 42.87% (Figures 2F and 3F).

There were significant differences across treatments concerning PAL enzyme activity at 4 haa sampling time for either the BMX Tornado and TMG 7062. The treatment containing mancozeb allowed for the highest PAL enzyme activity in both cultivars 1301% and 458% for BMX Tornado and TMG 7062, respectively (Figures 2G and 3G). However, it should be mentioned that both mancozeb and methylene urea allowed for statistically similar PAL enzyme activities quantified in TMG 7062 cultivar (an increase of 296.48%) (Fig.3G). In addition, at 12 hai methylene urea significantly increased PAL activity in NS 5445 (731.59%) (Fig. 1H) and TMG 7062 (81.08%) (Fig. 3H), but at the latter (TMG 7062) such results were not significantly different to the treatment containing mancozeb (89.66%) (Fig. 3H).

Higher TSP concentration occurred at 4 haa in ammonium thiosulphate-treated BMX Tornado (14.53%) and TMG 7062 (41.79%) plants (Figures 2I and 3I). At TMG 7062, methylene urea and mancozeb also increased statistically these phenolic compounds, differing of no treated plants (65.07% and 49.37%, respectively) (Fig. 3I). At sampling time 12 hai, the treatments that significantly increased TSP concentrations were mancozeb for NS 5445 (14.86%) (Fig. 1J), methylene urea for BMX Tornado (12.64%) (Fig. 2J) and, both cited treatments for TMG 7062, 12.07% and 17.86%, respectively to methylene urea and mancozeb (Fig. 3J).

Discussion

The lower ASR AUARPC in the leaves of TMG 7062 plants compared with NS 5445 and BMX Tornado is a good indication that this cultivar possesses the greatest level of resistance among all soybean cultivars used in this study. A greater level of ASR resistance was expected to be observed at soybean cultivar NS 5445 rather than at BMX Tornado, which has not been confirmed by the results shown above. Regardless of soybean cultivar, the application of foliar fertilizers significantly reduced AUARPC for ASR, also delaying the entry of the disease (NDAFS), as well as for mancozeb.

Pathogen infection increases ROS production in plants, which, in turn, need to activate a range of enzymes responsible for the synthesizes of compounds related to the prevention or alleviation of cellular damage (Debona et al. 2012; Fortunato et al. 2015). Mancozeb has been widely recognized for its fungicidal effect, while also been capable of modifying the plant's antioxidant system (Balardin et al., 2017), as it can favor ROS removal and reduce oxidative stress in plants. Despite methylene urea and ammonium thiosulphate not displaying direct fungicidal effects, these function as nitrogen and sulfur sources (Marschner, 2012), and provided significant ASR control in three susceptible levels used in this study. In addition, both were shown to also relieve *Phakopsora* *pachyrhizi*-induced oxidative stress, especially methylene urea, which behaved similarly to mancozeb.

There was difference among cultivars regarding biochemical parameters, mainly when cultivars NS 5445 and BMX Tornado are compared against TMG 7062. In no treated plants, an increase on the H_2O_2 concentration was perceived in all soybean cultivars tested in response to *P. pachyrhizi* inoculation. ROS production is an important plant defense response mechanism against pathogenic infection (Daub et al., 2013), which is well-documented on the literature. The H_2O_2 concentration is generated by a reaction catalyzed by SOD (superoxide dismutase), or even spontaneously (Lanubile et al., 2012). Fortunato et al. (2015) verified that the H_2O_2 concentration increased significantly in soybean plants upon *Corynespora cassiicola*, a necrotrophic fungus, infection in a cultivar showing susceptibility to this pathogen (TMG 132), corroborating with our results, although of the *P. pachyrhizi* be a biotrophic fungus, at which cultivars with increased susceptibility (NS 5445 – moderate susceptibility - and BMX Tornado - susceptible) had a greater increase on their H_2O_2 concentration upon *Phakopsora pachyrhizi* infection.

POX, an enzyme involved in H_2O_2 removal, also plays an important role in plant defense against pathogens due to its participation in lignin biosynthesis (Rauyaree et al., 2001). Therefore, an increase in POX activity is required to lower concentrations of H_2O_2 (Gill & Tujeta, 2010). Interestingly, our results indicated that the POX enzyme activity is reduced upon *P. pachyrhizi* infection in all cultivars employed in this research (Figures 1B, 2B and 3B); on no treated plants, this fact led directly to greatest concentrations of H_2O_2 (Figures 1D, 2D and 3D).

The increase of H_2O_2 can be related to high superoxide (O_2^-) concentration previously formed. Furthermore, excess of H_2O_2 can be transferred via the Haber-Weiss reaction to form the highly reactive oxidant hydroxyl radical (OH^-) which potentially reacts with all biologicals molecules (Mylona & Polidoros, 2010), influencing the higher MDA concentration. Such corroborates with our results, at which MDA concentrations were very high upon *P. pachyrhizi* infection only in no treated plants, regardless of cultivar. Dallagnol et al. (2011) verified that *Bipolaris oryzae* infection was found to increase the H_2O_2 concentration and most likely that of O_2^- at the same time that the MDA concentration was kept high, which was supported by the positive correlations that occurred among H_2O_2, MDA and brown spot severity.

The cultivar TMG 7062 showed a greater increase on the MDA concentration relative to other cultivars. This result confirms the occurrence of hypersensitive reaction with reddish-brown (RB) lesions observed in TMG 7062 cultivar with resistance genes to *P. pachyrhizi* (Miles et al., 2011). Biotrophic pathogen are entirely dependent on its host plant for nutrient supply and sporulate growing (Voegele et al., 2009). Plant breeding originated cultivars with the feature of reprograming cell death (hypersensitive reaction - HR) to avoid spread of pathogen within plant. To the best of our knowledge, this study provides the first biochemical evidence that the so-called "Inox" cultivars have a very high basal lipid membrane damage, which causes cell death and reduces fungal infection, hence increasing the pathogen´s latent period. In addition to limiting the spread of biotrophic pathogens, HR contributes to the activation of defense in adjacent cells and to the activation of systemic acquired resistance (SAR), a broad-spectrum form of disease resistance mediated by the action of Salicylic acid (SA), which is accompanied by the systemic activation of some defense responses (Vlot et al., 2008).

Soybean cultivar TMG 7062 showed an increase in phenylalanine ammonia-lyases (PAL) enzyme activity as compared to NS 5445 and BMX Tornado cultivars (Fig. 3H), reflecting in an increased proportionally TSP concentration. This is a major enzyme in the phenylpropanoid pathway that is responsible for the production of several phenols (coumaric, cafeic, ferulic, synaptic acids) with antimicrobial proprieties, salicylic acid, and lignin derivatives (Borges et al., 2012; Schuster & Rétey, 1995). However, the increased PAL activity did not proportionally increase phenolics compounds (Figure 3I and 3J), as TSP concentrations were greater in NS 5445 and BMX Tornado rather than TMG 7062.

A likely explanation for this is that phenol oxidation is a common event in host–pathogen interactions as shown by the browning of cells and tissues (e.g. hypersensitive reaction) (Heath, 1998), characteristic of TMG 7062. In addition, these oxidized phenolic species have enhanced antimicrobial activity and thus may be directly involved in stopping pathogen development (Urs & Dunleavy, 1975). However, oxidized phenols were not detected by the methodology utilized in this study.

Results regarding the use of foliar fertilizers and mancozeb indicated an increase on membrane damage upon spraying (Figures 1E, 2E and 3E), without the fungus presence, in NS 5445 and BMX Tornado – exception being cultivar TMG 7062, at which foliar fertilizers reduced the basal lipid membrane damage. Nilsen & Orcutt (1996) established that fungicide and foliar fertilizers can be the abiotic plant stress sources, causing damage oxidative. Plant vital processes are affected during these stress, those oxygen depents, aerobic respiration, photosynthesis and photorespiration, can contribute significantly to ROS formation and induce a generalized plant oxidative stress (Balardin et al., 2017) during the plant-treatment contact. This can explain our results, which treatments increase MDA concentrations without fungus interaction. However, interestingly upon *P. pachyrhizi* infection, treatments effects alleviated the oxidative stress caused during the pathogenesis (Figures 1F, 2F and 3F), such as ammonium thiosulphate for NS 5445, methylene urea and mancozeb for BMX Tornado and TMG 7062.

Sulfur (S) and nitrogen (N) are macronutrients that play a vital role in the regulation of plant growth and development (Marschner, 2012). S is found in the amino acids cysteine and methionine, in glutathione (GSH), and in a variety of metabolites, such as phytoalexins and glucosinolates. GSH, an important antioxidant compound, it participates on the ascorbate-glutathione cycle, which comprises a series of important redox reactions (Dinakar et al., 2012; Noctor et al. 2012). Debona & Rodrigues (2016) concluded that a sustained level of GSH at the late stages of fungal infection appeared to contribute to the reduced oxidative stress observed in azoxystrobin-sprayed plants. Cysteine also plays an important role as a signal to increase the activity of antioxidant enzymes and reduction of lipid peroxidation (Azarakhsh et al., 2015).

Nitrogen is also a structural element of all the proteins, which are the building blocks for the enzymes that are involved on the redox equilibrium, and also form the amino acids like glycine, phenylalanine, cysteine, glutamate, proline and others, which also have an antioxidant role. Teixeira et al. (2017) verified that some amino acids have direct roles on antioxidative system pathways by increasing the plant´s ability to deal with ROS. Ashraf & Foolad (2007) concluded that proline and glycine betaine provide protection to plants from stress by contributing to osmotic cellular adjustment, ROS detoxification, protection of membrane integrity and enzymes/protein stabilization. According with Hu et al. (2012), from the production of glycine betaine, several signaling processes start in plants, such as increased activity of antioxidant enzymes and reduction of lipid peroxidation.

Our findings corroborate with results by these authors, since the use of ammonium thiosulphate in NS 5445 and methylene urea in BMX Tornado and TMG 7062 alleviated oxidative stress caused by *P. pachyrhizi* in soybean leaves (Figures 1F, 2F and 3F). Similarly, mancozeb significantly reduced lipid membrane damage in BMX Tornado and TMG 7062. Mancozeb, besides having nitrogen and sulfur in its structure (with likely similar roles as previously cited), also has manganese (Mn) and zinc (Zn) atoms in its molecular structure. These are important elements with enzymatic (co-factor) roles, as antioxidant enzyme SOD (Gill & Tujeta, 2010). The isoforms of SOD catalyze O_2^- dismutation, hence generating H_2O_2 and O_2, and decreasing the overall likelihood of OH^- synthesis (Dubey, 2011; Dinakar et al., 2012).

In addition to alleviating oxidative stress, these products can significantly influence the production of phenolics compounds through PAL activity induction, since nitrogen and sulfur are structural elements of the PAL enzyme, as well as coenzyme A. In conclusion, this study provides evidence that *Phakopsora pachyrhizi* infection, regardless of treatments spraying, can induce oxidative stress in soybean leaves, but interestingly this infection was shown to inhibit POX enzyme activity. All treatments limited *P. pachyrhizi*-induced oxidative stress, not by POX activation, but probably by other antioxidative enzymes. However, a sustained level of TSP and PAL activity appeared to contribute to the greater level of disease control through of the activation of defense pathways observed in treated plants.

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Table 1. Analysis of variance of the effects of cultivars and treatments for area under the disease progress curve (AUARPC) and number of days for the appearance of the first symptoms (NDAFS).

Sources of Variation	**Df**	***F* values[a]**	
		AUARPC	NDAFS
Cultivars (C)	2	530.5807 *	18.1667 *
Treatments (T)	3	1020.2420 *	24.1111 *
C x T	6	150.7193 *	7.1111 *

[a] Levels of probability: ns = not significant, * = 0.05.

Table 2 - Area under asian rust progress curve (AUARPC) and number of days for the appearance of the first symptoms (NDAFS) for cultivars and treatments. Santa Maria/RS, 2018.

Treat.	**NS 5445**			**BMX Tornado**			**TMG 7062**		
	NDAFS		Ef(%)	NDAFS		Ef(%)	NDAFS		Ef(%)
No applic	4,33	bD	0,00	4,33	bC	0,00	7,67	Aa	0,00
AT	7,00	bB	38,10	9,00	aA	51,85	8,67	Aa	11,54
MU	6,00	bC	27,78	9,00	aA	51,85	9,00	Aa	14,81
Mz	8,67	aA	50,00	7,00	bB	38,10	8,67	Aa	11,54
Treat.	**NS 5445**			**BMX Tornado**			**TMG 7062**		
	AUARPC		Ef(%)	AUARPC		Ef(%)	AUARPC		Ef(%)
No applic	303,00	aA	0,00	246,67	bA	0,00	89,42	Ca	0,00
AT	92,75	bB	69,39	120,50	aB	51,15	60,85	Cb	31,95
MU	94,75	aB	68,73	103,17	aC	58,18	54,90	Bb	38,60
Mz	58,37	bC	80,74	69,93	aD	71,65	37,47	Cc	58,10

CV NDAFS: 10,97%, CV AUARPC: 5,91%. *Means followed by the same letter uppercase within columns and lowercase letter within lines are not significantly different at 5% probability by Scott-Knott test. No applic – Nontreated plants, AT – ammonium thiosulphate, MU – methylene urea, Mz – Mancozeb, Ef (%) – control effectiveness.

Table 3. Analysis of variance of the effects of cultivars (C) and treatments (T) on the activity of peroxidase (POX) and phenylalanine ammonia-lyases (PAL), on the total soluble phenolics (TSP) and hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations.

Sources of	**df**	***F* values**[a]				
		POX	PAL	TSP	H_2O_2	MDA
				4HAA		
C	2	6.6588 *	42.3961 *	175.20 *	25.54 *	52.5235 *
T	3	8.0365 *	44.1213 *	19.9481 *	6.3028 *	44.8266 *
C x T	6	46.3696 *	15.8131 *	28.2315 *	2.0039 ns	30.3101 *
				12HAI		
C	2	1.3705 ns	1959.33 *	20.0082 *	6.9321 *	137.39 *
T	3	15.7733 *	270.8475 *	4.8596 *	5.8258 *	19.7946*
C x T	6	5.8466 *	216.1345 *	10.9502 *	29.04 *	37.3324 *

[a] Levels of probability: ns = not significant, * = 0.05.

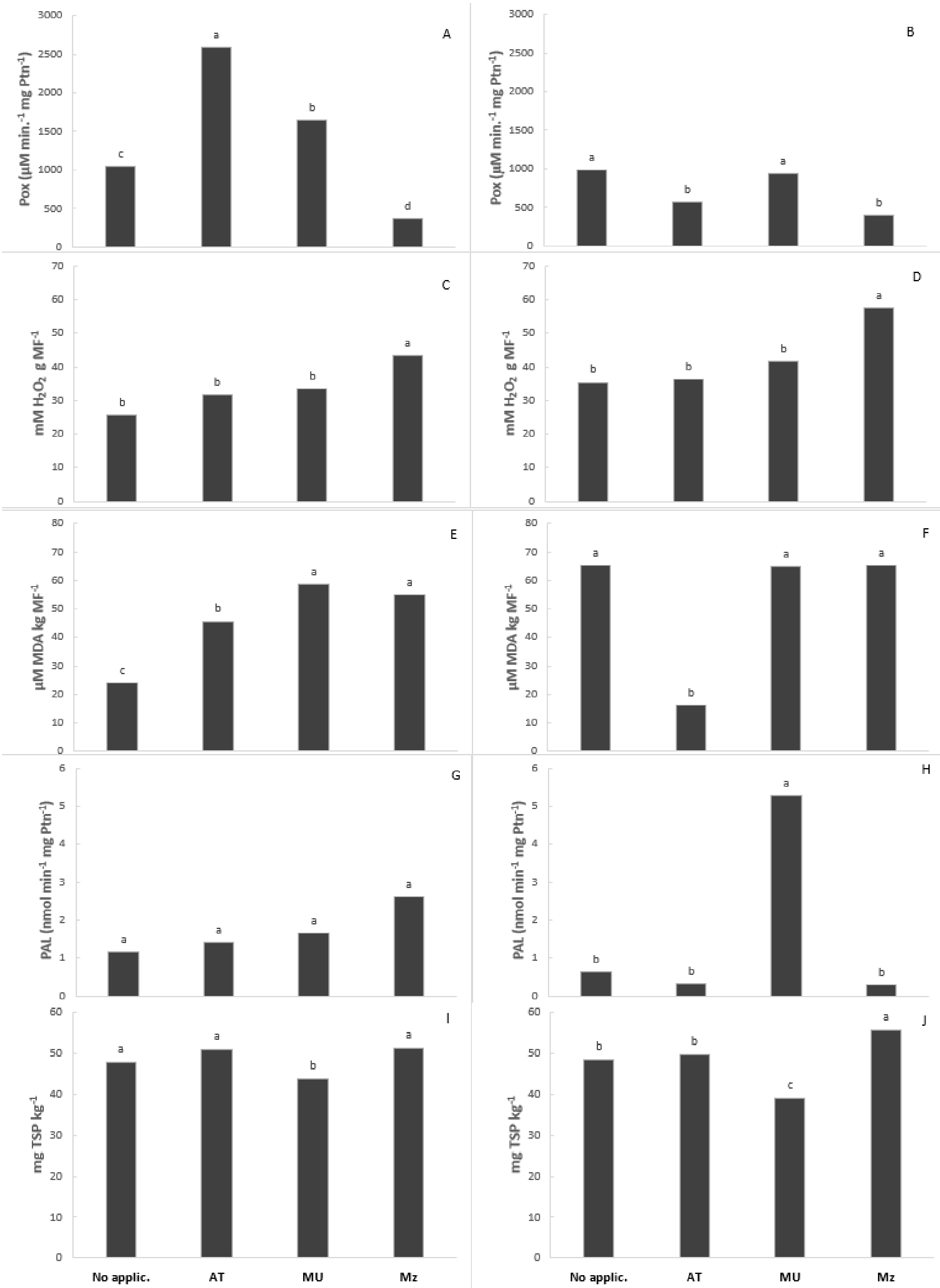

Figure 1 – Enzymes activities of Peroxidase (POX) (A and B) and phenylalanine ammonia-lyases (PAL) (G and H), concentration of hydrogen peroxide (H_2O_2) (C and D), malondialdehyde (MDA) (E and F) and total soluble phenolics (TSP) (I and J) concentrations 4 hours after application (A,C, E, G, I) and 12 hours after inoculation of *Phakopsora pachyrhizi* (B, D, F, H, J) for soybean cultivar NS 5445. AT – ammonium thiosulphate, MU – methylene urea, Mz – Mancozeb. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

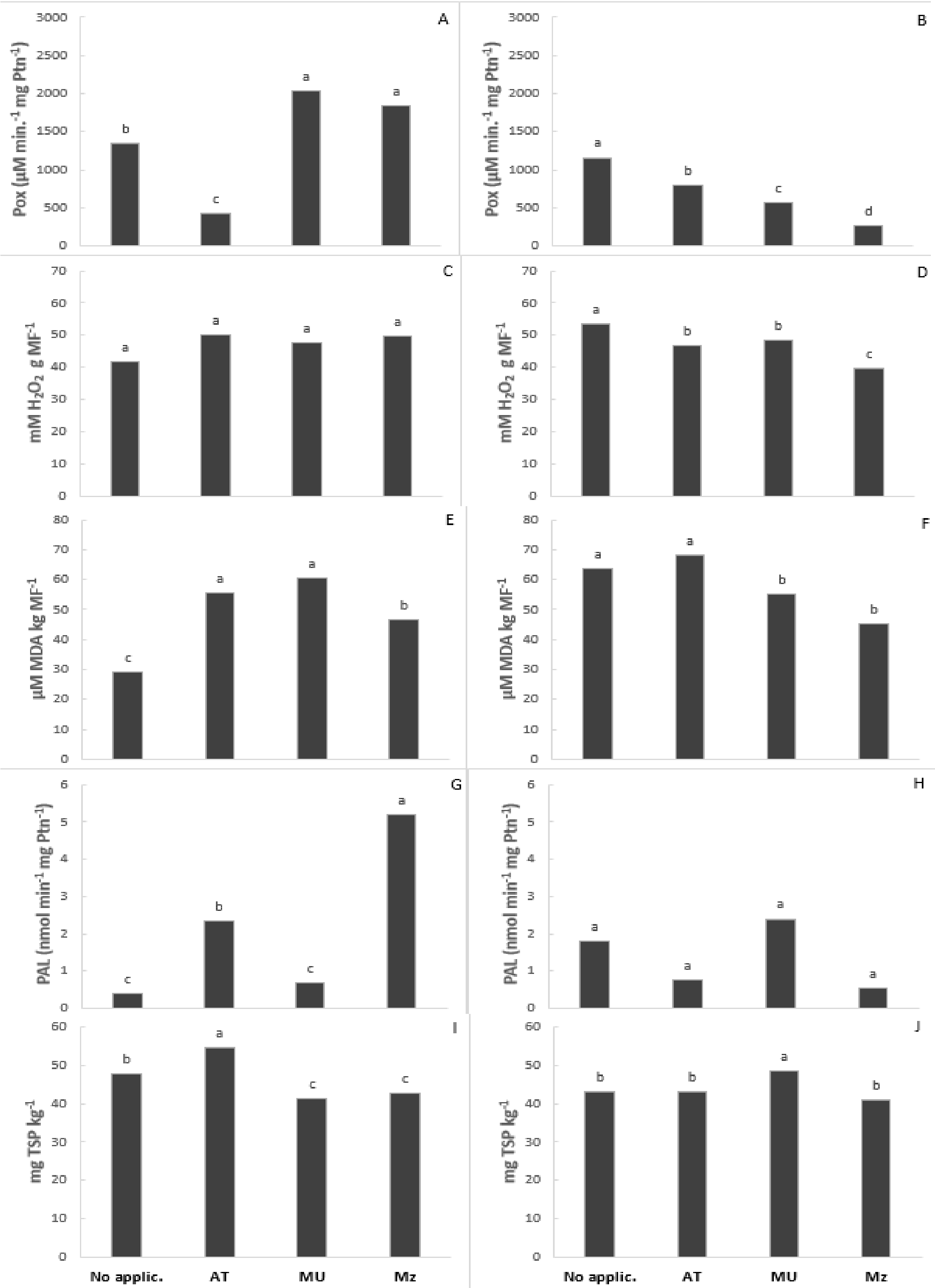

Figure 2 – Enzymes activities of Peroxidase (POX) (A and B) and phenylalanine ammonia-lyases (PAL) (G and H), concentration of hydrogen peroxide (H_2O_2) (C and D), malondialdehyde (MDA) (E and F) and total soluble phenolics (TSP) (I and J) concentrations 4 hours after application (A,C, E, G, I) and 12 hours after inoculation of *Phakopsora pachyrhizi* (B, D, F, H, J) for soybean cultivar BMX Tornado. AT – ammonium thiosulphate, MU – methylene urea, Mz – Mancozeb. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

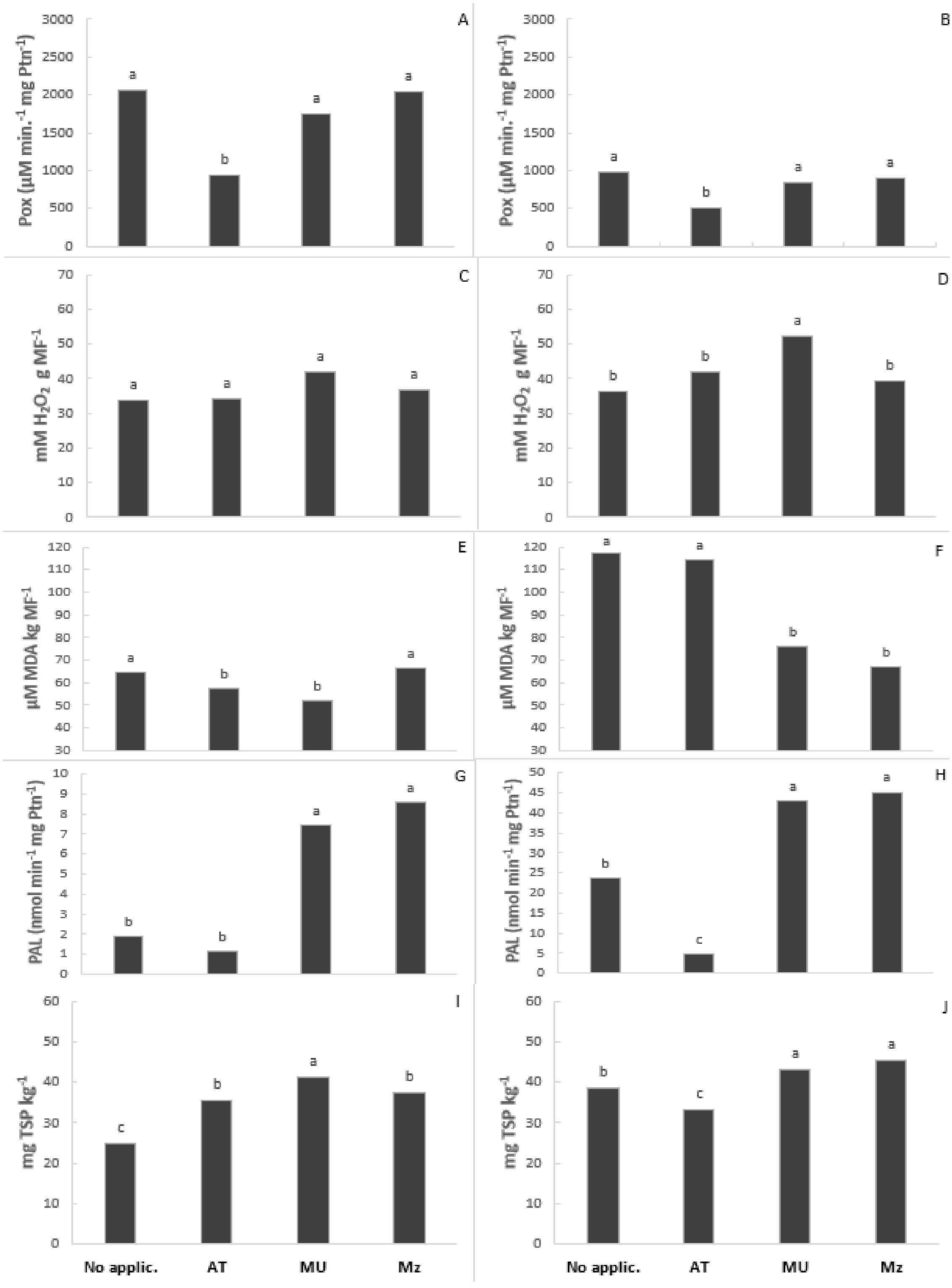

Figure 3 - Enzymes activities of Peroxidase (POX) (A and B) and phenylalanine ammonia-lyases (PAL) (G and H), concentration of hydrogen peroxide (H_2O_2) (C and D), malondialdehyde (MDA) (E and F) and total soluble phenolics (TSP) (I and J) concentrations 4 hours after application (A,C, E, G, I) and 12 hours after inoculation of *Phakopsora pachyrhizi* (B, D, F, H, J) for soybean cultivar TMG 7062. AT – ammonium thiosulphate, MU – methylene urea, Mz – Mancozeb. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

Manuscript II (Will be Submitted to Pesticide Biochemistry and Physiology)

Biochemical effects and *Phakopsora pachyrhizi* control of Picoxystrobin + Cyproconazole associated with Ammonium thiosulphate, methylene urea and mancozeb

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ABSTRACT

The major alternative to Asian Soybean Rust (ASR) control, caused by the plant pathogenic fungus *Phakopsora pachyrhizi*, has been achieved by fungicides, which cause oxidative stress in plants due to the production of reactive oxygen species (ROS). The use of foliar fertilizers associated to fungicides can decreased the oxidative damage, beside of increase the disease control. Therefore, at the present work our goal was to determine whether foliar fertilizers ammonium thiosulphate and methylene urea associated to picoxystrobin + cyproconazole alleviate the oxidative stress caused by fungicide isolated and increase ASR control. The activity of peroxidase (POX) and phenylalanine ammonia-lyase (PAL) enzymes, as well as the concentration of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and total soluble phenolics (TSP) were determined 4 hours after application and 12 hours after inoculation. The treatments were distilled water, picoxystrobin + cyproconazole isolated and associated to ammonium thiosulphate, methylene urea and mancozeb on three soybean cultivars, i.e. 'NS 5445', 'BMX Tornado' and 'TMG 7062', in which control parameters were obtained, number of days for the appearance of the first symptoms (NDAFS), and the area under asian rust progress curve (AUARPC). Results indicated that all the treatments reduced significantly the AUARPC

and increased the NDAFS, more expressively when picoxystrobin + cyproconazole was associated with ammonium thiosulphate or methylene urea, similar to mancozeb association. For biochemical parameters, picoxystrobin + cyproconazole spraying induced oxidative stress in soybean leaves, but foliar fertilizers associated with fungicide on the overall not reduced the oxidative stress relative to fungicide isolated, except on the BMX Tornado, and on the overall not activated defense pathway (PAL and TSP). Our study, provide the first biochemical evidences of picoxystrobin + cyproconazole isolated effects, as well as its interaction with foliar fertilizers and mancozeb on the ASR control and biochemical behaviors changes.

Keywords: soybean, Asian soybean rust, foliar fertilizer, control.

1. Introduction

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, is one of the most destructive diseases of soybean [1]. The typical symptom of the disease are small, tan-colored lesions formed mainly on the abaxial surface of soybean leaflets. Lesions are frequently associated with leaf chlorosis, and high lesion density leads to premature defoliation and early maturity, resulting in significant yield losses [2]. In the absence of control measures, yield losses of up to 90% have been reported [2, 3].

Since commercial soybean cultivars used in major soybean-growing countries are susceptible to ASR, its management has been primarily achieved via use of fungicides, although some cultural practices may also lower disease infection [4].

However, the application of fungicides, as well as other pesticides, causes a chemical toxicity in the plants that results in oxidative stress due to the production of ROS [5, 6, 7], among them, superoxide (O^-_2), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-). The imbalance between production and removal of ROS can result in oxidative damage [8, 9, 10], that results in lipid membrane peroxidation, damage to pigments, proteins, and nucleic acids, beside of leakage of electrolytes of cytoplasm and, consequently, cell death [11].

In order to relieve this stress, and to same time provide ASR control, has been found that the association with mancozeb bring benefits on oxidative stress reduction, which can increase antioxidative enzymes activity, as peroxidase (POX), as well as superoxide dismutase, catalase, among others, which maintain the concentration and production of ROS at tolerable levels [12]. Marques [14] demonstrated that plants with trifloxystrobin + prothioconazole application had a severe oxidative stress, but when associated with mancozeb, the plants presented alleviate oxidative stress and an increase of ASR control was observed.

In addition, the use of foliar fertilizer has been widely discussed about its effect when associated fungicides potentially harmful to plants, can also reduce oxidative stress also favoring the wheat disease control, as verified in studies with foliar fertilizer based on amino acids [15, 16, 17]. Furthermore, the introduction of foliar fertilizers can also influence on the phenylpropanoid pathway, increasing of phenylalanine ammonia lyase (PAL) activity as well as higher total soluble phenolics (TSP) concentration, which can contribute to disease resistance to the pathogen.

In the present study it was investigated control parameters and biochemical responses of soybean plants exposed to picoxystrobin + cyproconazole isolated and associated with ammonium thiosulphate (nitrogen and sulfur), methylene urea (nitrogen) or mancozeb with emphasis on the oxidative/antioxidative status, and activation of PAL and TSP concentration.

2. Material and Methods

2.1. Plant growth

The experiment was conducted during the 2016-2017 growing season in the municipality of Itaara, Rio Grande do Sul, Brazil, in partially controlled conditions in the greenhouse. Maximum and minimum temperatures recorded were 29 °C and 16 °C, respectively, and temperatures were regulated through hoods. Relative humidity was controlled by a computerized moisturizing system and arranged from 65 to 90%. Experiments included seeds from soybean cultivars “NS 5445”, “BMX Tornado”, and “TMG 7062”. Seeds were treated with a mixture of fipronil + pyraclostrobin + thiophanate-methyl (50 + 5 + 45 g a.i. 100 kg^{-1} of seed, respectively), and inoculated with *Bradyrhizobium japonicum* prior to sowing. Seeds were hand-sewn in 5-L pots containing rice husk + soil (2:1), and cultural and fertilization practices performed according to technical recommendations for soybean crop production.

2.2. Treatment application

Soybean plants were either sprayed with distilled water (control; hereinafter referred to as treatment T1), T2 – Fungicide: picoxystrobin + cyproconazole (60g a.i. ha^{-1} + 24g a.i. ha^{-1}), T3 - picoxystrobin + cyproconazole + ammonium thiosulphate (60g a.i. ha^{-1} + 24g a.i. ha^{-1} + 510g sulfur ha^{-1}), T4 - picoxystrobin + cyproconazole + methylene urea (60g a.i. ha^{-1} + 24g a.i. ha^{-1} + 3300g nitrogen ha^{-1}), T5 - picoxystrobin + cyproconazole + mancozeb (60g a.i. ha^{-1} + 24g a.i. ha^{-1} + 1.125g a.i. ha^{-1}). Spraying took place as a preventive measure at early flowering (R1) [18], that is, prior to any ASR disease symptoms being noticeable. Treatments were applied using a knapsack sprayer, and the spray boom equipped with four XR 11002 flat-fan nozzles spaced 0.5 m apart, and calibrated to deliver 150 L ha-1 at 30 psi (206.8 kPa).

2.3. Inoculation of plants with Phakopsora pachyrhizi

On treatment spraying day, six hours after spray, soybean leaves were artificially inoculated following the methodology presented by Lenz et al. [19] using a uredospores suspension of *P. pachyrhizi* ($4x10^4$ spores mL^{-1}) with water, adhesive spreader Tween 80 ppm (for uredospores adhesion to the leaf), and uredospores of *P. pachyrhizi* mixture at R1 stage (early flowering) [18]. Uredospores were previously collected from ASR-naturally infected plants. Immediately after inoculation, pots were transferred to a growth chamber with an average temperature of 25 ± 2 ºC and a relative humidity of 90 ± 5% and subjected to an initial 12 h dark period.

2.4. Assessment of number of days for the appearance of the first symptoms (NDAFS) and area under asian rust progress curve of ASR.

The last two fully expanded leaflets of each plant were marked with colored tape and repeatedly evaluated, and the actual number of days for the appearance of the first symptoms (NDAFS) evaluated. In order to do so, daily observations were made from the second day of inoculation on using a 20X magnifier to examine initial symptoms. This methodology also allowed for the estimation of residual length under each treatment. Severity was determined by assigning visual scores of diseased area percentage relative to the healthy area in each leaflet, as proposed by Godoy [20]. Afterwards, the AUARPC parameter was calculated according to Campbell & Madden [21].

2.5. Biochemical analysis

For all biochemical essays, the fourth and fifth trifoliate leaves, as counted from the plant bottom up, were collected from two plants within each replication either at four hours after spray (4HAA) or 12 hours after inoculation (12HAI). Leaf samples were kept in liquid nitrogen during sampling, and subsequently stored at -80ºC until further analysis.

2.5.1. Determination of peroxidase (POX) enzyme activity

The POX enzyme activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra [22]. In total, 0.5 g of leaf tissue was ground into a fine powder using a mortar and pestle to which liquid nitrogen was added. Upon grinding, the fine powder was homogenized in 2000 µl of a solution containing 50 mM of a potassium phosphate buffer (pH 6.8). The homogenate was then centrifuged at 12,000 x *g* for 15 min at 4ºC, and the supernatant used as a crude enzyme extract. The reaction was started after the addition of 15 µl of the crude enzyme extract to 985 µl of reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2. Activity of the POX enzyme was determined through absorbance of colored purpurogallin at 420 nm for 1 min at 25°C. An extinction coefficient of 2.47 mM^{-1} cm^{-1} [23] was used to calculate the POX enzyme activity, which was expressed as micromoles of purpurogallin produced per minute, per milligram of protein.

2.5.2. Determination of malondialdehyde (MDA) concentration

Oxidative damage in the leaf cells was estimated as the concentration of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA) following Cakmak and Horst [24]. In total, 0.1 g of leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was homogenized in 2000 µl of 0.1% (wt vol^{-1}) trichloracetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant used as a crude enzyme extract. After centrifugation, 500 µl of the supernatant was added into 1500 µl of TBA solution (0.5% in 20% TCA), and the reaction proceeded for 30 min in a boiling water bath at 95°C. After this period, the reaction was stopped by transferring Eppendorfs to an ice bath for 15 min. Samples were centrifuged at 9,000 × *g* for 10 min, and the specific absorbance was determined at 532 nm. The nonspecific absorbance was estimated at 600 nm and subtracted from the specific absorbance value.

An extinction coefficient of 155 mM^{-1} cm^{-1} [25] was used to calculate the MDA concentration, which was expressed as micromoles per kilogram of fresh weight.

2.5.3. Determination of hydrogen peroxide (H_2O_2) concentration

In total, 0.1 g of leaf tissue was ground into a fine powder following a similar procedure as the one described previously. The fine powder was homogenized in a volume of 1,500 µl of TCA 0,1%. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C [26]. In total, 500 µL of the supernatant were added to a reaction mixture containing 500 µL of potassium phosphate buffer 10 mM (pH 7.0) and 1000 µl of potassium iodide (1M). The absorbance of the samples was determined at 390 nm. The concentration of H_2O_2 in the samples was estimated based on a standard curve of H_2O_2 and expressed as millimoles per gram of fresh weight.

2.5.4. Determination of phenylalanine ammonia lyase (PAL) enzyme activity

For the determination of PAL enzyme activity, 0.3 g of leaf tissue was ground following the same procedures described previously. The fine powder obtained was homogenized in 2000 µL of a solution containing 50 mM potassium phosphate buffer (pH 6.8) and 1 mM phenyl-methyl sulfonyl fluoride (PMSF) in an ice bath. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant used to determine the PAL enzyme activity. The latter was achieved by following the methodology proposed by Guo et al. [27] with some modifications. The reaction was started by adding 100 µL of the crude enzyme extract to a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 20 mM *L*-phenylalanine; the final volume was 1000 µL. The reaction mixture was incubated in a water bath at 30°C for 1 h, and 50 µl of cloridric acid (HCl) 6 N were added afterwards to stop the reaction. The absorbance of *trans*-cinnamic acid derivatives was measured at 290 nm. A similar procedure was used for the control samples, but the reaction was immediately stopped with 50 µl of HCl 6 N after the addition of the crude enzyme extract to the reaction mixture. The extinction coefficient of 100 M^{-1} cm^{-1} was used to calculate PAL activity [28].

2.5.5. Determination of Total Soluble Phenolics (TSP)

A total of 0.1 g of leaf tissue was ground into a fine powder with liquid nitrogen in a mortar and pestle and homogenized in 1000 µl of a solution containing 80% (vol/vol) methanol in an ice bath. The homogenate was centrifuged at 17,000 × *g* for 30 min, and the supernatant used to determine TSP concentration by following the methodology proposed by Zieslin and Ben-Zaken [29], with modifications proposed by Rodrigues et al. [30]. The reaction was started after the addition of 0.2 M Folin-Ciocalteu phenol reagent to 150 µl of the methanolic extract and kept at 25°C for 5 min. Next, 0.1 M sodium carbonate was added to the solution, which was maintained at 25°C for 10 min. Afterwards, 1000 µL of deionized water was added to the mixture and it was incubated at 25°C for 1 h. The absorbance was read at 725 nm, and TSP concentration calculated based on a calibration curve using pyrogallol (Sigma-Aldrich, São Paulo, Brazil) as a standard.

2.6. Experimental design and data analysis

A two-by-two factorial experiment, consisting of three soybean cultivars (NS 5445, BMX Tornado and TMG 7062) as well as five treatments (picoxystrobin + cyproconazole premixed isolated and associated with ammonium thiosulphate, methylene urea, mancozeb, and untreated control) was arranged in a completely randomized design with three replications. Each experimental unit consisted of 5L pots containing two soybean plants. Data were subjected to analysis of variance (ANOVA) and average compared by performing the Scott-Knott test at 5% probability using the Assistat software [31].

3. Results

ANOVA results indicated that all the factors in this study were significant for AUARPC of ASR, for which the treatment effect varying according with cultivar. Factors cultivars and treatments were also significant for NDAFS (Table 1), except for factors interaction. The ASR AUARPC was significantly lower in TMG 7062 relative to soybean cultivars NS 5445 and BMX Tornado, regardless of the treatments, as well NDAFS was greater overall, but only significantly different of the other cultivars on picoxystrobin + cyproconazole treatment (Table 2). Moreover, regardless of the actual cultivar used, there was a significant decrease in the ASR AUARPC when untreated plants were compared to those treated with pixoxystrobin + cyproconazole isolated and associated with foliar fertilizers and mancozeb. The latter achieved the greatest control effectiveness of all treatments and genotypes 88.36% for NS 5445, 83.42% for BMX Tornado, and 87.74% for TMG 7062.

Similarly, to control parameters, all the isolated factors as well as their interactions were statistically significant for POX and PAL enzyme activity, and H_2O_2, MDA and TSP concentration (Table 3).

POX enzyme activities 4haa did not significantly differ among the association treatments relative fungicide isolated (T2) or no treated plants for NS 5445 (Fig. 1A), however the association of fungicide with methylene urea and mancozeb for BMX Tornado (Fig. 2A) and, with ammonium thiosulphate and mancozeb for TMG 7062 (Fig. 3A) reduced significantly the activity of this antioxidant enzyme related with fungicide isolated (T2) and no treated plants (T1). However, at 12 hai, picoxystrobin + cyproconazole + mancozeb treatment significantly reduced POX activity 61.63% (Fig. 1B), compared to NS 5445 no treated plants and to pixoystrobin + cyproconazole. In contrast on the BMX Tornado, the same treatment increased significantly under *P. pachyrhizi* infection (Fig. 2B), though fungicide isolated and associated with ammonium thiosulphate or methylene urea have reduced significantly relative no treated plants, not differing among them (Fig. 2B). Under infection, on the TMG 7062 cultivar, should be noticed that all treatments led to a significantly reduced POX activity related with no treated plants (Fig. 3B), did not differing of picoxystrobin + cyproconazole isolated.

Regarding H_2O_2 concentration, there were significant difference for all the association treatments in both sampling periods for NS 5445 comparing to fungicide isolated (Figures 1C and 1D). However, there were no significant differences between each treatment compared to no treated plants at 4 haa for either BMX Tornado (Fig. 2C) and TMG 7062 (Fig. 3C). All treatments reduced significantly the H_2O_2 concentration 12hai relative to no treated plants for BMX Tornado, but only the associations of fungicide (T2) with ammonium thiosulphate or mancozeb differed of fungicide isolated (Fig. 2D), while at 12 hai, only the fungicide + methylene urea association significantly increased H_2O_2 concentration (43.82%) relative to fungicide quantified in the TMG 7062 cultivar (Fig. 3D).

Regarding the MDA concentration, significant differences among the treatments occurred 4haa for NS 5445 and BMX Tornado cultivars, at which all treatments significantly increased MDA concentrations compared to no treated plants (Figures 1E and 2E). However, if compare fungicide isolated to its mancozeb association, there was significantly reduced the membrane lipid damage when associated in both cited cultivars (Figures 1E and 2E), reductions more pronounced were verified on associations with foliar fertilizers for BMX Tornado (Fig. 2E). On the other hand, treatments did not reduce the MDA concentrations relative to picoxystrobin + cyproconazole for TMG 7062 4haa (Fig. 3E). On sampling time 12 hai, on the overall the associations significantly increased MDA concentration or equaled compared to no treated plants for NS 5445 and BMX Tornado (Fig. 1F and 2F, respectively), while at TMG 7062, all the associations significantly increased, 24.89%, 33.29% and 35.76%, respectively for fungicide associated with ammonium thiosulphate, methylene urea and mancozeb (Fig. 3F), but reduced significantly relative to no treated plants.

There were significant differences across treatments concerning PAL enzyme activity at sampling time 4 haa for all the cultivars. The associations treatments significantly reduced the PAL activities relative to fungicide for NS 5445 (Fig. 1G), while the association with ammonium thiosulphate significantly increased (293.94%) for BMX Tornado (Fig. 2G), whereas the associations with methylene urea or mancozeb allowed for the highest PAL enzyme activity at TMG 7062 (564.45% and 548.49%, respectively) (Fig. 3G). In addition, at 12 hai the associations of picoxystrobin + cyproconazole with ammonium thiosulphate and methylene urea significantly increased PAL activity in NS 5445 (54.40% and 96.27%, respectively) (Fig. 1H). At BMX Tornado did not have significantly difference among treatments (Fig. 2H). At TMG 7062 the associations significantly reduced PAL activities relative to picoxystrobin + cyproconazole (Fig. 3H), but if compare these associations in both evaluation time (Fig. 3G and 3H), it is perceived continuous increase on the activity of this enzyme provided by treatments.

Higher TSP concentration occurred at 4 haa in association with mancozeb for NS 5445, however the treatments for BMX Tornado and TMG 7062 did not increase significantly relative to picoxystrobin + cyproconazole, only compared to no treated plants (Figures 2I and 3I). At sampling time 12 hai, the associations did not allow significant higher TSP concentrations compared to fungicide for NS 5445 (Fig. 1J) and BMX Tornado (Fig. 2J), but an increase was observed at association with ammonium thiosulphate for TMG 7062 (14.48%) (Fig. 3J).

4. Discussion

The associations of foliar fertilizers or mancozeb with picoxystrobin + cyproconazole provided increase on the ASR control, have been found lower AUARPC and greater NDAFS when there was association relative to fungicide isolated, in other words, delayed the entry of the disease and reduced the severity of *Phakopsora pachyrhizi* on the three soybean cultivars. Among the associations, have not been verified difference between foliar fertilizers and mancozeb for BMX Tornado and TMG 7062, being for NS 5445 only methylene urea was significantly similar to mancozeb (Table 2).

Corroborating with our results, Marques [15] verified reduction on severity and AUARPC of rust and leaf spots on wheat when associated azoxystrobin + cyproconazole with foliar fertilizer based on amino acids. Morales et al. [32] also demonstrated that application of fungicide with foliar fertilizer associated there was reduction of 18.4% on the AUARPC of leaf spot on wheat. On the soybean, was verified reductions on the incidence and severity of powdery mildew and rust when associated fungicide with foliar fertilizer [33]. The same authors affirmed that there is complementary action between fungicide and nutrients, increasing the defense response of plant to disease infection. In addition, Marques [14] also concluded that the association pyraclostrobin + epoxiconazole with mancozeb increased the NDAFS and ASR control.

Plants have a well-developed antioxidative machinery to prevent cellular membranes from toxic effects caused by reactive oxygen species [34]. It is reported that reactive oxygen species (ROS) are responsible for various stress-induced damages to cellular structures. It is widely accepted that chemical toxicity results in oxidative stress due to the production of ROS [5, 7], among them, superoxide (O^-_2), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-).

Oxidative stress causes lipid peroxidation in the cell membrane and damage to pigments, proteins, and nucleic acids [11]. Under stress conditions plants may alter the activities of ROS scavenging enzymes, such as peroxidase, superoxide dismutase, catalase, glutathione-S-transferase, ascorbate peroxidase, glutathione reductase, and glutathione peroxidase [35].

On the NS 5445 cultivar, increases on the H_2O_2 concentrations 4haa were found with fungicide isolated relative no treated plants, but even more under associations effects (Fig. 1C). This increase relates with high MDA concentrations found in the treatments with associations (Fig. 1E), except for fungicide + mancozeb (relative to fungicide insolated), because H_2O_2 can be related with high superoxide (O_2^-) concentrations previously formed, besides of the excess H_2O_2 can be transferred via the Haber-Weiss reaction to form the highly reactive oxidant hydroxyl radical (OH^-) which potentially reacts with all biologicals molecules [36]. In this cultivar, it was not observed differences on the POX activity for the treatments (Fig. 1A), since an increase in POX activity would be required to lower concentrations of H_2O_2 [37], what, therefore favored to lipid membrane damage.

Despite of the results for BMX Tornado not show overall increases significant on the H_2O_2 concentrations, which would be related more specifically with protein oxidation of cellular lipid membrane, for fungicide isolated or associated relative to no treated plants (Fig. 2A), high MDA concentrations were detected (Fig. 2E). This suggests that the other reactive oxygen species are degrading the lipid membrane, as O_2^- and OH^- concentrations, which are potentially more harmful [12]. However, the associations with both foliar fertilizers or mancozeb reduced the oxidative damage, more pronounced for foliar fertilizers.

A probable explanation for this is based on the increase of mineral elements on base of nitrogen and sulfur provided by fertilizers, which are essentials minerals play a vital role in the regulation of plant growth and development [38]. Sulfur is found in the glutathione (GSH) tripeptide that has recognized antioxidant action [35] and is the substrate for peroxidase glutathione and reductase glutathione action, which are important antioxidative enzymes [39]. Debona & Rodrigues [40] concluded that a sustained level of GSH at the late stages of fungal infection appeared to contribute to the reduced oxidative stress observed in azoxystrobin-sprayed plants. Cysteine, another amino acid with sulfur and also nitrogen, also plays an important role as a signal to increase the activity of antioxidant enzymes and reduction of lipid peroxidation [41].

Nitrogen is also a structural element of all the proteins, which are the building blocks for the enzymes that are involved on the redox equilibrium, and also form the amino acids like glycine, phenylalanine, methionine, glutamate, proline and others, which also have an antioxidant role. Teixeira et al. [42] verified that some amino acids have direct roles on antioxidative system pathways by increasing the plant´s ability to deal with ROS.

Mancozeb, besides having nitrogen and sulfur in its structure (with likely similar roles as previously cited), also has manganese (Mn) and zinc (Zn) atoms in its molecular structure. These are important elements with enzymatic (co-factor) roles, as antioxidant enzyme SOD [37]. The isoforms of SOD catalyze O_2^- dismutation, hence generating H_2O_2 and O_2, and decreasing the overall likelihood of OH^- synthesis [43, 44].

Corroborating with our results, Marques [14] concluded that association of the mancozeb to fungicide systemic trifloxystrobin + prothioconazole played an important role relieving the damages, reducing the MDA concentrations. Besides this, the association of foliar fertilizer (based on amino acids) with fungicide (azoxystrobin + cyproconazole) also was beneficial reducing oxidative stress in wheat [15].

Interestingly, TMG 7062 not suffered oxidative stress neither with fungicide isolated nor associated with methylene urea, in which both presented reduction on the high basal energy expenditure in this cultivar, translated in MDA concentrations of no treated plants of TMG 7062 relative to NS 5445 and BMX Tornado. However, the associations with ammonium thiosulphate or mancozeb reduced significantly the POX activity (Fig. 3A), consequently greater H_2O_2 concentrations can be observed. Wu and Tiedmann [45] demonstrated that high H_2O_2 levels on the epoxiconazole treated plants was directly relative lack of POX activity, in contrast with azoxystrobin effect under ozone exposure. This fact favored the occurrence of membrane lipid damage (Fig. 3E).

Under *P. pachyrhizi* infection presents a strong impact on several of the plant's physiological processes, including great oxidative stress and lipid membrane damage, due increases ROS production in plants, which, in turn, need to activate a range of enzymes responsible for the synthesizes of compounds related to the prevention or alleviation of cellular damage [8, 9]. It perceives in all the cultivars that overall the treatments not reduced the oxidative damage relative to no treated plants, probably due the lower POX activation and greater H_2O_2 concentrations compared to no treated plants and also to picoxystrobin + cyproconazole. Except for TMG 7062, in which the treatments providing stabilization redox equilibrium relative to no treated plants (Fig. 3F), even with lower peroxidase activity, and thus, the avoiding expenditure of energy when under infection.

It seems reasonable to assume that the treatments spraying might have reduced the ASR infection by activating the defense biochemical machinery. Thus, reduced *P. pachyrhizi*-triggered ROS production, mainly O_2^-, can exhibited lower SOD activity, and lower MDA concentration was obtained. The O_2^- dismutation by the SOD represents a front-line defence against oxidative stress. Debona & Rodrigues [40] demonstrated that azoxystrobin spray under inoculated rice plants with *Bipolaris oryzae* exhibited lower SOD activity than no treated plants, by reducing brown spot symptoms, O_2^- production, and with this lower concentration of MDA was verified.

The PAL activities varied greatly among the cultivars and treatments, at overall the associations reduced or equaled the activity of this enzyme compared to fungicide isolated on evaluated periods 4haa. Exceptions refer to association with ammonium thiosulphate on BMX Tornado and, with methylene urea or mancozeb on TMG 7062, that is cultivar effect responding differently to treatments. Under infection - 12hai, the PAL activation seems to be more pronounced on the foliar fertilizer associations for NS 5445, in contrast for TMG 7062. For BMX Tornado, there were not differences significant among treatments compared no treated plants under infection, thus did not bring benefits on the PAL pathway activation.

Corroborating with Nadernejad et al. [46] that demonstrated larger differences among the three pistachio cultivars and concluded that the greater resistance to environmental stresses on the Ahmadaghaii cultivar grafted on Mutica is positively correlated with greater PAL activity. Its provides additional support to our hypothesis of greater resistance to *P. pachyrhizi* for TMG 7062, due the enormous PAL activation, regardless of the treatments applied, than NS 5445 and BMX Tornado, considered more susceptible, on the treatments average to each cultivar, 21.90, 2.88 and 2.20 nmol $min^{-1}mg^{-1}$, respectively. Liang et al. [47] also demonstrated strong protein-related pathogenesis (PRPs) activation, among them PAL, related to high level of basal resistance of cucumber cultivars to *Podosphaera xanthii*.

It was observed greater PAL activation on the picoxystrobin + cyproconazole isolated observed on the NS 5445 (4haa), and on the TMG 7062, upon *P. pachyrhizi* infection (12hai). To our knowledge, this study provides the first biochemical evidence that this fungicide has an important role on the PAL activation on these cultivars, being a reinforcement in the fight against the disease. Phenylalanine ammonia lyase a major enzyme in the phenylpropanoid pathway that is responsible for the production of several phenols (coumaric, cafeic, ferulic, synaptic acids) with antimicrobial proprieties, salicylic acid, and lignin derivatives [48, 49].

Interestingly, the phenols concentrations not responded proportionally to PAL activity in our results. A probable explanation for this is PAL pathway can has directed to salicylic acid production, directly involved on the reaction hypersensitive (HR) or by phenol oxidation, which has enhanced antimicrobial activity and thus may be directly involved in stopping pathogen development [50].

In conclusion, the associations of foliar fertilizers or mancozeb with picoxystrobin + cyproconazole provided increase on the ASR control, with lower AUARPC and greater NDAFS when there was association relative to fungicide isolated. To our knowledge, this study provides the first biochemical evidence that picoxystrobin + cyproconazole fungicide induced oxidative stress in soybean plants. Meanwhile the associations with foliar fertilizers and mancozeb on the overall not reduced oxidative stress, in which the cultivar NS 5445 seems has had a negative response to associations with foliar fertilizers relative to picoxystrobin + cyproconazole isolated. In contrast, the BMX Tornado had its oxidative damage reversed by the foliar fertilizers addition, but under *P. pachyrhizi* infection, both cultivar not helped to maintain the redox equilibrium. Although the TMG 7062 has had an intermediate response of treatments associated relative to oxidative stress of the fungicide isolated, upon *Phakopsora pachyrhizi* infection a reduction on the MDA concentration has helped to combat the fungus.

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Table 1 - Analysis of variance of the effects of cultivars and treatments for area under the disease progress curve (AUARPC) and number of days for the appearance of the first symptoms (NDAFS).

Sources of Variation	**df**	***F* values**[a]	
		AUARPC	NDAFS
Cultivars (C)	2	620.6754 *	6.9698 *
Treatments (T)	4	1881.2718 *	21.4010 *
C x T	8	181.4184 *	0.7534 ns

[a] Levels of probability: ns = not significant, * = 0.05.

Table 2 - Area under the disease progress curve*(*AUARPC*)* and number of days for the appearance of the first symptoms (NDAFS) for cultivars and treatments. Santa Maria/RS, 2018.

Treat.	**NS 5445**			**BMX Tornado**			**TMG 7062**		
	NDAFS		**Ef.**	**NDAFS**		**Ef.**	**NDAFS**		**Ef.**
No appl.	4,3	aB	0,0	4,3	aC	0,0	7,7	aB	0,0
PC	8,3	bB	48,0	9,0	bB	51,9	13,7	aA	43,9
PC + AT	13,7	aA	68,3	13,3	aA	67,5	16,3	aA	53,1
PC + MEU	13,3	aA	67,5	16,3	aA	73,5	15,0	aA	48,9
PC + MZ	11,3	aA	61,8	14,3	aA	69,8	15,7	aA	51,1
Treat.	**NS 5445**			**BMX Tornado**			**TMG 7062**		
	AUARPC		**Ef.**	**AUARPC**		**Ef.**	**AUARPC**		**Ef.**
No appl.	303,00	aA	0,0	246,67	bA	0,0	89,42	cA	0,0
PC	51,37	aB	83,5	55,88	aB	77,3	25,17	bB	71,9
PC + AT	52,83	aB	82,6	47,12	aC	80,9	17,10	bC	80,9
PC + MEU	40,95	aC	86,5	46,75	aC	81,1	15,68	bC	82,5
PC + MZ	35,27	aC	88,4	40,90	aC	83,4	10,97	bC	87,7

CV NDAFS: 21.85%, CV AUARPC: 7,60%. *Means followed by the same letter uppercase within columns and lowercase letter within lines are not significantly different at 5% probability by Scott-Knott test. Ef. – control effectiveness (%), PC – picoxystrobin + cyproconazole, AT – ammonium thiosulphate, MEU – methylene urea, MZ – mancozeb.

Table 3. Analysis of variance of the effects of cultivars (C) and treatments (T) on the activity of peroxidase (POX) and phenylalanine ammonia-lyases (PAL), on the total soluble phenolics (TSP) and hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations.

Sources of Variation	df	*F* values[a]				
		POX	PAL	TSP	H_2O_2	MDA
4HAA						
C	2	16.514*	30.972*	326.441*	16.692*	44.656*
T	4	7.591*	21.311*	13.508*	5.112*	60.511*
C x T	8	4.213*	38.126*	11.022*	5.117*	65.973*
12HAI						
C	2	27.286*	1030.075*	204.240*	3.622*	60.823*
T	4	10.154*	110.515*	19.432*	18.682*	3.417*
C x T	8	18.057*	131.025*	27.838*	14.388*	15.899*

[a] Levels of probability: ns = not significant, * = 0.05.

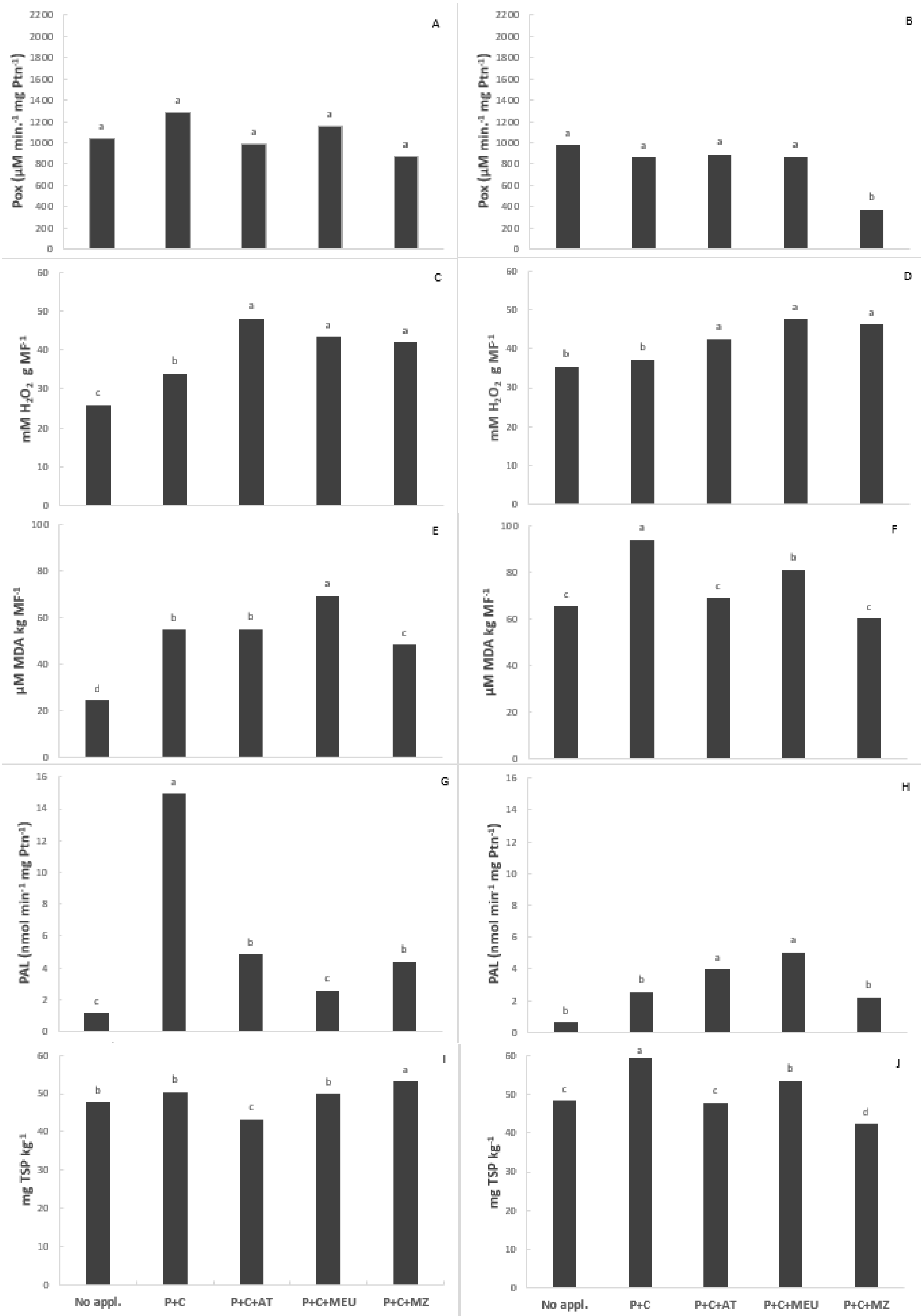

Figure 1 – Enzymes activities of POX (A and B) and PAL (G and H), H_2O_2 (C and D), MDA (E and F) and TSP (I and J) concentrations 4 hours after application (A, C, E, G, I) and 12 hours after inoculation (B, D, F, H, J) for cultivar NS 5445. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

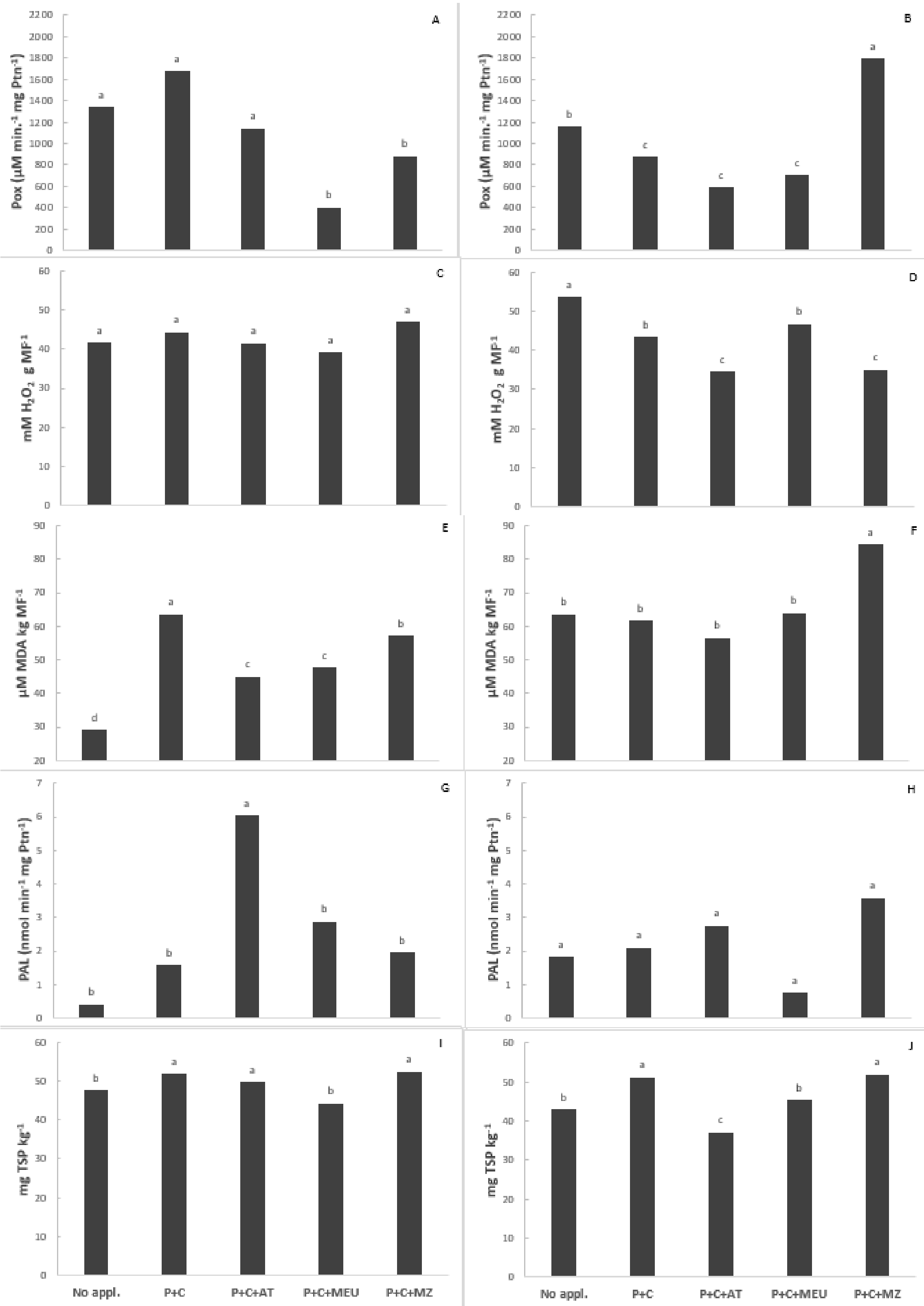

Figure 2 – Enzymes activities of POX (A and B) and PAL (G and H), H_2O_2 (C and D), MDA (E and F) and TSP (I and J) concentrations 4 hours after application (A, C, E, G, I) and 12 hours after inoculation (B, D, F, H, J) for cultivar BMX Tornado. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

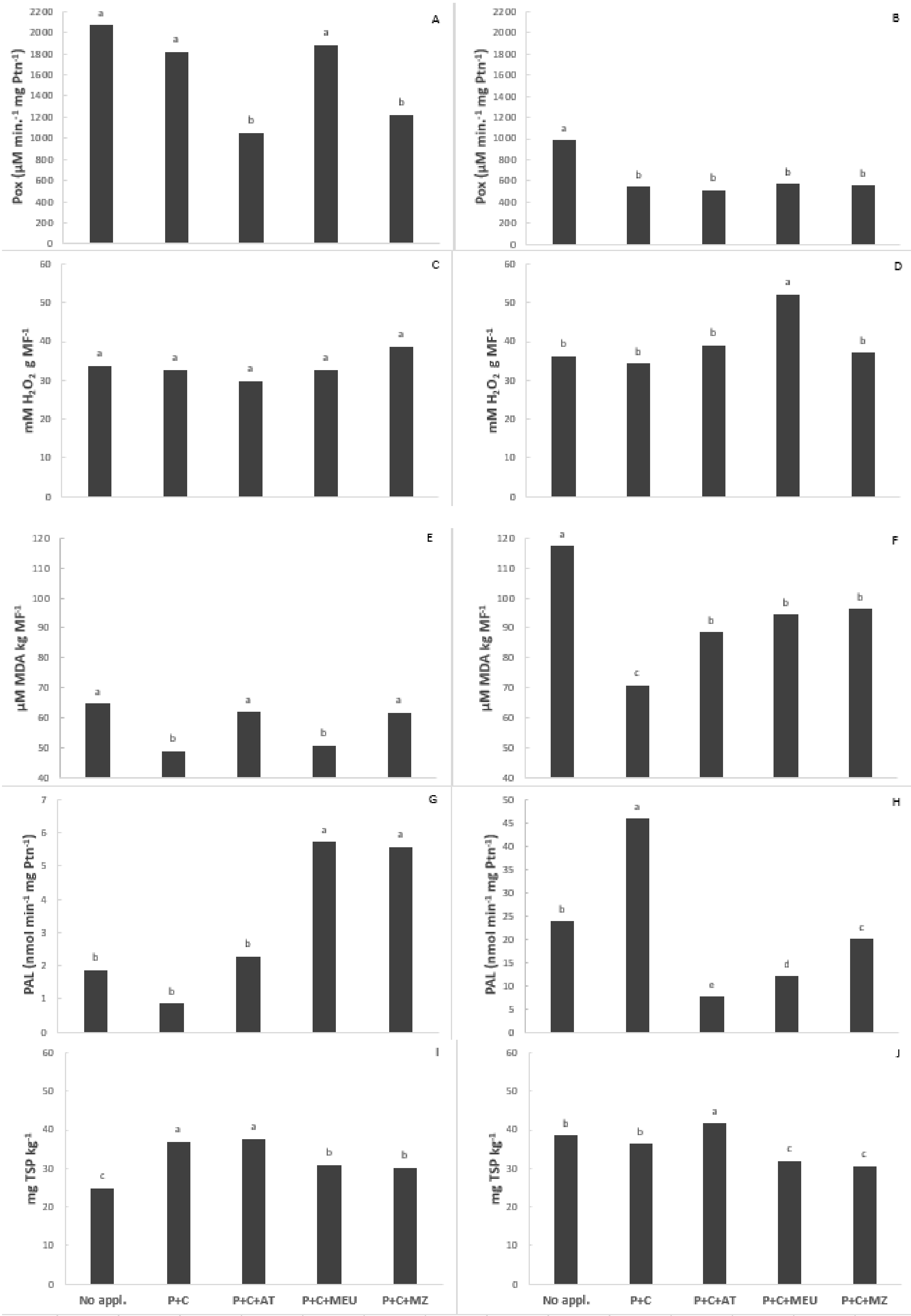

Figure 3 – Enzymes activities of POX (A and B) and PAL (G and H), H_2O_2 (C and D), MDA (E and F) and TSP (I and J) concentrations 4 hours after application (A, C, E, G, I) and 12 hours after inoculation (B, D, F, H, J) for cultivar TMG 7062. Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test.

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Foliar fertilizers and fungicide for Asian Soybean Rust control under different disease pressure levels and edaphoclimatic conditions in Brazil

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RESUMO

O presente estudo tem como objetivo avaliar o efeito de fertilizantes foliares à base de tiossulfato de amônio e metileno ureia sobre a interação *Glycine-max* - *Phakopsora pachyrhizi*, sob diferentes condições edafoclimáticas e de pressão da doença. Foram conduzidos experimentos em dois locais com distintas condições edafoclimáticas (Planaltina/DF e Itaara/RS), usando as cultivares NS 5445 e BMX Tornado, parcialmente suscetível e suscetível a ferrugem asiática da soja, respectivamente, e sete tratamentos aplicados no começo do florescimento e nas primeiras vagens visíveis, sendo eles: Tiossulfato de amônio, metileno ureia e mancozebe isolados e associados com picoxistrobina + ciproconazol e picoxistrobina + ciproconazol isolado, além de plantas não tratadas. Os parâmetros avaliados foram produtividade, área abaixo da curva de progresso da doença (AACPD), e massa de mil sementes. As cultivares responderam diferentemente conforme os locais e tratamentos. Independentemente da cultivar e dos locais o tratamento que proporcionou maior controle foi a associação picoxistrobina + ciproconazol + mancozebe, que por sua vez, obteve maiores produtividades. Os fertilizantes foliares não aumentaram o controle de *Phakopsora pachyrhizi*, tanto isolado como em associação ao fungicida dentro do programa de manejo da doença em ambas condições edafoclimáticas.

Palavras-chave: *Glycine max, Phakopsora pachyrhizi,* pressão da doença, nitrogênio, enxofre.

ABSTRACT

The aim of this study was to evaluate the effect of foliar fertilizers ammonium thiosulphate and methylene urea on the *Glycine-max-Phakopsora pachyrhizi*, particularly under different disease pressure and edaphoclimatic conditions. In the present work, experiments were carried out at two locations with distinct edaphoclimatic conditions (Planaltina/ DF, and Itaara/RS), using soybean cultivars 'NS 5445' and 'BMX Tornado', partially susceptible and susceptible to Asian Soybean Rust, respectively, and seven treatments applied beginning of flowering and first pods visible, being methylene urea, ammonium thiosulphate and mancozeb isolated and associated with Picoxystrobin + Cyproconazole and Picoxystrobin + Cyproconazole isolated, and no treated plants. The assessments were in crop yield, area under asian rust progress curve (AUARPC), and thousand seed mass were calculated. The cultivars responded differently according to site and treatments. Regardless of soybean cultivar and across both locations, the treatment displaying the greater overall performance was the picoxystrobin + cyproconazole + mancozeb mixture, which allowed for the highest yields. The efficiency control of ammonium thiosulphate and methylene urea associated with fungicide varied according the environment, where, differently of Planaltina, in Itaara the foliar fertilizers associated with fungicide did not increase the *Phakopsora pachyrhizi* control relative to fungicide.

Keywords: *Glycine max, Phakopsora pachyrhizi,* disease pressure, nitrogen, sulfur.

INTRODUCTION

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* Syd. & P. Syd, is considered the most damaging foliar disease of soybeans [*Glycine max* (L.) Merr.]. Since its introduction in Brazil, ASR has greatly affected this crop´s profitability due to its large negative impact on grain productivity - yield losses of up to 90% have been reported in the country in the absence of control measures (Hartman, Sikora, & Rupe, 2015).

Edaphoclimatic conditions are known to greatly influence ASR epidemics. In general, optimum climate conditions for growing soybeans are also considered favorable for ASR´s pathogen establishment and development. Disease infection takes place when temperatures range from 10 ºC to 27.5 ºC (optimum 20-23 ºC) and a minimum dew period of 6h (Melching et al. 1989). Typical ASR infection symptoms include sporulating lesions on the abaxial leaf surface, which is usually associated with leaf chlorosis. Lesions first appear in the lower canopy, and then advance up to the mid and upper portions of the plant. As the disease progresses, high-density lesions can develop, leading to premature defoliation and early maturity (Goellner et al., 2010).

Since commercial soybean cultivars used in major soybean-growing countries are susceptible to ASR, fungicides are the main strategy, although some cultural practices may also lower disease infection likelihood within local and regional scales (Miles, Frederick, & Hartman, 2006). However, a total of 100 fungicides have recently been deregistered for ASR control in Brazil - reduction of *Phakopsora pachyrhizi* sensitivity to these fungicides as well as ASR-induced yield losses in treated were the primary drivers for this decision. Therefore, it is crucial that new disease management strategies be investigated, allowing for the adoption of integrated measures aimed to control the spread of this disease, and ultimately reassuring soybean production sustainability.

An overview of current knowledge on the effect of mineral nutrition on plant diseases was compiled by Datnoff, Elmer, e Huber (2007). All plant nutrients have a direct impact on plants, pathogens, and microbial growth so that all of them as well as their proportions are important in disease control and will affect disease incidence or severity (Huber & Haneklaus 2007). In this sense, the foliar fertilizers introduction with sulfur and/or nitrogen can be efficient tools within disease integrated management on soybean crop systems. Sulfur (S) metabolites such as cysteine, glutathione, gaseous S emissions, phytoalexins, glucosinolates, and elemental S depositions have been investigated for their role in plant defense and how targeted S applications may prompt and enhance crop resistance to fungal pathogens (Bloem et al. 2007; Haneklaus, Bloem, & Schnug, 2007; 2009). Recently, for most S containing metabolites a direct antifungal mode of action was proven (Bloem, Haneklaus, & Schnug, 2015).

For nitrogen (N), it was shown that fertilizer application above recommended rates can lead to significantly greater disease incidences (Walters & Bingham 2007). However, positive results also are found, Hofer et al. (2016) concluded that nitrogen fertilization restricts Fusarium grain infection of barley by influencing canopy characteristics and possibly plant physiology. Despite the researches focus mainly nitrogen fertilizers in cereals because of poor biological nitrogen fixation, recently study was published by La Menza et al. (2017), which N limitations in field-grown soybean leads to lower yield in high-yield soybean cropping systems. In adition, the nitrogen can improve the plant physiology, helping in the disease control.

In this sense, one of the greatest benefits of sulfur (S) and Nitrogen (N) to many plant species is them are incorporated in the formation of secondary metabolites (Hirai & Saito 2008; Marschner 2012; Taiz & Zeiger 2013) and bring benefits to the reduction of the intensities of several diseases caused by pathogens. The aim of this study was to verify if disease pressure levels and edaphoclimatic conditions influence on the responses of the soybean cultivars to foliar fertilization isolated and associated with fungicide on the ASR control and its reflection on crop productivity.

MATERIAL AND METHODS

Experiments were carried out in two locations, Itaara and Planaltina (Rio Grande do Sul and Distrito Federal states, respectively) during the 2016/2017 growing season. The first location, Itaara/RS, displays a humid subtropical climate (Koppen classification: Cfa) and litholic neosol soil, and is located at 29º35’15.79” S latitude, 53º48’33.63” W longitude and an elevation of 462 m above sea level. At this location, average minimum and maximum temperatures equaled 16 ºC and 25 ºC, respectively, with a relative humidity of 76% and average rain of 6.87 mm day^{-1}.

The second location, Planaltina/DF lies within a tropical climate location (Koppen classification: Aw) at 15º39’59.6” S latitude and 47º20’09.4” W longitude, and an average elevation of 877 m; soil in the experimental area has been characterized as a red Latossol soil. Despite similar average minimum temperatures to those recorded at Itaara (17 ºC), a warmer maximum temperature (30 ºC) was recorded instead; average relative humidity (79%) and average daily rain amounts (5.12 mm day^{-1}) were also somewhat similar to data collected in Itaara. Soybean was sown in Itaara in November 17, 2016 whereas crop sowing in Planaltina took place in January 10, 2017.

The experimental design in both locations consisted of randomized blocks in a 2 x 8 factorial, at which factor A was soybean cultivars NS 5445 (moderate susceptibility to ASR) and BMX Tornado (susceptible). Factor B consisted of 7 fungicide and nutrient combinations for ASR control plus an untreated check, as follows: 1 - no disease control, 2 – ammonium thiosulphate, 3 – methylene urea, 4 - picoxystrobin + cyproconazole, 5 – mancozeb, 6 - picoxystrobin + cyproconazole + mancozeb, 7 - picoxystrobin + cyproconazole + ammonium thiosulphate, 8 - picoxystrobin + cyproconazole + methylene urea. Factor B treatments were sprayed at R1 (beginning flower; number 61 on the BBCH scale) and R5.3 (74, BBCH) growth stages. Each cultivar x treatment combination was replicated four times.

Except for the untreated control plants, all plots were first sprayed with the fungicides trifloxystrobin + prothioconazole when the crop had reached the sixth trifoliate-leaf growth stage (e.g. V7; number 18, BBCH scale) and again at the end of flowering: first pods visible (e.g. R3; number 69, BBCH).

Parameters evaluated included ASR severity and area under asian rust progress curve (AUARPC) (Campbell & Madden, 1990), as well as grain yield and thousand seed mass (TSM). Disease severity was estimated by visual assessment and grading of the extent by which the crop foliage developed ASR symptoms within each treatment. An area equal to 15 m^2 was harvested per experimental unit, and crop yields determined afterwards. Later on, thousand seed mass and seed moisture were determined and data normalized to 13% seed moisture concentration.

Data were subject to analysis of variance (ANOVA), and means separatedby perfoming the Scott-Knott test at 5% probability using the Assistat software (Silva & Azevedo, 2002).

RESULTS

There was a significant interaction between factors A (soybean cultivar) and B (treatments) for the variables AUARPC and TSM, regardless of experimental site (Itaara/RS and Planaltina/DF) (Table 1). Moreover, factors had a significant interaction at the Planaltina site for the yield variable as well; this response, however, differed from what was recorded at Itaara, where yield differences between cultivars were not significant (Table 1).

Even though significant differences for the variable AUARPC were found in both regions regardless of soybean cultivar, opposite responses were recorded across experimental sites (Table 2). That is, in Itaara, the AUARPC score was 713.33 for soybean cv. NS 5445 and 553.88 for cv. BMX Tornado. However, given that Planaltina conditions allowed for a lower disease intensity relative to Itaara, cv. BMX Tornado displayed a larger AUARPC score in comparison to cv. NS 5445.

Table 1 - ANOVA results for the interaction between factor A (soybean cultivar) and factor B (treatments for ASR control) and variables area under the disease progress curve (AUARPC), crop yield (Y), and thousand seed mass (TSM), in Itaara/RS and Planaltina/DF.

Sources of Variation	df	F values[a]		
		Itaara/RS		
		AUARPC	Y	TSM
Cultivars (C)	1	64.8712*	0.0635ns	1760.9771*
Treatments (T)	7	2585.7570*	12.7766*	106.3568*
C x T	7	51.3087*	1.0669ns	3.6541*
		Planaltina/DF		
Cultivars (C)	1	97.3467*	85.7028*	519.2386*
Treatments (T)	7	95.1042*	17.2373*	10.1039*
C x T	7	6.3810*	9.0807*	7.8132*

[a] Levels of probability: ns = not significant, * = 0.05.

Table 2 – Area under the disease progress curve (AUARPC) for *Phakopsora pachyrhizi* development on soybean cultivars NS 5445 and BMX Tornado cultivars, as affected by a range of fungicides treatments sprayed in beginning flower (R1) and when about 40% of pods have reached final length (R5.3) in Itaara/RS and Planaltina/DF during the 2016/2017 growing season.

Treatments	**Itaara/RS**			
	NS 5445		**BMX Tornado**	
No fungicide	713,13	aA[1]	553,88	bA[1]
Ammonium thiosulphate (AT)	214,56	aB	217,06	aB
Methylene urea (MEU)	177,50	aC	182,56	aC
Fungicide	72,56	aD	73,13	aD
Mancozeb (Mz)	58,88	aD	64,51	aD
Fungicide + Mz	34,24	aE	20,83	aE
Fungicide + AT	78,94	aD	56,81	bD
fungicide + MEU	70,38	aD	72,68	aD
				CV % = 6.67
Treatments	**Planaltina/DF**			

	NS 5445		BMX Tornado	
No fungicide	215,65	bA[1]	378,50	aA[1]
Ammonium thiosulphate (AT)	45,38	bB	117,48	aB
Methylene urea (MEU)	37,70	bB	109,90	aB
Fungicide	32,70	bB	76,08	aC
Mancozeb (Mz)	32,33	bB	80,43	aC
Fungicide + Mz	30,81	bB	75,40	aC
Fungicide + AT	27,91	aB	52,03	aC
Fungicide + MEU	26,09	aB	52,03	aC
				CV % = 10.67

[1] Means followed by the same letter uppercase within columns and lowercase letter within lines are not significantly different at 5% probability by Scott-Knott test. Fungicide - Picoxystrobin + Cyproconazole. All the treatments had alternated applications with Trifloxystrobin + Prothioconazole (sixth trifoliate-leaf growth stage-V7 and end of flowering: first pods visible-R3).

In addition to significant differences between soybean cultivars, it was determined that factor B treatments provided significant ASR control, since disease levels differed to those recorded on untreated, in both regions. In Itaara, treatments provided an average control effectiveness (Figure 1) of 90.13% (cv. NS 5445) and 86.88% (cv. BMX Tornado), whereas in Planaltina, disease control levels of 87.90% and 86.25% were recorded for cv. NS 5445 and cv. BMX Tornado, respectively, underlining the possibility of achieving great disease control when treatments are sprayed at the right timing and fungicide rates.

The treatment which provided the greatest ASR control level was the combination of picoxystrobin + cyproconazole + mancozeb at the Itaara site, which allowed for ASR control levels of 95.20% and 96.24% for cvs. NS 5445 and BMX Tornado, respectively (Fig. 1). Adding foliar fertilizers at such location worsened efficacy (Figure 1) relative to association with mancozeb. However, did not significant differ of the picoxystrobin + cyproconazole pre-mixture (Table 2).

In Planaltina, the picoxystrobin + cyproconazole combination associated with both foliar fertilizers treatments achieved greater for ASR control, but did not significant differ of the fungicide isolated in both cultivars (Table 2). Treatments that only contained foliar fertilizers, in alternated applications with Trifloxystrobin + Prothioconazole, achieved lower disease control levels than no treated plants, but greater disease severity than other treatments, exception being cv. NS 5445 at Planaltina which did not differ statistically from the others (Table 2).

Similarly, to results obtained for ASR control, in Itaara, the treatment consistent of a combination of picoxystrobin + cyproconazole + mancozeb allowed for a crop yield increase of 1.526 kg ha^{-1} for soybean cv NS 5445 and 1.096 kg ha^{-1} for cv. BMX Tornado relative to untreated (no fungicide) plots (Fig. 2A). Furthermore, the treatments with fungicide associated with mancozeb or methylene urea were superior to the use of isolated fungicide, leading to a yield increase of 742 and 615 kg ha^{-1}, respectively, for cultivar NS 5445. However, for BMX Tornado, the associations did not differ significant of picoxystrobin + cyproconazole pre-mixtured yield, although lead to increases of 209 and 69 kg ha^{-1}, respectively to associations with Mancozeb and Ammonium thiosulphate, at the Itaara site (Figure 2A).

At the Planaltina experimental site, despite the fact that the use of foliar fertilizers in association with fungicide allowed lower AUARPC (Table 2), this treatment did not increase significantly crop yields (Fig. 2B), leading to an actual decrease significant of 170 kg ha^{-1} (fungicide + ammonium thiosulphate) and 323 kg ha^{-1} (fungicide + methylene urea) for cv. NS 5445 and a 188 kg ha^{-1} (fungicide + methylene urea) decrease for soybean cv. BMX Tornado. At this location, fungicide + ammonium thiosulphate ultimately brought a yield increase of 317 kg ha^{-1} (Figure 2B).

Interestingly, in Itaara applications of methylene urea alternated with trifloxystrobin + Prothioconazole provided a yield increase of 580 kg ha^{-1} (NS 5445) and 737 kg ha^{-1} (BMX Tornado); the use of ammonium thiosulphate, on the other hand, led to an increase of 239 kg ha^{-1} (NS 5445) and 106 kg ha^{-1} (BMX Tornado), thus being less efficient than methylene urea (Figures 2A). However, such treatments had an inverted crop yield response in Planaltina in comparison to their performances in Itaara, for ammonium thiosulphate allowed for the greatest productivity for the NS 5445 cultivar (an increase of 356 kg ha^{-1}) relative to the untreated plots, and an increase of 692 kg ha^{-1} for cv. BMX Tornado (Figure 2B). Overall, the use of methylene urea led to a yield gain of 243 kg ha^{-1} and 353 kg ha^{-1} for NS 5445 and BMX Tornado, respectively relative to no treated plants (Fig. 2B).

Throughout all trials, cv. BMX Tornado´s thousand seed mass (TSM) was lower than NS 5445´s (Figure 2C and 2D). In Itaara, the treatments picoxystrobin + cyproconazole + mancozeb, and mancozeb applied alone displayed the highest values, across both cultivars (Fig. 2C). In Planaltina, the same treatments were shown to have a better response for cv. NS 5445, whereas cv. BMX Tornado´s results were improved when picoxystrobin + cyproconazole were applied either isolated or in association with mancozeb (Fig. 2D).

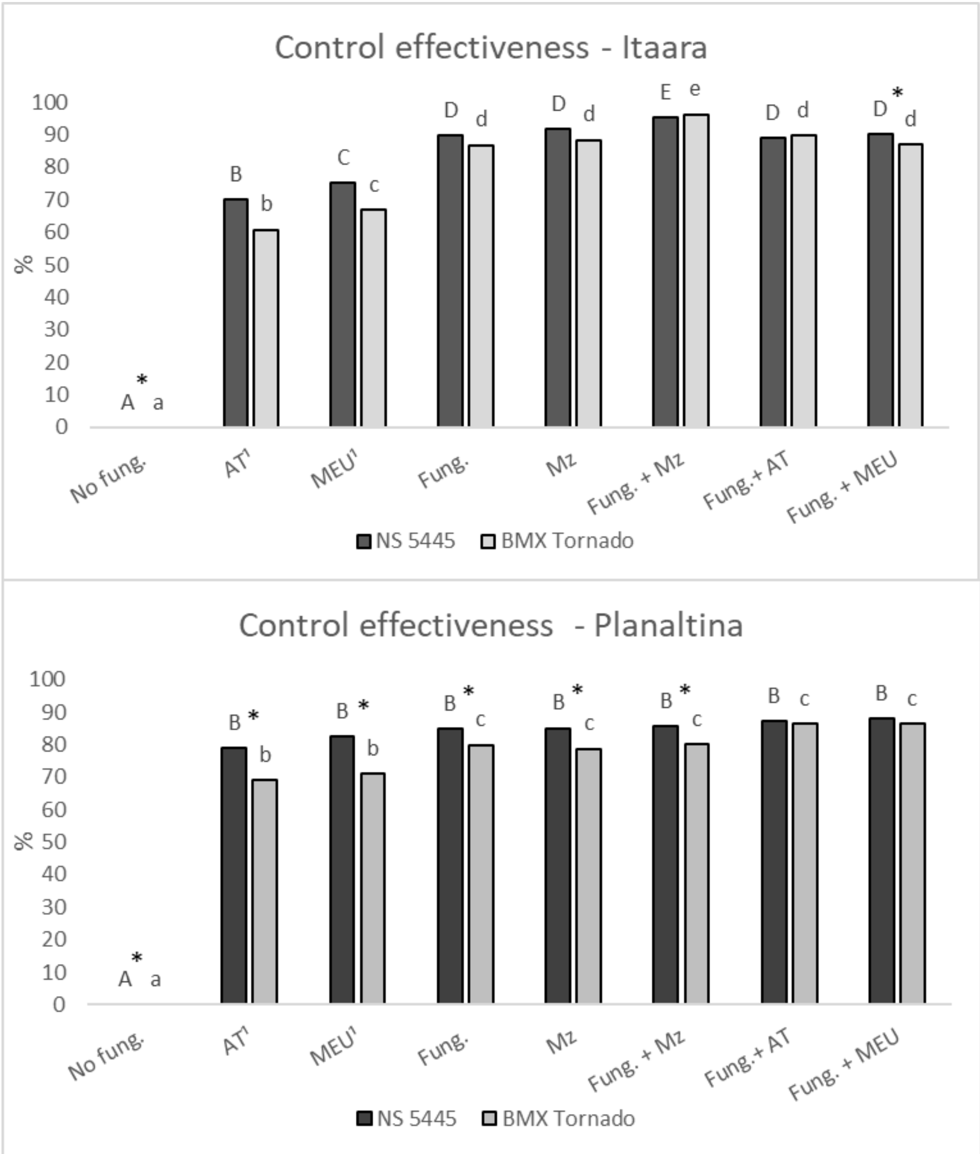

Figure 1 – Treatments *Phakopsora pachyrhizi* control effectiveness in two soybean cultivar (NS 5445 and BMX Tornado) for both sites. AT – Ammonium thiosulphate, MEU – Methylene urea, Fung. – fungicide (Picoxystrobin + Cyproconazole), Mz – Mancozebe. Means followed by the same letter uppercase within NS 5445 and lowercase letter within BMX Tornado are not significantly different at 5% probability by Scott-Knott test. Means followed by the asterisk (*) within each treatment are significantly different at 5% probability by Scott-Knott test. ¹ All the treatments had alternated applications with Trifloxystrobin + Prothioconazole (sixth trifoliate-leaf growth stage-V7 and end of flowering: first pods visible-R3), except to no fungicide treatment.

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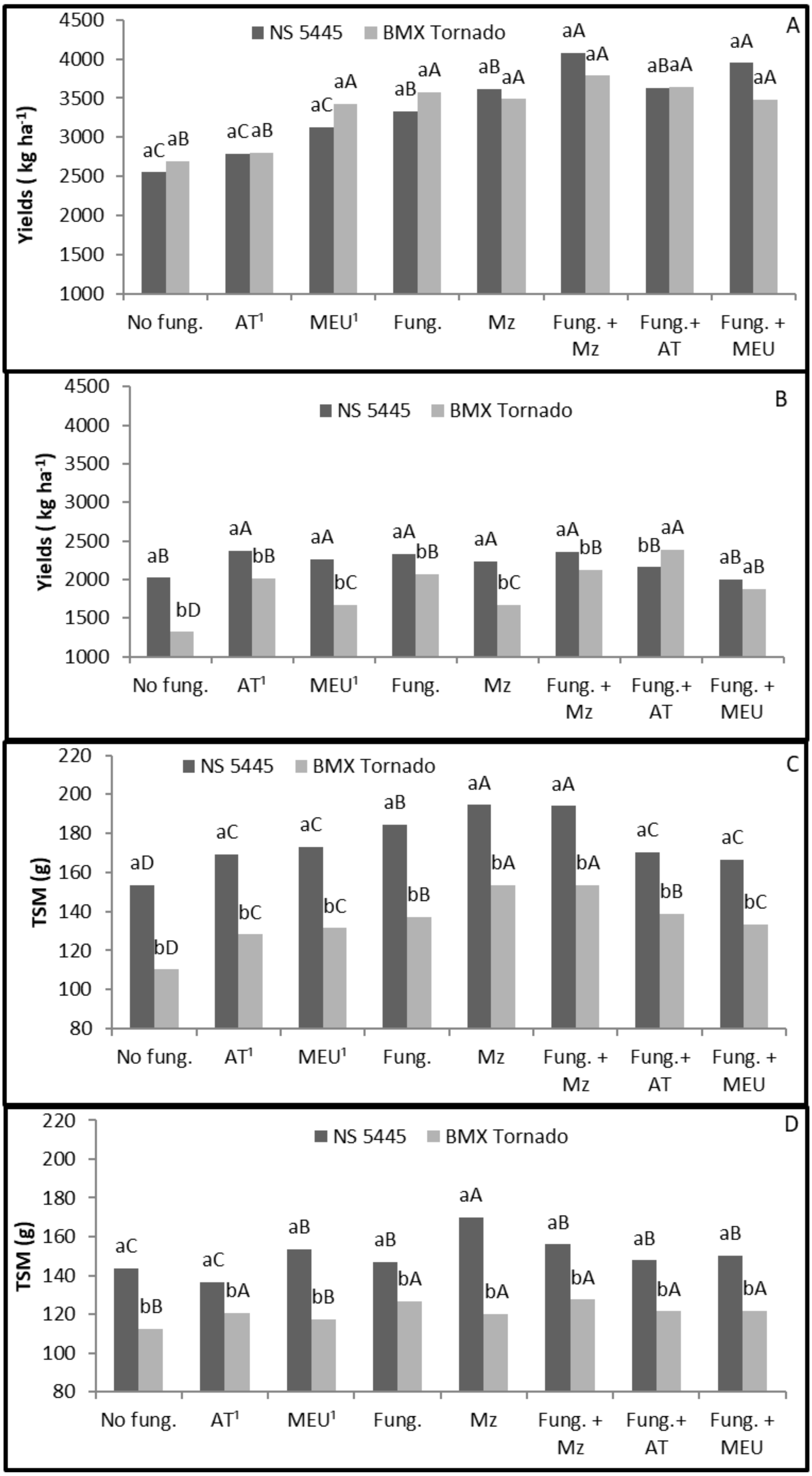
A
B
C
D
NS 5445
BMX Tornado
Yields (kg ha-1)
TSM (g)
4500
4000
3500
3000
2500
2000
1500
1000
220
200
180
160
140
120
100
80
No fung.
AT1
MEU1
Fung.
Mz
Fung. + Mz
Fung.+ AT
Fung. + MEU
aC aB aC aB aC aA aB aA aB aA aA aA aB aA aA aA
aB bD aA bB aA bC aA bB aA bC aA bB bB aA aB aB
aD bD aC bC aC bC aB bB aA bA aA bA aC bB aC bC
aC bB aC bA aB bB aB bA aA bA aB bA aB bA aB bA

Figure 2 – Yield and thousand seed mass in Itaara/RS (A and C, respectively) and in Planaltina/DF (C and D, respectively) at 2016/2017 cropping season in two soybean cultivar (NS 5445 and BMX Tornado). AT – Ammonium thiosulphate, MEU – Methylene urea, Fung. – fungicide (Picoxystrobin + Cyproconazole), Mz – Mancozebe. *Means followed by the same letter uppercase, among treatments, and lowercase, between the cultivars, are not significantly different at 5% probability by Scott-Knott test. [1] All the treatments had alternated applications with Trifloxystrobin + Prothioconazole (sixth trifoliate-leaf growth stage-V7 and end of flowering: first pods visible-R3), except to no fungicide treatment.

DISCUSSION

Distinct edaphoclimatic conditions between experimental sites, as temperature, altitude and water availability allowed for a more intense severity of ASR in soybean grown at the Itaara location relative to Planaltina. Besides climate differences, the greater occurrence of ASR in Itaara is also explained by the fact that growers nearby this location have not adopted the technical recommendation by which fields should be left soybean-free for 90 days between growing seasons (Godoy et. al 2016), thus allowing for a more favorable condition for the occurrence of *Phakopsora pachyrhizi,* hence the disease pressure was greater in Itaara relative to Planaltina.

It should be noted that in Planaltina, the experiment was sown late and, in addition, there was a water deficit during the development of the crop, leading to underdevelopment of the plants. This condition reduced the occurrence of the disease in the area and also influenced the responses of the plants to the treatments, since no great differences between the treatments were found.

In Planaltina, the foliar fertilizers employed in this study were more effective when used isolated, but intervealed with trifloxystrobin + prothioconazole, than noticed in Itaara, possibly due to a lower disease pressure at such location. Moreover, any foliar treatment against *P. pachyrhizi* promoted disease suppression in Planaltina, especially for the NS 5445 cultivar, there were not differ significant among foliar fertilizers isolated or associated with fungicide, which displays a lower susceptibility to ASR than BMX Tornado (Table 2). Balardin et al. (2006) concluded that the association of cultivars with elevated partial resistance and balanced mineral nutrition are the primary elements in an integrated management program aimed to enable larger ASR control efficiency. However, the use of foliar fertilizers for ASR control in soybean cv. BMX Tornado was less effective, which can be explained by its greater susceptibility to ASR, underscoring the need for more effective treatments and fungicides for ASR control. This was successfully achieved via applications of picoxystrobin + cyproconazole either isolated and in combination with foliar fertilizers, or mancozeb. Silva, Juliatti e Silva (2007) and Silva et al. (2011) also verified differential responses of soybean cultivars to a range of fungicides for ASR control.

Despite of the associations with foliar fertilizers, in Itaara, with greater disease pressure, did not increase the ASR control relative to fungicide isolated (Table 2), the association with methylene urea increased yields significantly relative to fungicides applied alone on the NS 5445, however not promoting increases for BMX Tornado (Fig. 2A).

Silva et al. (2013) worked with commercial sources of phosphite and acibenzolar-S-methyl (ASM) in two separate growing seasons (i.e. 2006/2007 and 2007/2008) and verified that a larger disease pressure in the 2006/2007 season meant that treatments containing at least two fungicide spraying events allowed for greater ASR disease control levels relative to treatments which only involved one spraying for disease management, even when such applications were associated with ASM and sources of phosphite. In 2007/08, lower disease pressures allowed for similar results across treatments containing one or two fungicide applications either applied alone or in association with ASM and phosphite sources. Therefore, under high disease severity, the ASR control program must be more robust.

Accordingly, under conditions present at the Itaara site (high disease pressure), soybean cultivar NS 5445, which is moderately susceptible to ASR as was determined in a preliminary experiment (data not shown), had breakdown in less susceptibility become susceptible, and with this, had higher severity than BMX Tornado. Tolerant varieties are useful tools to reduce economic losses associated with severe infections by Asian soybean rust. However, disease tolerance in cultivars displaying a single resistance gene tend to be easily disrupted (Yorinori 2008), particularly in biotrophic pathogens showing high virulence and variability such as *P. pachyrhizi*.

However, differential responses among treatments regarding crop yields and ASR control levels were better visualized under high disease pressure. This observation is in agreement with Scherm et al. (2009), who established that the actual efficacy of disease control set forth by fungicides is directly correlated with the overall disease pressure, meaning that the largest differences in yield responses across fungicide treatments happen at high disease pressure situations.

Larger thousand seed mass (TSM) values were obtained with applications of mancozeb either isolated and in association with Picoxystrobin + Cyproconazole for cultivars NS 5445 (both Itaara and Planaltina sites) and BMX Tornado (Itaara only). The combination of Picoxystrobin + Cyproconazole + Mancozeb also allowed for the largest TSM score for the BMX Tornado cultivar in Planaltina, followed by Picoxystrobin + Cyproconazole applications.

Godoy et al. (2009) highlighted the importance of incorporating as many variables as possible when designing disease management strategies, taking into account regional- and local-specific conditions such that the use of fungicides is determined by assessing risk factors that can be monitored during the current growing season. Such infers that the adoption of a single model for disease management is not suitable considering the large range of edaphoclimatic conditions in Brazil and also the actual level of disease inoculum present in the field, which is known to vary throughout growing seasons. Thereby the results of the present study indicate that the efficient use of ammonium thiosulphate and methylene urea for ASR control vary according to the environment at which soybean is grown, either isolated or associated with Picoxystrobin + Cyproconazole fungicide to fight the Asian Soybean Rust epidemics.

CONCLUSION

In conclusion, the results indicate that foliar application of ammonium thiosulphate and methylene urea have potential for reducing soybean rust severity under low disease pressure conditions (Planaltina). The association them with picoxystrobin + cyproconazole did not bring benefits to control of *Phakopsora pachyrhizi* under high disease pressure (Itaara) conditions, requiring robust control program for Asian Soybean Rust. The best treatment was pycoxistrobin + cyproconazole + mancozeb providing disease reduction and yield increase. Under high disease pressure the BMX Tornado showed lower severity than NS 5445, but under low disease pressure the NS 5445 showed superior genetic control for *Phakopsora pachyrhizi.*

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CONSIDERAÇÕES FINAIS

A utilização dos fertilizantes foliares a base de tiossulfato de amônio e metileno ureia isolados proporcionaram controle (reduziram a severidade) da ferrugem asiática da soja tendo apresentado menor eficácia de controle se comparado ao mancozebe. Tiossulfato de amônio e metileno ureia limitaram o estresse oxidativo das plantas sob infecção de *Phakopsora pachyhizi* em todas as cultivares avaliadas. A maior atividade da fenilalanina amônia liase e a maior concentração de compostos fenólicos proporcionado pelos fertilizantes foliares e pelo mancozebe, parece terem contribuído significativamente para maiores níveis de controle da doença em todas as cultivares. Foram apresentadas, também, as primeiras evidências bioquímicas relacionadas à ativação da fenilalanina amônia liase e peroxidação lipídica na cultivar TMG 7062 que possui genes de resistência à FAS cuja expressão possa ser através de reação hipersensível.

Ficou evidente que o fungicida picoxistrobina + ciproconazol acarretou estresse oxidativo nas plantas sob efeito deste produto. Salienta-se que este estresse, quando levado à nível de campo, pode ser maior, visto a grande quantidade de aplicações feitas durante o desenvolvimento da cultura. Com o intuito de amenizar esse estresse, o fungicida associado aos fertilizantes foliares não reduziram o estresse oxidativo nas plantas nas cultivares NS 5445 e TMG 7062, porém benefícios em controle da FAS são observados. Presume-se que o incremento de controle proporcionado nas associações possa ser devido à barreira química estabelecida na superfície foliar promovendo a redução da infecção pelo patógeno no tecido foliar.

Foi verificado que sob condições de baixa pressão da doença (Planaltina), as associações de picoxistrobina + ciproconazol com os fertilizantes foliares tenderam a proporcionar aumento de controle da FAS. Por outro lado, sob alta pressão da doença (Itaara) os mesmos não produziram incrementos no controle da doença. A necessidade de um programa de controle mais robusto, com produtos de maior eficácia, é necessária a fim de aumentar o controle e manter o potencial produtivo da cultura. Exceção foi verificada na associação do fertilizante a base de metileno ureia com o fungicida, no qual foi verificado aumento significativo no rendimento de grãos da cultivar NS 5445.

Pesquisas adicionais que visem investigar o sistema antioxidativo e a ativação de rotas e compostos de defesa em cultivares de soja desempenharão um papel fundamental no desenvolvimento de marcadores bioquímicos que podem ser usados em programas de melhoramento para selecionar cultivares resistentes ou tolerantes a *Phakopsora* *pachyrhizi*. Novas investigações serão necessárias a fim de elucidar o mecanismo de ação dos fertilizantes estudados, assim como outros fertilizantes foliares, para utilização no controle de doenças e/ou na ativação de rotas de defesa das plantas que ajudem as mesmas a suportar algumas infecções. Somado a isso, a associação de outros fungicidas com os fertilizantes foliares estudados promove novas linhas de pesquisa que necessitam de esclarecimentos acerca da eficácia de controle, estresse oxidativo, ativação de rotas de defesa, bem como proteínas expressadas quando da aplicação dessas associações.

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