ALINE VIEIRA DE BARROS

PHOTOSYNTHETIC PERFORMANCE IN SOYBEAN LEAVES CAUSED BY EPOXICONAZOLE + PYRACLOSTROBIN AND ACIBENZOLAR-S-METHYL AND *Phakopsora pachyrhizi* INFECTION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 16 de fevereiro de 2016

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Jorge Luis Badel Pacheco

Paulo Cezar Cavatte

Fabrício de Ávila Rodrigues
(Orientador)

Aos meus amados pais, Rosani e Sebastião,

Aos meus irmãos Lívia e Davi,

Ao meu companheiro de jornada Franklin,

DEDICO

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BIOGRAFIA

Aline Vieira de Barros, filha de Sebastião Eurestes de Barros e Rosani Fernandes Vieira de Barros, nasceu em 22 de abril de 1991, em Senador Firmino, Estado de Minas Gerais.

Em 2009, ingressou no curso de Agronomia da Universidade Federal de Viçosa (UFV) onde lhe foi conferido o título de Engenheiro Agrônomo, no ano de 2014. Nesta instituição, foi bolsista de Iniciação Científica em Fitopatologia sob orientação do Prof. Laércio Zambolim.

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SUMÁRIO

RESUMO

BARROS, Aline Vieira de, M. Sc., Universidade Federal de Viçosa, Fevereiro de 2016. **Photosynthetic Performance in Soybean Leaves Caused by Epoxiconazole + Pyraclostrobin and Acibenzolar-S-Methyl and *Phakopsora pachyrhizi* infection.** Orientador: Fabrício de Ávila Rodrigues.

A ferrugem asiática da soja (FAS), causada pelo fungo *Phakopsora pachyrhizi,* é uma das doenças foliares mais importantes que afetam a produção de soja em todo o mundo. Este estudo teve como objetivo investigar o desempenho fotossintético (trocas gasosas, imagens da fluorescência da clorofila (Chl) *a* e concentração de pigmentos fotossintéticos) de plantas de soja pulverizadas com Acibenzolar-S-Metil (ASM) e com o fungicida Epoxiconazol + Piraclostrobina (Epo + Pyr) e inoculadas com *P. pachyrhizi*. Os sintomas da FAS progrediram muito mais rapidamente nas folhas das plantas do tratamento controle (inoculadas com água) em comparação com os tratamentos ASM e Epo + Pyr. Em geral, os valores para os parâmetros de trocas gasosas taxa líquida de assimilação de carbono, condutância estomática, concentração interna de CO_2 e taxa de transpiração aumentaram para as plantas infectadas pulverizadas com ASM ou Epo + Pyr em relação às plantas do tratamento controle. Os valores para os parâmetros de fluorescência inicial, fluorescência máxima, eficiência quântica máxima do fotossistema II, rendimento quântico efetivo do fotossistema II Y (II) e rendimento quântico de dissipação de energia regulado Y (NPQ) foram mantidos sempre superiores para os tratamentos ASM e Epo + Pyr em comparação ao tratamento controle nos estágios mais avançados da infecção pelo fungo. Em contraste, os valores para o parâmetro de rendimento quântico de dissipação de energia não regulado (Y(NO) reduziu significativamente para os tratamentos ASM e Epo + Pyr. As curvas para Y(II) diminuíram enquanto que as curvas para Y(NPQ) e Y(NO) aumentaram para as plantas dos tratamentos controle, ASM e Epo + Pyr em função da densidade de fluxo de fótons fotossintéticos. As concentrações de clorofilas e de carotenóides aumentaram significativamente para as plantas infectadas dos tratamentos ASM e Epo + Pyr em comparação com as plantas do tratamento de controle. Em conclusão, os resultados do presente estudo demonstram que a aplicação de ASM ou Pyr + Epo em pantas de soja, mas especialmente do fungicida, contribuíram para reduzir os efeitos negativos causados pela infecção por *P. pachyrhizi* e permitiu a manutenção do desempenho fotossintético das plantas infectadas.

ABSTRACT

BARROS, Aline Vieira de, M. Sc., Universidade Federal de Viçosa, February 2016. **Photosynthetic Performance in Soybean Leaves Caused by Epoxiconazole + Pyraclostrobin and Acibenzolar-S-Methyl and *Phakopsora pachyrhizi* infection.** Adviser: Fabrício de Ávila Rodrigues.

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is one of the most important foliar diseases affecting soybean production worldwide. This study aimed to investigate the photosynthetic performance (leaf gas exchange, chlorophyll (Chl) *a* fluorescence images and photosynthetic pigment pools) of soybean plants sprayed with Acibenzolar-S-Methyl (ASM) and with the fungicide Epoxiconazole + Pyraclostrobin (Epo + Pyr) and further inoculated with *P. pachyrhizi*. The ASR symptoms progressed much faster on the leaves of plants from the control treatment (water spray) in comparison to the ASM and Epo +Pyr treatments. In general, the values for the leaf gas exchange parameters net carbon assimilation rate, stomatal conductance to water vapor, internal CO_2 concentration and transpiration rate increased for the infected plants sprayed with ASM or Epo +Pyr in comparison to plants from the control treatment. The values for the parameters initial fluorescence, maximal fluorescence, maximal photosystem II quantum efficiency, effective photosystem II quantum yield (Y(II)) and quantum yield of regulated energy dissipation (Y(NPQ)) were consistently higher for the ASM and Epo +Pyr treatments in comparison to the control treatment at advanced stages of fungal infection. By contrast, the values for the parameter quantum yield of non-regulated energy dissipation (Y(NO) were significantly lower for the ASM and Epo +Pyr treatments. The curves for Y(II) decreased while the curves for Y(NPQ) and Y(NO) increased for plants from the control, ASM and Epo +Pyr treatments as a function of the photosynthetic photon flux density. The concentrations of total Chl and carotenoids significantly increased for the infected plants from the ASM and Epo +Pyr treatments in comparison to plants from the control treatment. In conclusion, the results of the present study demonstrat that the spray of either soybean plants with Epo + Pyr or ASM, but especially the former, contributed to reduce the negative effects caused by *P. pachyrhizi* infection and allowed a good photosynthetic performance of the infected plants

Introduction

Asian soybean rust (ASR), caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd., has caused significant losses in all major soybean (*Glycine max* (L.) Merr.) producing regions of Brazil (Yorinori and Morel, 2002; Sinclair and Hartman, 1999). The initial disease symptoms appear on leaves as chlorotic polygonal areas restricted by the veins, which become necrotic and where uredia containing urediniospores are formed abundantly (Reis et al., 2006). The greater ASR severity in function of intense necrosis and drying of the leaves can negatively impair the photosynthesis (Rodrigues et al., 2009). Plants suffering from intense defoliation show reduced number of pods with seeds, less grains per pod and lower weight grains mainly when *P. pachyrhizi* infection on leaves occur at plant growth stages of pre-flowering or grain filling (Bromfield, 1984; Hartman et al., 1991; Ogle et al., 1979). The unavailability of commercial cultivars with acceptable levels of ASR resistance and a high genetic variability of the *P. pachyrhizi* population are the major causes of the failure to control ASR (Reis et al., 2006; Yamanaka et al., 2010). On soybean producing regions with frequent occurrence of ASR epidemics, the disease has been mananged with fungicides (Reis et al., 2006). However, the use of inducers of resistance such as the Acibenzolar-S-Methyl (ASM), an analog of the salicylic acid, and the spray of soluble silicon, alone or in combination with fungicides, could be alternatives for the management of ASR (Dallagnol et al., 2006; Duarte et al. 2009; Rodrigues et al., 2009; Cruz et al., 2013, 2014).

Alterations in leaf gas exchange, energy dissipation via chlorophyll *a* (Chl *a*) fluorescence, foliar temperature as well as carbon and or nitrogen metabolism have been reported to occur in plants during the infection process of pathogens mainly when the application of fungicides is made irrationally (Petit et al., 2012; Baker et al., 2008; Petit et al., 2006; Resende et al., 2012; Zhao et al., 2012). It is known that in the presence of

ozone injury and chilling stresses, plants sprayed with some fungicides, especially those from the triazole and strobilurin groups, have their antioxidative system enhanced or increase their capacity to produce hormones and pigments (Diaz-Espejo et al., 2012; Wu et al., 2001; Fletcher et al., 2000; Jaleel et al., 2008; Sujatha et al., 1999; Buchenauer et al., 1981; Muthukumarasamy et al., 1997; Gopi et al., 1999; Gao et al., 1988).

On the other hand, foliar spray of epoxiconazole caused alterations in the biosynthesis of phytosterol and loss of thylakoid integrity on leaves of cleavers plants that also suffering from a growth delay (Benton et al., 1997). Plants sprayed with strobilurins had more photosynthetically active green tissues; high foliar concentrations of nitrogen, pigments and proteins that contributed to retard senescence; and enhanced in the hormonal regulation of mitochondrial energy production (Wu et al., 2001; Bertelsen et al., 2001; Bartlett et al., 2002; Ruske et al., 2003; Grossmann et al., 1999).

Suitable assessment of photosynthesis in cultivated plants infected by pathogens can bring new insights into the mechanisms underlying host-pathogen interactions, may help to predict any imperceptible alteration in crop growth that, consequently, can result in yield losses as well as any novel strategy for disease management (Resende et al., 2012; Bastiaans et al., 1991; Bastiaans et al., 1993; Rolfe and Scholes at al., 2010). Non-invasive methods such as Chl *a* fluorescence imaging, which provides a detailed spatio-temporal analysis of the alterations in photosynthesis, particularly when combined with leaf gas exchange measurements, have been used to improve our knowledge about how an infected leaf responds to pathogen infection (Baker et al., 2001; Rolfe and Scholes, 2010; Iqbal et al., 2012; Rousseau et al., 2013). Several studies have used Chl *a* fluorescence imaging tecnique to assess talterations in photosynthesis of plants infected by biotrophic, hemibiotrophic and necrotrophic fungal

pathogens (Rolfe and Scholes, 2010; Tatagiba et al., 2014; Bermúdez-Cardona et al., 2015; Honorato et al., 2015).

In the present study, an in-depth analysis of the photosynthetic performance of soybean plants challenged with *P. pachyrhizi* and sprayed with ASM or epoxiconazole + pyraclostrobin was performed by analyzing Chl *a* fluorescence images and photosynthetic pigment pools. The main goals was to investigate fungus-induced perturbations in photosynthesis as ASR progressed.

Materials and Methods

Planth growth

Soybean seeds (cultivar Anta 82 RR) were surface sterilized in 10% NaOCl for 2 min and sown in plastic pots (20-cm diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) filled with 3 kg of substrate made from a 1:1:1 mixture of pine bark, peat and expanded vermiculite (Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). A total of 1.63 g of calcium phosphate monobasic was added to each plastic pot. A total of five seeds per pot were sown and at five days after seedlings emergence, each pot was thinned to three seedlings. Fifteen days after sowing, the plants received 30 ml of nutrient solution containing 2,5 mM $Ca(NO_3)_2.4H_2O$, 1 mM NH_4NO_3, 1 mM KNO_3, 1 mM KH_2PO_4, 1 mM KCl,1 mM $MgSO_4.7H_2O$,0,2 µM H_3BO_3, 0,2 µM $ZnSO_4.7H_2O$, 0,2 µM $CuSO_4.5H_2O$, 0,2 µM $MnCl_2.4H_2O$, 0,2 µM $Na_2MoO_4.4H_2O$, 0,5 mM $Na_2EDTA2.H_2O$ and 0,5 mM $FeSO_4.7H_2O$. A total of 30 ml of nutrient solution was applied to each pot weekly. The nutrient solution was prepared using deionized water. Plants were kept in the greenhouse during the experiments and were watered as needed.

Treatments, inoculum preparation and plant inoculation

The treatments used in the experiments were: distilled water (control treatment), Acibenzolar-S-Methyl (ASM) (300 mg L^{-1}; Syngenta, São Paulo, Brazil) and the

fungicide mixture 13.3% epoxiconazole + 5% pyraclostrobin (Epo + Pyr; 0.5 L ha^{-1}; Opera, Basf S.A., São Paulo, Brazil). These products were applied at 48 hours before plant inoculation using a VL Airbrush atomizer (Paasche Airbrush Co, Chicago, IL). Plants at the V6 growth stage (Caviness and Fehr, 1977) were inoculated with a uredospores suspension of a monopustular isolate of *P. pachyrhizi* (UFV-DFP *Pp*018) at a concentration of 10^5 uredospores mL^{-1}. The ureiniospores of isolate of *P. pachyrhizi* were previously multiplied on plants from the cultivar BR/MG 46 (Conquista). The urediniospores showed 89% of germination in the *in vitro* tests. Twenty-five milliliters of suspension was sprayed as a fine mist on the leaves of each plant until runoff using a VL Airbrush atomizer. Gelatin (1%, w v^{-1}) was added to the suspension to aid uredospores adhesion to the leaf blades. Immediately after inoculation, the plants were transferred to a growth chamber with a temperature of 25 ± 2°C and a relative humidity of 90 ± 5% and were subjected a 24-h darkness period. After this period, the plants were transferred to a mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The MGC was made of wood (2 m wide, 1.5 m high and 5 m long) and covered with 100-µm thick transparent plastic. The temperature inside the MGC ranged from 24 ± 1°C (day) to 18 ± 2°C (night). The relative humidity was maintained at 90 ± 5% using a system whose nozzles (model NEB-100; KGF Company, São Paulo, Brazil) that sprayed mist above the plant canopy every 30 min. The relative humidity and the temperature were measured with a thermo-hygrograph (TH-508, Impac, São Paulo, Brazil). The maximum natural photon flux density at plant canopy height was approximately 950 µmol m^{-2} s^{-1}.

Disease evaluations

Rust severity was assessed at 9, 13, 17 and 21 days after inoculation (dai) using the scale proposed Godoy et al. (2006). The area under disease progress curve

(AUDPC) for each plant was calculated using the trapezoidal integration of the disease progress curves according to Shaner and Finney (1997). The number of lesions (NL) per cm^2 of trifoliate leaf was counted at threevplaces randomly chosen on the first fully-expanded trifoliate leaf (from the apex to the base) of each plant at 21 dai.

Determination of the leaf gas exchange parameters

The gas exchange parameters were measured on the first leaf (from the apex to the base) of each plant at 9, 13, 17 and 21 dai. The net carbon assimilation rate (*A*), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and transpiration rate (*E*) were measured from 09:00 to 12:00 h under artificial and saturating photon irradiance (1000 μmol m^{-2} s^{-1}) and an external CO_2 concentration of 400 μmol mol^{-1} using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA).

Imaging of the Chl *a* fluorescence parameters

Images and parameters of Chl *a* fluorescence were obtained from the the first trifoliolate leaf (from the apex to the base) of each plant using the MAXI version of the Imaging-PAM fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany). The Chl *a* fluorescence emission transients were captured by a CCD (charge-coupled device) camera with a resolution of 640 × 480 pixels in a visible sample area of 24 × 32 mm on each leaf. The leaves were initially adapted to darkness for 60 min, after which they were carefully and individually fixed in a support at a distance of 18.5 cm from the CCD camera. The leaves were then exposed to a weak, modulated measuring beam (0.5 μmol m^{-2} s^{-1}, 100 μs, 1 Hz) to determine the initial fluorescence (F_0) when all the PSII reaction centers are to be "open". Next, a saturating white light pulse of 2,400 μmol m^{-2} s^{-1} (10 Hz) was applied for 0.8 s to ensure maximum fluorescence emission (F_m) when all the PSII reaction centers are expected to be

"closed". From these initial measurements, the maximum PSII photochemical efficiency of the dark-adapted leaves was estimated through the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. The leaf tissues were subsequently exposed to actinic photon irradiance (110 μmol m^{-2} s^{-1}) for 120 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (2,400 μmol m^{-2} s^{-1}; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). Following the calculations proposed by Kramer et al. (2004), the energy absorbed by the PSII for the following two yield components for dissipative processes were determined: the yield of photochemistry [(YII = $(F_m' - F)/F_m')$], the yield for dissipation by down-regulation [Y(NPQ) = $(F_s/F_m') - (F_s/F_m)$] and the yield for other non-photochemical (non-regulated) losses [Y(NO) = F_s/F_m]. These parameters were calculated using the Imaging Win software (Kramer et al., 2004).

Determination of the concentration of photosynthetic pigments

The concentrations of chlorophyll (Chl) *a*, Chl *b* and carotenoids were determined using dimethyl sulfoxide (DMSO) as solvent (Santos et al., 2008). Five leaf disks (1 cm in diameter) were obtained from the first trifoliolate leaf (from the apex to the base) of each plant. The collected disks were immersed in glass tubes containing 5 ml of saturated DMSO solution and calcium carbonate ($CaCO_3$) (5 g/L) (Wellburn, 1994) and kept in the dark at room temperature for 24 h. The absorbances of the extracts were read at 480, 649.1 and 665.1 nm using a saturated solution of DMSO and $CaCO_3$ as a blank.

Experimental design and data analysis

A three-by-two factor experiment, consisting of three treatments (distilled water, ASM and fungicide) and non-inoculated or inoculated plants, was arranged in a completely randomized design with six replications. The experiment was repeated once. Each experimental unit corresponded to a plastic pot containing three plants. Data for all

the variables were subjected to analysis of variance and the means from the treatments were compared by Tukey's test ($P \leq 0.05$) using SAS software (SAS Institute Inc., Cary, NC).

Results

Rust severity, AUDPC and NL

Rust severity progressed much faster on the leaves of plants from the control treatment in comparison to the other treatments (Fig. 1A). The AUDPC was significantly reduced by 32.5 and 88.7% for the treatments ASM and fungicide, respectively, in comparison to the control treatment (Fig. 1B). There were significant reductions of 57.9 and 84.9% for the NL for the ASM and fungicide treatments, respectively, in comparison to the control treatment (Fig. 1C).

Leaf gas exchange parameters

There were significant increases in *A* of 41.6 and 57.3 for the ASM treatment and of 48.8, 70.7 and 44.8% for the fungicide treatmentat 13, 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 2A). There were significant increases in g_s of 41.1 and 54.4 for the ASM treatmentat 9 and17dai and of 35.4, 30.4, 69.5, respectively, and 33.8% for the fungicide treatmentat 9, 13, 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 2B). On the leaves of non-inoculated plants, *A* was significantly reduced by 8.9% for the fungicide treatment and g_s by 13.4 and 18.4% for the ASM and fungicide treatments, respectively, in comparison to the control treatment (Fig. 2A and B). The C_i significantly increased by 13.3 and 9.3% at 9 dai, by 5.4 and 7.4% at 17 dai and by 8.6 and 10.7% at 21 dai for the control treatment in comparison to the ASM and fungicide treatments (Fig. 2C). The *E* significantly increased by 6.1, 54.7 and 30.5% for the ASM treatment and by 20.3, 73.4 and 26.7%

for the fungicide treatment, respectively, at 13, 17 and 21 dai in comparison to the control treatment (Fig. 2D).

Imaging of Chl *a* fluorescence

In addition to the quantitative assessment of Chl *a* fluorescence parameters, semi-quantitative analysis of photosynthesis using Chl *a* fluorescence imaging (Figs. 3 and 4) of plants sprayed with water (control treatment) and with ASM and fungicide that were non-inoculated or inoculated was performed. Notably, the magnitude of the changes in F_0, F_m, F_v/F_m (Fig. 3) as well as in Y(II), Y(NO) and Y(NPQ) (Fig 4) for the control treatment were more evident in contrast to the others treatments. Indeed, the first detectable changes fluorescence of Chl *a* in the leaves of soybean plants from the control treatment were evident at 9 dai (Figs. 3 and 4) and increased thereafter as the necrotic lesions expanded (Figs. 3 and 4). On the other hand, on leaves of plants from the fungicide treatment, which showed reduced ASR symptoms, the detectable changes were less pronouced indicating lower damage to the photosynthetic apparatus (Fig. 3B and C). The values for the F_0, F_m, F_v/F_m, Y(II) and Y(NPQ) parameters were consistently higher for the ASM and fungicide treatments in comparison to the control treatment during most of the time course evaluated (Figs. 5 and 6). There were significant increases in F_0 of 21.7, 55.6 and 55.4% for the ASM treatment and of 24.0, 56.5 and 66.4% for the fungicide treatmentat 13, 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 5A). The F_m values showed significant increases of 11.8, 19.9, 70.03 and 56.5% for the ASM treatment and of 6.3, 26.7, 75.5 and 77.1% for the fungicide treatmentat 9, 13, 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 3B). The F_v/F_m values were significantly higher by 14 and 17% for the ASM treatment and by 18.9 and 15.7% for the fungicide treatmentat 17 and 21 dai, respectively, in comparison to the control treatment (Fig.

5C). In comparison to the control treatment, there were significant increases of 13.4 and 31.9% in the Y(II) values at 13 and 21 dai for the ASM treatment and of 20.4, 14.0, 39.9 and 36.6% for the fungicide treatment during the time course evaluated (Fig. 6A). For Y(NPQ), there were significant increases of 28.5 and 26.5% in the non-inoculated plants for the ASM and fungicide treatments, respectively, in comparison to the control treatment (Fig. 6B). There were also significant increases for Y(NPQ) of 32.1 and 24.1% for the ASM treatment at 17 and 21 dai, respectively. For the fungicide treatment, Y(NPQ) significantly increased by 13.6, 27.0 and 29.4% at 13, 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 6B). Conversely, the Y(NO) values were significantly lower by 15.9% in the non-inoculated plants for the fungicide treatment in comparison to the control treatment (Fig. 6C). For the Y(NO), there were significant reductions of 34.9 and 29.3% for the ASM treatment, respectively, at 17 and 21 dai, respectively, in comparison to the control treatment. Similarly, there were significant reductions of 26.9, 16.7 and 37.2% for the fungicide treatmentat 13, 17 and 21 dai. The curves for Y(II) decreased (Fig. 7A) while the curves for Y(NPQ) and Y(NO) (Fig. 7B and C) increased for all treatments as a function of the PPFD. The values of Y(II) were lower and for Y(NPQ) and Y(NO) were higher for the control treatment in comparison to the other treatments (Fig. 7A-C). The Y(NO) values were significantly lower by 14.1 and 24.3%, for the ASM and fungicide treatments, respectively, in comparison to the control treatment (Fig. 7D). The Y(NPQ) was significantly higher for the fungicide treatment by 19.3 and 20.6%, respectively, in comparison to the control and ASM treatments (Fig. 7D).

Concentration of pigments

The concentration of Chl*a*+*b* significantly increased by 32.6 and 55.9% for the ASM treatment and by 12.3 and 25.2% for the fungicide treatmentat at 17 and 21 dai,

respectively, in comparison to the control treatment (Fig. 8A). The concentration of carotenoids significantly increased by 23.1 and 30.9% for the ASM treatment and by 23.3 and 27.1% for the fungicide treatmentat 17 and 21 dai, respectively, in comparison to the control treatment (Fig. 8B).

Discussion

This study provides direct evidence that the negative effect caused by *P. pachyrhizi* on infection the photosynthetic apparatus of soybean leaves was greatly reduced by foliar tratament with Epo + Pyr, and to a lesser extend with ASM, a known inducer of host resistance. Indeed, the photosynthesis was up-regulated at the photosynthetic machinery level coupled with lower destruction of chlorophyll molecules and higher leaf gas exchange capacity. The findings of the present study support the concept that both ASM and Epo + Pyr enhance soybean resistance to ASR by decreasing disease severity and the number of pustules per leaf (Duarte et al., 2009; Rodrigues et al., 2009; Cruz et al., 2013, 2014; Miles et al., 2007). It is known that QoI fungicides affect both spore germination and fungal penetration into the host tissue (Bartlett et al., 2002), both of which are of detrimental importance for successful fungal infection; the DMI fungicides also affect fungal mycelial growth inside plant tissues (Kuck et al., 2012). According to Cruz et al. (2013), besides reducing the ASR symptoms in soybean pants, ASM causes increase in the activities of chitinases and phenylalanine ammonia-lyases.

Alterations in photosynthesis have been reported to occur in several plant species in response to pathogen infection (Batista et al., 2012; Aucique-Perez et al., 2014; Bermúdez-Cardona et al., 2014; Dallagnol et al., 2011). However, an interesting aspect of spraying fungicides such those of as the triazole and strobilurins groups onto plants aiming disease control is that they may interfer with plant metabolism (Petit et al.,

2012). In general, the reduced symptoms of ASR on soybean plants sprayed with ASM or Epo + Pyr contributed to a better response of *A*, g_s and *E* in comparison to plants from the control treatment. Research with cereals (*e.g*. barley, rice and wheat) have shown that reduction in photosynthesis caused by the spray of strobilurin was primarily of non-stomatal origin therefore hindering, the CO_2 flow into the intercellular spaces and compromising carboxylation to occur in the chloroplasts (Nason et al., 2007; Debona et al., 2016). Rice plants sprayed with azoxystrobin and inoculated with *Bipolaris oryzae* showed lower reductions in the values of leaf gas exchange parameters in comparison to the non-sprayed infected plants (Debona et al., 2016). In the case of triazole compounds, depending of their chemical characteristics, several plants responses at the photosynthesis level can be obtained, especially with epoxiconazole which is known to reduce oxygen evolution from thylakoids, decreasing the electron transport rate in the thylakoids and limiting the photochemical phase (Benton and Cobb, 1997; Petit et al., 2012). Unexpectedly, the results of the present study showed that the infected plants sprayed with Epo + Pyr were favored in terms of stomatal opening based on the greater g_s values observed, resulting in increases in both *A* and *E*.

There were progressive decreases in *A* and g_s as the ASR developed being the magnitude of the reduction lower in plants sprayed with ASM in comparison to Epo + Pyr. According to Alves et al. (2011), reduction in *A* in the leaves of *Eucalyptus urophylla* in response to *Puccinia psidii* infection was related to damage in the green leaf area. However, despite the reduced stomatal aperture, which should reduce the CO_2 influx, the C_i values increased for the non-infected leaves. Reductions in both *A* and g_s and increases in C_i have been reported to occur for *Eucalyptus urophylla-Puccinia psidii* (Alves et al., 2011), bean-*Uromyces appendiculatus* (Bassanezi et al., 2002) and barley-*Rhynchosporium secalis* interactions (Martin et al., 1968). According to these

authors, the reduction in *A* was unlikely to have been solely associated with less CO_2 entry into leaves, but rather with some biochemical limitation in CO_2 fixation at the chloroplast level. Frequently, alterations in leaf photochemistry and carbon metabolism are related to lower activity of ribulose bisphosphate carboxylase and changes in the capacity for ribulose bisphosphate regeneration (Shaner and Finney, 1977; Hiscox and Israelstam, 1979). Reductions in *E* for the infected soybean plants from the control treatment can be linked to reductions in g_s and, therefore, can be associated with stomatal closure. Resende et al. (2012) reported that *E* dramatically decreased in sorghum leaves infected by *Colletotrichum sublineolum* as a mechanism to keep the stomata closed and to maintain a favorable water status within the infected leaves. Concomitant reductions in both *E* and g_s have been also reported to occur for other host-pathogen interactions (McGrath and Pennypacker, 1990; Duniway and Durbin, 1971; Bassanezi et al., 2002; Alves et al., 2011).

In comparison to infected plants sprayed with either ASM or Epo + Pyr, a sharp decrease in the values of F_0 and F_m were obtained for the infected plants from the control treatment indicating functional alterations in the photosystems followed by drastic reductions in the concentration of photosynthetic pigments that ultimately affected the efficiency of excitation energy from the light collecting. Maximum efficiency at which light is absorbed by the PSII to reduce Q_A is represented by the F_v/F_m parameter, which is a sensitive indicator of the photosynthetic performance of plants when the values are close to 0.8 for most plant species (Krause and Weis, 1991). The F_v/F_m values for leaves of infected plants from the control treatment were less than 0.8 indicating damage to the reaction centers associated with the photosystems. In the case of the infected plants sprayed with Epo + Pyr, the F_v/F_m values remained closer to the values obtained for the non-infected plants suggesting, that the action of Epo + Pyr

on fungal infection decreased the effect on the function of the photosynthetic apparatus. The severity of coffee leaf rust was reduced on coffee plants sprayed with Epo + Pyr, and this reduction was accompanied by a better photosynthetic capacity because of a lower photochemical damage as demonstrated by higher F_0, F_m and F_v/F_m values (Honorato et al., 2015). For infected plants sprayed with ASM, the F_0, F_m and F_v/F_m values showed minor reductions in comparison to the infected plants from the control treatment indicating a delay in the manifestation of operational damage in the photosystems. The Y(II) values were higher for plants sprayed either with AMS or Epo + Pyr demonstrating that energy dissipation was channeled into the photochemical process regardless of the infection by *P. pachyrhizi*. The increase in the photooxidative damage to leaf tissues is often followed by progressive increases on Y(NO) values suggesting, therefore, that the protection regulatory mechanisms become physiologically inefficient (Klughammer and Schreiber, 2008). The Y(NO) values for plants sprayed either with AMS or Epo + Pyr were lower in comparison to plants from the control treatment. Meanwhile, values for the Y(NPQ) parameter for plants sprayed either with AMS or Epo + Pyr remained higher at advanced stages of fungal infection in comparison to plants from the control treatment. This finding suggests the occurrence of dissipation of excess excitation energy as heat as a physiological mechanism of photoprotection (Krause and Weis, 1991). The greater ASR symptoms on the leaves of plants from the control treatment in comparison to plants from the other treatments caused a continuous losts in the use of light energy through carbon fixation reactions. Considering that these responses were also accompanied by concomitant reductions in the values of F_v/F_m, Y(II) and Y(NPQ) on the leaves of inoculated plants from the control treatment in comparison to plants from the other treatments, it is likely that the capacity for photoprotection was also lost, which ultimately resulted in an increase of

the photooxidative damage in leaf tissues, which could also be depicted from the progressive increases in the Y(NO) values as reported by Klughammer and Schreiber (2008). Therefore, both the excitation energy directed to photochemical conversion and the regulatory mechanisms of protection become ineffective (Klughammer and Schreiber, 2008). Finally, when the Y(II) values approached zero in a high PDPP, high values of Y(NPQ) were obtained for plants sprayed either with AMS or Epo + Pyr indicating, a high photoprotective capacity in association with a high concentration of carotenoids. In contrast, the high Y(NO) values obtained for the inoculated plants reflected their inability to protect themselves against the damage caused by an excess of illumination, which is closely linked to the production of reactive oxygen species (Klughammer and Schreiber, 2008).

An adequate concentration of photosynthetic pigments in plant cells and the integrity of the plasm membrane are of detrimental importance for the maintenance of photosynthesis (Matsuda et al., 2004). The concentrations of Chl *a*+*b* and carotenoids were higher for plants sprayed with either Epo + Pyr or ASM, but especially for the former, confirming, the potential of the strobilurin and triazole fungicides to increase the concentrations of photosynthetic pigments in plants (Bartlett et al., 2002; Pinhero et al., 1994). It has been reported that the synthesis of chlorophylls, a phenomenon known as the green effect, which is associated with delayed leaf senescence and yield maximization, has been achieved with the spray of strobilurins therefore, disease control and yield increase can be obtained (Bartlett et al., 2002). Additionally, higher concentration of Chl *a*+*b* would lead to an increase in the ability and the efficiency of the leaves to capture the energy required for the photochemical reactions that occurs in the photosynthesis (Buchenauer and Rohner, 1981). Reduction in the concentration of pigments in the leaves of soybean plants infected with *P. pachyrhizi* as well as for other

host-pathogen interactions such as *Stenocarpella macrospora* on maize, *Phakopsora pachyrhizi* on soybean, *Hemileia vastatrix* on coffee, *Pyricularia oryzeae* on wheat, and *Monographella albescens* on rice has been reported (Bermúdez-Cardona et al., 2014; Kumudini et al., 2008; Honorato et al., 2014; Rios et al., 2013; Tatagiba et al., 2014).

In conclusion, the results of the present study demonstrate that the spray of either Epo + Pyr or ASM, but especially the former, contributed to reduce the negative effects caused by *P. pachyrhizi* infection and allowed a good photosynthetic performance of the infected plants.

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Fig. 1. Severity of soybean rust (A), area under disease progress curve (AUDPC) (B) and number of lesions per cm^2 of trifoliolate leaf (C) for soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). For AUDPC and NL, means of the treatments labeled with different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical line indicate standard deviations of the means. $n = 6$.

Fig. 2. Net carbon assimilation rate (A) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C) and transpiration rate (E) (D) determined in trifoliolate leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). Means of treatments at each evaluation time that are labeled with different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical lines indicate the standard deviations of the means. $n = 6$.

Fig. 3. Initial fluorescence (F_0) (B), maximal fluorescence (F_m) (A) and maximal photosystem II quantum efficiency (F_v/F_m) (C) on the photosynthetic photon flux density (PPFD) of chlorophyll *a* fluorescence in leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr).

Fig. 4. Effective photosystem II quantum yield (Y(II)) (A), quantum yield of non-regulated energy dissipation Y(NO) (B) and quantum yield of regulated energy dissipation Y(NPQ) (C) determined in leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr).

Fig. 5. Initial fluorescence (F_0) (A), maximal fluorescence (F_m) (B) and maximal photosystemII quantum efficiency (F_v/F_m) (C) determined in leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). Means of treatments at each evaluation time that are labeled with different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical lines indicate the standard deviations of the means. $n = 6$.

Fig. 6. Effective photosystem II quantum yield (Y(II)) (A), quantum yield of regulated energy dissipation (Y(NPQ)) (B) and quantum yield of non-regulated energydissipation (Y(NO) (C) determined in leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). Means from the three treatments at each evaluation time that labeled with by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical lines the standards deviations of the means. $n = 6$

Fig. 7. Effective photosystem II quantum yield (Y(II)) (A), quantum yield of regulated energy dissipation Y(NPQ) (B) and quantum yield of non-regulated energy dissipation Y(NO) (C) on the photosynthetic photon flux density (PPFD) (D) of chlorophyll *a* fluorescence in the leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). For AUDPC, means of the treatments labeled with different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical line indicate standard deviations of the means. $n = 6$.

Fig. 8. Total chlorophyll (Chl_{a+b}) (A), and carotenoids (B) in the leaves of soybean plants non-inoculated with *Phakopsora pachyrhizi* (control), inoculated with *P. pachyrhizi* and sprayed with Acibenzolar-S-Methyl (ASM) and inoculated with *P. pachyrhizi* and sprayed with Epoxiconazole (Epo) + Pyraclostrobin (Pyr). Means of treatments at each evaluation time that are labeled with different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent means and vertical lines indicate standard error of the mean. $n = 6$.

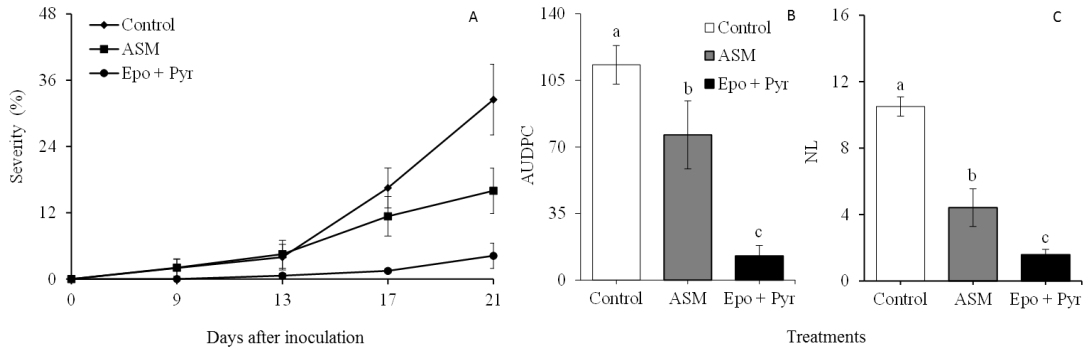
A
Control
ASM
Epo + Pyr
Severity (%)
48
36
24
12
0
0
9
13
17
21
Days after inoculation
B
Control
ASM
Epo + Pyr
AUDPC
140
105
70
35
0
a
b
c
Control
ASM
Epo + Pyr
Treatments
C
NL
16
12
8
4
0
a
b
c
Control
ASM
Epo + Pyr

Figure 1

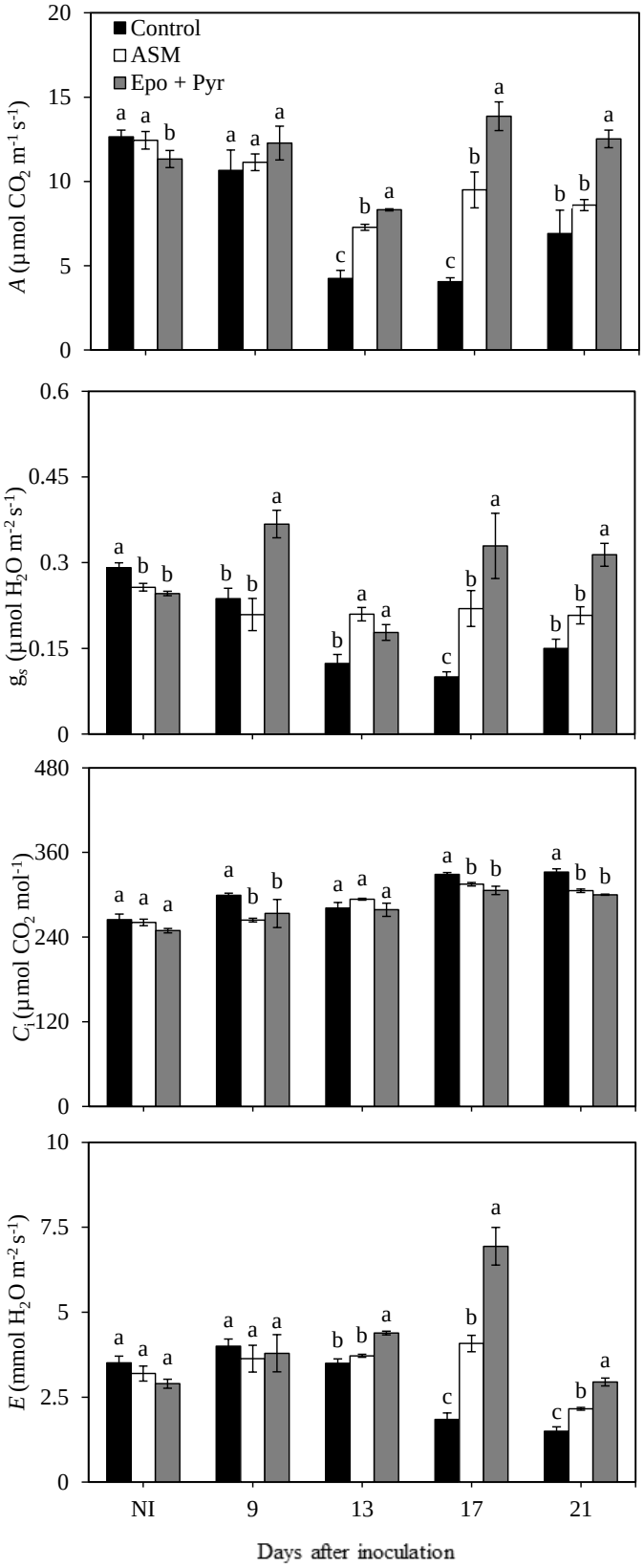
Control
ASM
Epo + Pyr
A (µmol CO2 m-1 s-1)
gs (µmol H2O m-2 s-1)
Ci (µmol CO2 mol-1)
E (mmol H2O m-2 s-1)
NI
9
13
17
21
Days after inoculation

Figure 2

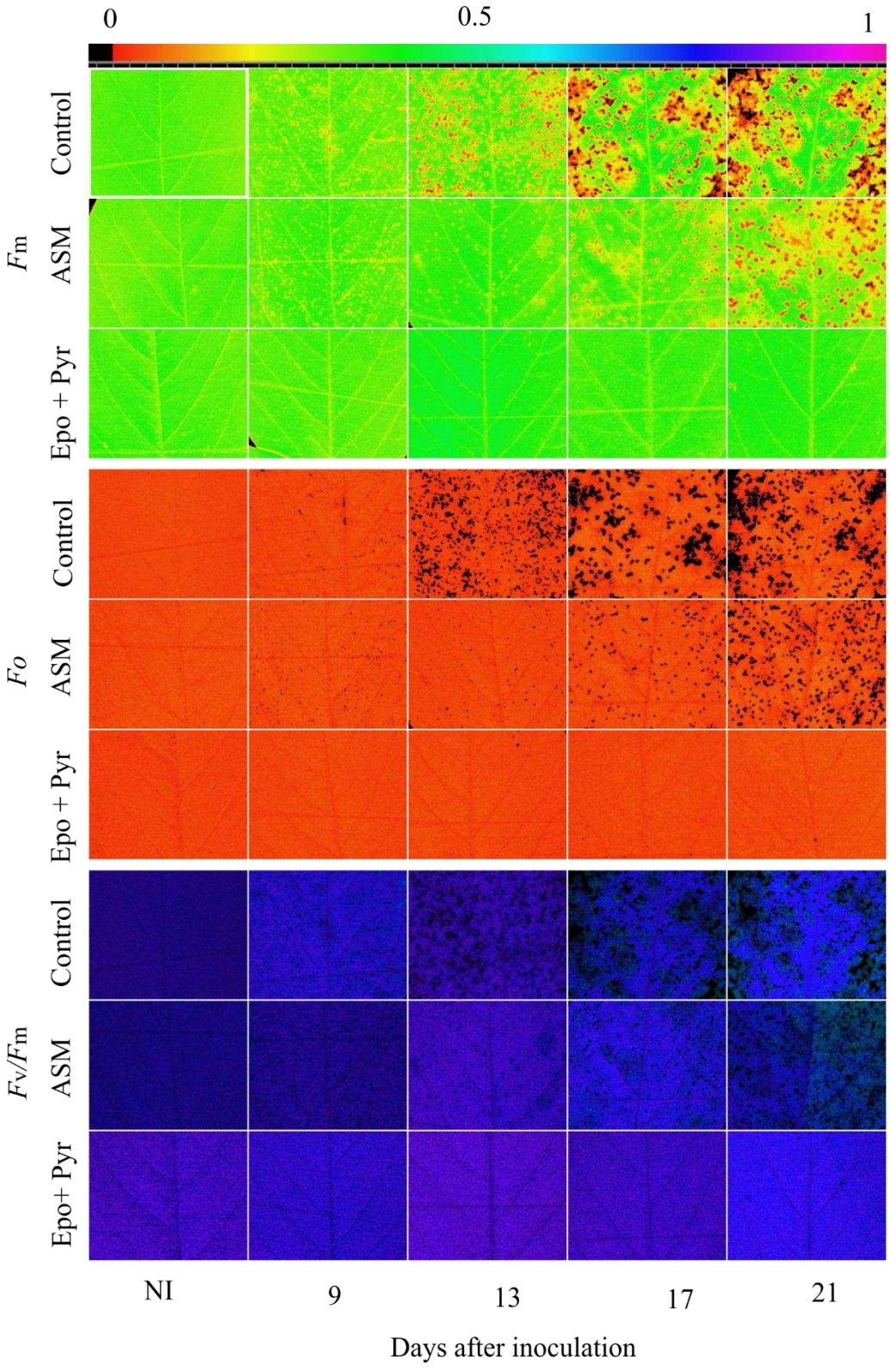
0
0.5
1
Fm
Fo
Fv/Fm
Control
ASM
Epo + Pyr
Epo+ Pyr
NI
9
13
17
21
Days after inoculation

Figure 3

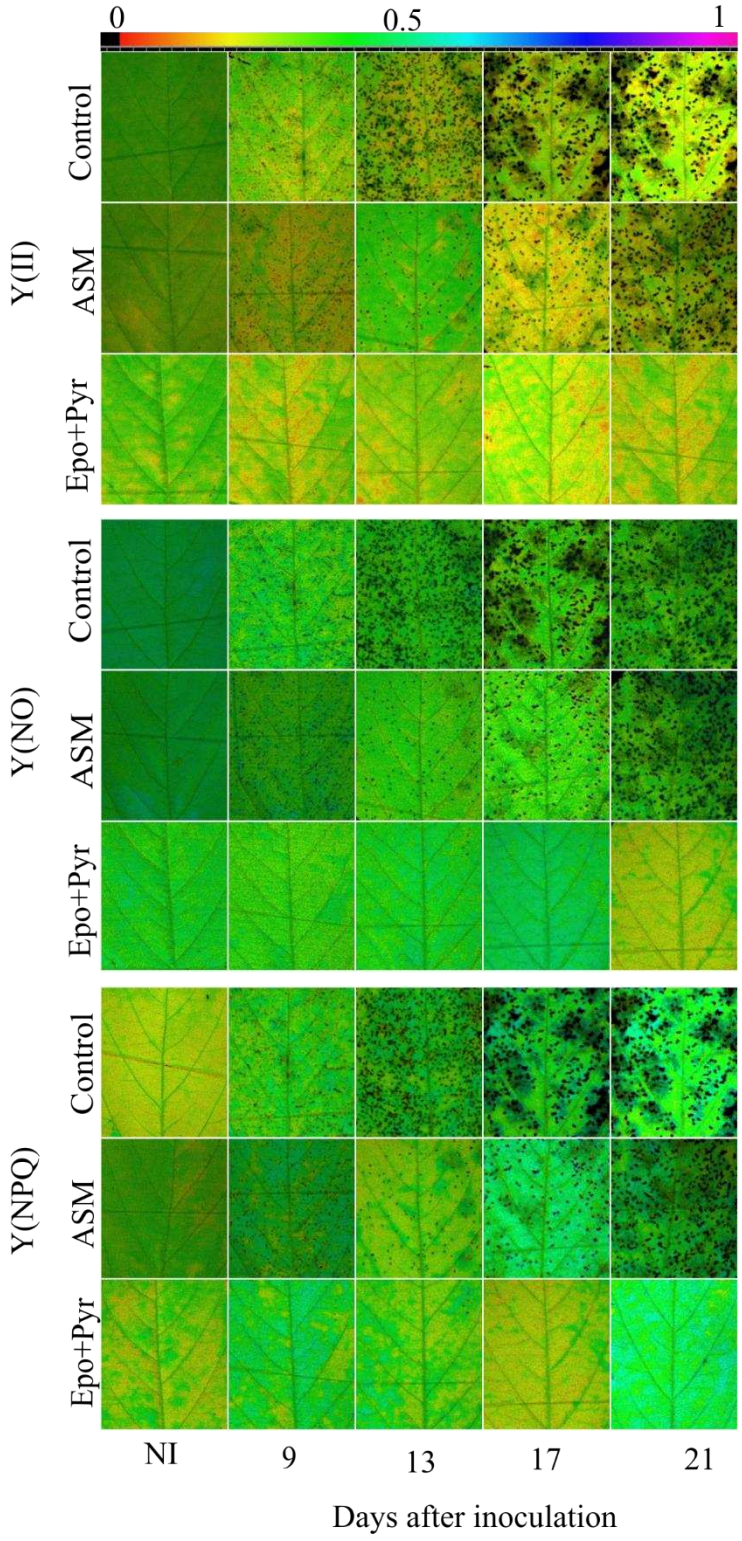
0
0.5
1
Y(II)
Y(NO)
Y(NPQ)
Control
ASM
Epo+Pyr
NI
9
13
17
21
Days after inoculation

Figure 4

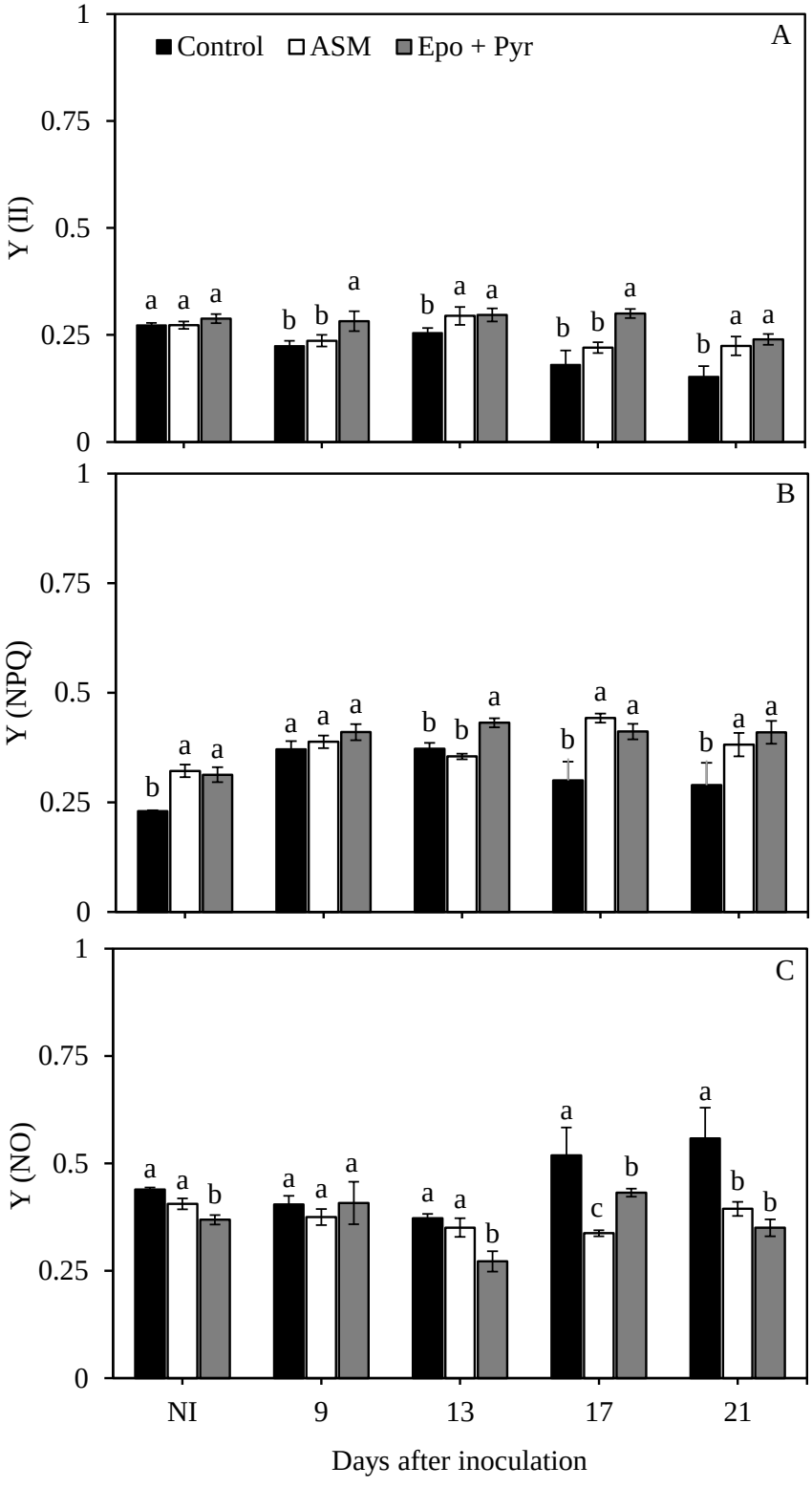
Control
ASM
Epo + Pyr
A
B
C
Y (II)
Y (NPQ)
Y (NO)
1
0.75
0.5
0.25
0
NI
9
13
17
21
Days after inoculation

Figure 6

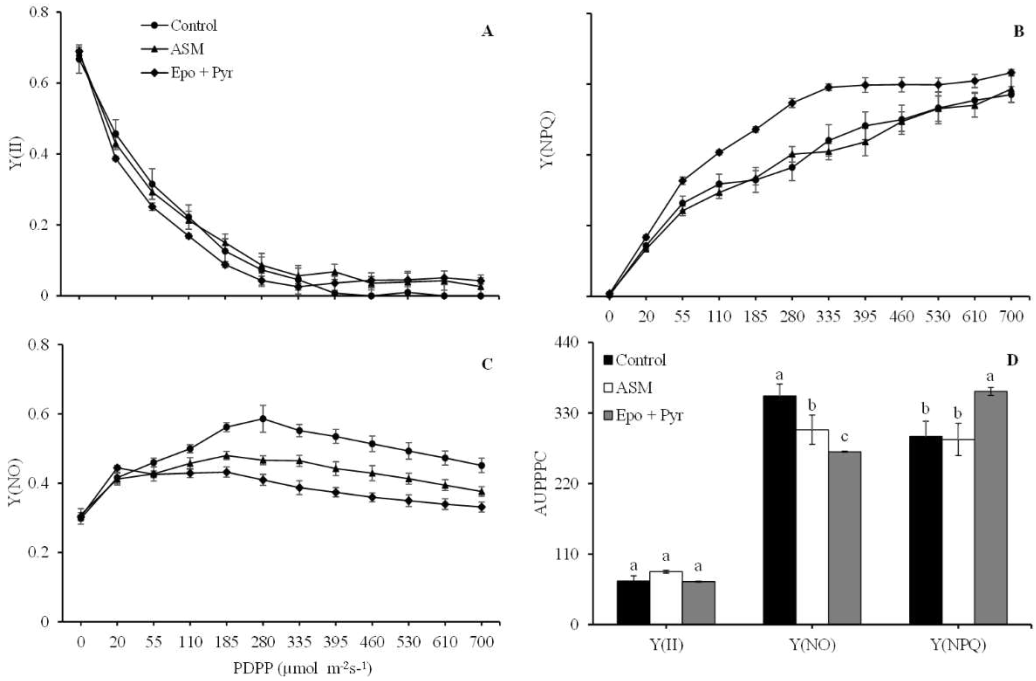
A
B
C
D
Control
ASM
Epo + Pyr
Y(II)
Y(NPQ)
Y(NO)
AUPPPC
PDPP (µmol m-2s-1)
0 20 55 110 185 280 335 395 460 530 610 700
0 0.2 0.4 0.6 0.8
0 110 220 330 440
a a a b c b b a
Y(II) Y(NO) Y(NPQ)

Figure 7

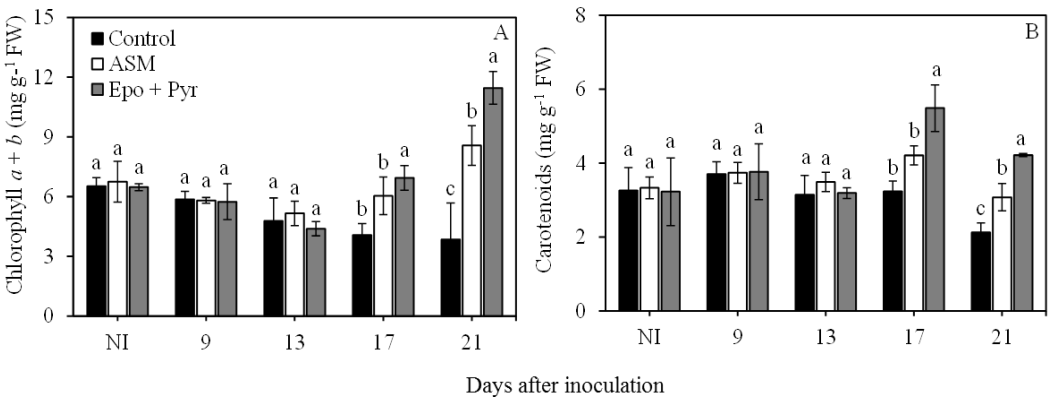
Control
ASM
Epo + Pyr
A
B
Chlorophyll a + b (mg g-1 FW)
Carotenoids (mg g-1 FW)
NI
9
13
17
21
Days after inoculation

Figure 8