

ROBERTO BARUCH PONCE ORTIZ

**MULTI-SITE FUNGICIDES ASSOCIATED WITH DMIs AND QoIs:
A NEW STRATEGY TO CONTROL ASIAN SOYBEAN RUST**

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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Alessandro Antônio Fortunato

Jorge Luis Badel Pacheco

Laércio Zambolim
(Orientador)

*A meu filho Roberto Eliud quem é meu pedacinho
de céu na terra, para você com amor...*

DEDICO

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me dado, meus amados pais Roberto Ponce e
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na vida, para vocês meu
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BIOGRAFIA

ROBERTO BARUCH PONCE ORTIZ, Filho de Guadalupe Ortiz Ávila e Roberto Ponce Loaéza, nasceu em Coyoacán, D.F. – México o dia 03 de fevereiro de 1982. Ingresso na Universidade Autônoma de Chapingo, (U.A.Ch.) em Chapingo, Estado de México, em agosto 2001, concluindo o curso de Engenheiro Agrônomo com Especialidade em Parasitologia Agrícola em junho 2006, no ano 2005 e 2006 fiz estagio na região sudeste do México, assessorando produtores de melancia e milho amarelo, durante os anos 2006 até 2009 trabalho como pesquisador de comercialização na Unidade Regional de ASERCA Noreste, no estado de Tamaulipas administrando a compra e venda de commodities agrícolas, nos anos 2009 até 2014 trabalho como Subdiretor de área na Direção de Mercados Financeiros de ASERCA nas oficinas centrais da SAGARPA, México, Distrito Federal, dando atenção as diversas Direções Regionais de ASERCA. Em fevereiro de 2014, iniciou o curso de Mestrado do Programa de Pós-Graduação em Fitopatologia na Universidade Federal de Viçosa, Viçosa-MG, submetendo-se à defesa em 31 de julho de 2015.

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RESUMO

ORTIZ, Roberto Baruch Ponce, M. Sc., Universidade Federal de Viçosa, julho de 2015. **Fungicidas multisítios associados com DMIs e QoIs: uma nova estratégia de controle da ferrugem asiática da soja.** Orientador: Laércio Zambolim.

A ferrugem asiática da soja, causada pelo fungo *Phakopsora pachyrhizi*, desde que foi relatada pela primeira vez no Brasil, tem sido alvo de estudos quanto ao emprego de fungicidas para seu controle. Entretanto, devido ao uso indiscriminado destes produtos, em especial provenientes do grupo DMI (“Demethylation inhibitor”) e QoI (“Quinone outside inhibitor”), também conhecidos respectivamente como triazóis e estrubirulinas, relatos de resistência estão cada vez mais comuns atualmente. Com intuito de estudar novas estratégias para o controle químico da ferrugem, experimentos foram conduzidos a nível de laboratório, casa-de-vegetação e campo para avaliar o efeito da associação de fungicidas pertencentes ao grupos dos DMIs e QoIs com fungicidas de múlti-sítios. Os experimentos realizados a nível de laboratório e casa-de-vegetação estudaram o efeito sistêmico e translaminar de diferentes fungicidas comerciais à base de triazóis (Epoxiconazol, Ciproconazol, Protioconazol, Flutriafol e Tebuconazol) e estrubirulinas (Piraclostrobina e Trifloxistrobina), sendo que para o experimento em casa-vegetação foi adicionado diferentes fungicidas multi-sítios (Mancozebe e Chlorothalonil), Com base nos resultados obtidos em laboratório e em casa-de-vegetação, foram realizados experimentos a nível de campo para avaliar o efeito de fungicidas à base dos triazóis; Epoxiconazol e Ciproconazol, e das estrubirulinas Piraclostrobina e Azoxistrobina, em associação com Mancozebe, Metiram e Chlorothalonil. A ferrugem asiática foi avaliada por médio da severidade com auxílio de escala diagramática e os dados obtidos foram integrados para calcular a área abaixo da curva de progresso da doença (AACPD). Além da avaliação de doença, foi determinado rendimento de grãos (kg/ha) de cada parcela. O bioensaio realizado no laboratório e estufa mostraram que os tratamentos fungicidas utilizados obtiveram uma eficiência mínima de controle da ferrugem asiática da soja de 92 %, comparado com a testemunha. Ensaio de campo, demonstraram que todas as combinações fungicidas empregadas foram eficazes para controlar a ferrugem asiática e consequentemente favorecer um maior ganho de produtividade em comparação com a testemunha. Ao analisar a eficácia de controle da ferrugem asiática nos terços inferior, médio e superior, mostrou que a aplicação de Epoxiconazole com piraclostrobina e Ciproconazol com azoxistrobina reduz ASR (inferior, médio e superior) a queda de folha (%), e aumento no rendimento (kg/ha). Observou-se que a incidência do ASR diferiu entre as partes de dossel. A gravidade aumentou de inferior para a parte superior

Ficou evidenciado nesse trabalho, que a aplicação das misturas entre DMI, QoI e multi-sítios promoveu de forma eficaz a redução da doença, propiciou ganho de produtividade e evitou a desfolha das plantas. O emprego de misturas que combinam fungicidas com alto risco de resistência (DMI e QoI) com fungicidas de baixo risco (multi-sítios) podem ser uma nova estratégia para o manejo da ferrugem a curto e a longo prazo. Além disso, em função do efeito residual e protetor de fungicidas multi-sítios promover maior longevidade de moléculas e redução do número de aplicação de fungicidas sistêmicos do grupo dos DMI e QoI.

ABSTRACT

ORTIZ, Roberto Baruch Ponce, M.Sc., Universidade Federal de Viçosa, july 2015. **Multi-site fungicides associated with DMIs and QoIs: a new strategy to control Asian soybean rust.** Advisor: Laércio Zambolim.

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, since it was first reported in Brazil, has been the subject of studies regarding the use of fungicides to its control. However, due to the indiscriminate use of this products, especially from the DMIs ("demethylation inhibitors") and QoIs ("Quinone outside inhibitor") groups, also known respectively as triazoles and strobilurins, resistance reports are increasingly common nowadays. In order to study new strategies for chemical control of ASR, experiments were conducted in laboratory, greenhouse and field to evaluate the effect of fungicides belonging to the DMIs and QoIs groups associated with multi-site fungicides. Experiments were carried out at laboratory and greenhouse in order to study the systemic and translaminar effect of triazoles (Epoconazole, Cyproconazole, Prothioconazole, Flutriafol and Tebuconazole) and Strobilurins (Pyraclostrobin and Trifloxystrobin), and for the experiment in greenhouse were mixed these lasts with multi-site fungicides like (Mancozeb and Chlorothalonil). Based on the results obtained in the laboratory and greenhouse, field trial experiments were conducted to evaluate the effect of fungicides compounded with triazole like Epoconazole and Cyproconazole, and strobilurins like Pyraclostrobin and Azoxystrobin with Mancozeb, Metiram and Chlorothalonil. ASR was assessed by the severity using a diagrammatic scale and the data were integrated to calculate the area under the disease progress curve (AUDPC). Besides the evaluation of disease, it was determined grain yield (kg/ha) for each plot. The bioassay conducted in the laboratory and greenhouse showed that the fungicide treatments obtained a minimum efficacy of 92 % against ASR, compared to the control. The field trials showed that all combinations of fungicides used were effective to control ASR and promoted greater yield gain compared to the control. Analyzing the effectiveness to control ASR in the lower, medium and upper part of the canopy, showed that application of Epoconazole with Pyraclostrobin, and Cyproconazole with Azoxystrobin reduced ASR (lower, medium and upper) and leaf fall (%), and increased yield gain (kg/ha). It was observed that the incidence of the ASR differed between the canopy parts. The severity increased from lower to the upper part. It was evidenced in this work, that the application of DMI, QoI and multi-site fungicides in association promoted effectively the disease reduction, increased yield and avoided the

leaf fall. The use of commercial formulation fungicides with high risk of resistance (DMI and QoI) combined to low risk ones (multi-site) could be a new strategy for the management of the ASR at short and long term. Furthermore, due to the residual effect and protective of multi-site fungicides like Mancozeb, Chlorothalonil and Metiram, can promote higher longevity of DMI and QoI molecules and reduce the number of application of systemic fungicides.

GENERAL INTRODUCTION

Brazil is the second soybean (*Glycine max* (L.) Merrill) producer in the world and largest exporter (FAO, 2015). In the 2013/14 growing season, the country produced about 89 million tons, accounting for 30 % of the global production (CONAB, 2015). Global soybean production for 2014/15 is projected at 304.8 million tons, up 4.8 million than last season (USDA, 2015). However, among the diseases affecting soybean production, Asian soybean rust (ASR) caused by the fungus *Phakopsora pachyrhizi* (Syd. and P. Syd.) is the most important. *P. pachyrhizi* was first described in Japan in 1902. Due to the dissemination by the air currents and wind-blown rain the pathogen was spread to several countries around the world (Bromfield, 1984; Caldwell and Laing, 2002; Giordani, 2001; Hartman et al., 2007; Navarini et al., 2007; Ogle, 1979; Reis and Bresolin, 2004; Yorinori et al., 2004). In addition to its high capacity to spread, *P. pachyrhizi* has been documented on many legume hosts (Bromfield, 1984; Miles et al., 2003; Ono et al., 1992; Rytter et al., 1984; Vakili, 1979), as well as on over-wintering kudzu (*Pueraria lobata*) which may be found in the Southeastern United States, Caribbean Basin, Central America, and South America (Navarini et al., 2007). In 2015, the pathogen was found in ‘jacatupe’ (*Pachyrhizus erosus*) nearby a soybean field in Viçosa (state of Minas Gerais, Brazil). Cross inoculation of the uredospores produced in ‘jacatupe’ and soybean it was possible to prove that *P. erosus* is also a secondary host of *P. pachyrhizi* in Brazil (Zambolim, 2015; unpublished data).

After infection, the fungus produces the uredinia and the urediniospores between seven to 14 days according to the environmental conditions (Dorrance et al., 2005; Koch et al., 1983). The symptoms are grouped into tanned coloring lesions (2 to 5 mm in diameter) with two to five uredinia and abundant sporulation or forming brown-red lesions with zero to two uredinia and sparse sporulation (Bromfield, 1984). The pathogen has short life cycle, which means, when under the right environmental conditions (frequent rain events, long dew periods and temperatures ranging from 15 to 29°C), soybean rust epidemics can quickly build up from almost undetectable levels to very high levels, and the urediniospore production can last for at least three weeks (Dorrance et al., 2005; Koch et al., 1988; Marchetti et al., 1975; Melching et al., 1989; Rupe and Sconyers, 2008). *P. pachyrhizi* in favorable conditions was reported to causes damages varying from 10 to 90 % in soybean fields in Asian (Sinclair and Hartman, 1999) and from 10 to 80 % in some growing areas in South America (Navarini et al., 2007).

Rapid disease development has been correlated with canopy closure and bloom stage (R1+) (Dorrance et al., 2005; Fehr et al., 1971; Koch et al., 1983; McLean, 1979). For this reason ASR shows a high defoliation rate of the crop and keep going until the environment is no longer conducive for the development of the disease (Rupe and Sconyers, 2008). According Bromfield (1984) infection at flowering produce high levels of damage, affecting the protein content (Ogle, 1979). Moreover, severely infected plants show early defoliation, compromising pod formation and filling and final grain weight (Yang et al., 1991).

The first step to the ASR control is based on the destruction of secondary hosts, an early sowing, taking into account on the avoidance period, and soybean cultivars resistance (Rupe and Sconyers, 2008). However, due to the absence of cultivars with an adequate level of basal resistance to soybean rust and the low efficiency of prophylactic strategies, the chemical control is far ahead the most effective alternative to control this disease.

Since soybean rust first report in Brazil, the chemical control of this disease was based on fungicides belonging to triazoles or “demethylation inhibitors” (DMI) group (Yorinori, 2004). These fungicides inhibit the sterol C-14 α -demethylation of 24-methylenedihydrolanosterol, a precursor of the cell membrane component ergosterol in fungi (Brent, 1995). The DMI group is classified as single-site fungicide and is associated to be a high risk factor in the development of resistance in fungi population (FRAC, 2015). Consequently, the massive use for many years of this chemical group in soybean fields had forced to the selection of resistant strains of *P. pachyrhizi* in the fungus population leading to the loss in control efficacy of DMIs. In 2005/06 soybean season, ASR average control by DMIs was 90.3 %, whereas in 2014/15, DMI control was 24 % (67 % reduction in the control efficacy) (Godoy *et al.*, 2013). Due to the fact that DMIs alone were not efficient to control this disease no longer in the field, triazole fungicide manufacturers started to produce mixtures between DMIs and “quinone outside inhibitors” (QoIs) aiming to increase the efficacy of control (Andrade and Andrade, 2006). The QoI fungicides are very effective against a broad spectrum of fungi and work by inhibiting mitochondrial respiration, effectively stopping energy production of the fungus, and result in death (FRAC, 2015). QoI fungicides also have a very specific site of action, then the risk of resistance development is high and currently there are more than 20 plant pathogens with some level of resistance to QoI fungicides as well as *P. pachyrhizi* (FRAC, 2015).

In the last years, new fungicides belonging to Carboxamide group, a single-site molecule that acts inhibiting the complex II fungal respiration, has been developed to control ASR (FRAC, 2015). However cases of resistance to other pathogens, had already been documented for these fungicides (FRAC, 2015). Fungicides with single-site mode of action are at relatively high risk for resistance development compared to those with multi-site mode of action. It is well known that multi-site fungicides have multiple modes of action, so they affect multiple target sites.

The difficulty to control soybean rust with fungicides as reported above is increasing, that shows that the fungus have a high adaptability in soybean fields (Schmitz et al., 2014). Due to the high risk of resistance to fungicides associated to the DMIs and QoIs, researches are trying to find new molecules to be incorporated in a better integrated and effective management of the ASR control. For this reason, the objective of this study was to evaluate a new strategy of chemical control using a multi-site and broad spectrum fungicide in different combinations with DMI and QoI fungicides to reduce significantly the ASR.

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Multi-site fungicides associated with DMIs and QoIs: a new strategy to control Asian soybean rust

Roberto B.O. Ponce, Laércio Zambolim, Alessandro A. Fortunato and Luan F. de Queiroz

Universidade Federal de Viçosa, Departamento de Fitopatologia, Laboratório Proteção de Plantas, Viçosa, Minas Gerais, 36570-900, Brasil.

ABSTRACT

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* and the use of fungicides for its control have been the subject of several studies since ASR was first reported in Brazil. However, reports of fungicide resistance have increased considerably due to the indiscriminate use of specific site fungicides for ASR control, especially DMIs (demethylation inhibitors) and QoIs (quinone outside inhibitors). To study new strategies for the chemical control of ASR, experiments were conducted at the laboratory, greenhouse and field levels to evaluate the effect of DMIs and QoIs combined with multi-site fungicides. In the laboratory, experiments were carried out to study the systemic and translaminar effects of triazoles (Epoconazole, Cyproconazole, Prothioconazole, Flutriafol and Tebuconazole) and strobilurins (Pyraclostrobin and Azoxystrobin) on the control of ASR. In the greenhouse, the mixture of systemic + strobilurins and multi-site fungicides Mancozeb, Chlorothalonil and Metiram + Pyraclostrobin were tested for the control of ASR. Based on the results obtained in the laboratory and greenhouse, field experiments were conducted to evaluate the multi-site fungicides (Mancozeb, Chlorothalonil and Metiram) in association with triazoles (Epoconazole and Cyproconazole) and strobilurins (Pyraclostrobin and Azoxystrobin). ASR infection was assessed by a diagrammatic scale of severity on a scale of R1 to R7. Data were integrated to calculate the area under the disease progress curve (AUDPC) and the infection rate of the disease. The grain yield data (kg/ha) of each plot and defoliation percentage were taken at the R 8.2 stage. The translaminar bioassay in the laboratory showed a minimum efficacy of 92 %, the protectant effect in the greenhouse varied from 69 to 91 %, and the multi-site fungicides varied from 82 to 86 % when compared to the control treatment. The field trials showed that all of the fungicide combinations used were effective in controlling ASR and promoted a greater soybean yield gain relative to the control. Analyzing the efficacy of ASR control in the lower, medium and upper part of the canopy showed that the application of

Expoconazole with Pyraclostrobin, and Cyproconazole with Azoxystrobin reduced ASR and leaf fall (%) and increased the soybean grain yield (kg/ha). The severity of ASR increased from the lower to the upper part of the plant. The application of DMI, QoI and multi-site fungicides promoted the effective reduction of ASR, increased yield and reduced the leaf fall. Overall, the DMIs with QoIs combined with multi-site fungicides were 68 % and 70.2 % more efficient for ASR control and soybean grain yield than the control at a disease severity level of 2.0 % at the time of fungicide application. The protectant multi-site fungicides Mancozeb (2400 g a.i./ha) and Chlorothalonil (1500 g a.i./ha) in concert with the lowest dose of Epoconazole + Pyraclostrobin (16.5 + 10.1 g a.i./ha, respectively) or Cyproconazole + Azoxystrobin (9.0 + 3.6 g a.i./ha) increased soybean grain yield at least 45.0 %, and the same combination of fungicides at the dose just above the lowest increased 89.5 % and 109.0%. The use of a commercial formulation of high risk (DMI and QoI) fungicides combined with low risk (multi-site) fungicides could be a new strategy for the short and long term management of ASR. Furthermore, due to residual effects and multi-site action, fungicides such as Mancozeb, Chlorothalonil and Metiram can promote the higher longevity of DMI and QoI molecules and decrease the frequency of applications of these systemic fungicides.

Key words: *Phakopsora pachyrhizi*, triazole, strobilurin, protectant fungicide.

INTRODUCTION

Brazil is the second largest soybean (*Glycine max* (L.) Merrill) producer in the world and the largest exporter (FAO, 2015). However, the real potential soybean yield is limited due to the losses caused by insects and diseases (Godoy et al., 2013). Among the diseases affecting the soybean, Asian soybean rust (ASR) caused by the fungus *Phakopsora pachyrhizi* (Syd. and P. Syd.) can currently be considered the main threat to the crop production in Brazil (Godoy et al., 2013). The fungus is considered highly aggressive, causing damages varying from 10 to 90 % in soybean fields in Asia (Sinclair and Hartman, 1999) and yield losses ranging from 10 to 80 % in some growing regions in South America (Navarini et al., 2007). The first symptoms of ASR begin as very small brown or brick-red spots on leaves, grouped into tan colored lesions (2-5 mm in diameter) that develop into a pustule (uredinia), where abundant sporulation occurs to help the fungus disseminate (Bromfield, 1984). In the field, these spots usually begin in the lower canopy at or after flowering, and often the first lesions appear toward the base of the leaflet near the petiole and leaf veins (Rupe and Sconyers, 2008; Sinclair and Hartman, 1999).

The fungus possesses a short life cycle which means, under optimal conditions (frequent rain events, long dew periods and temperatures ranging from 15 to 29°C), ASR epidemics can quickly build up from almost undetectable levels to very high prevalence, and the urediniospore production can last at least three weeks (Dorrance et al., 2005; Marchetti et al., 1975; Melching et al., 1989). Rapid disease development has been correlated with canopy closure and bloom stage (R1+) (Dorrance et al., 2005; Fehr et al., 1971; Koch et al., 1983; McLean, 1979). Then, ASR progresses until there is complete canopy defoliation or until the environment is no longer conducive to disease development (Rupe and Sconyers, 2008; Sinclair and Hartman, 1999). According to Bromfield (1984), infection at flowering can produce high levels of damage, compromising pod formation and filling, final grain weight (Yang et al., 1991), and also affecting the protein content (Ogle, 1979).

Since ASR was first reported in Brazil, the chemical control of this disease has been based on fungicides belonging to triazoles or the demethylation inhibitors (DMI) group (Yorinori and Lazzarotto, 2004). These fungicides inhibit the sterol C-14 α -demethylation of 24-methylenedihydrolanosterol, a precursor of the cell membrane component ergosterol in fungi (Brent, 1995). The DMI group is classified as a single-site fungicide and is identified as a high risk factor in the development of resistance in

fungi populations (FRAC, 2015). Consequently, due to the widespread use of this chemical group in soybean fields for many years, the selection of resistant strains of *P. pachyrhizi* from the fungus population has occurred, and consequently, the loss in efficacy of DMIs to be used to control ASR. In the 2005/06 soybean season, the average degree of ASR control by DMIs was 90.3 %, whereas in 2012/13, DMI control was 52.0 % (a 42.0 % reduction in the efficacy of control) (Godoy et al., 2013). For this reason, triazole fungicide manufacturers have started to produce mixtures between DMIs and quinone outside inhibitors (QoIs) aiming to increase the efficacy of control treatments (Andrade and Andrade, 2006). The QoI fungicides are very effective against a broad spectrum of fungi and inhibit mitochondrial respiration, effectively stopping energy production of the fungus, resulting in cell death. QoI fungicides also have a very specific site of action, thus the risk of developing resistance is high. Currently, there are more than 20 plant pathogens, including *P. pachyrhizi*, with some level of resistance to QoI fungicides (FRAC, 2015).

In recent years, new fungicides have been developed to control ASR that belong to the Carboxamide group, a single-site molecule that inhibits complex II, of the respiration process inside the fungal cell. However, resistance to other pathogens had already been documented for this new group (FRAC, 2015). Fungicides with a single-site mode of action have a relatively high risk of promoting resistance compared to those with multi-site mode of action. Most fungicides being developed today have a single-site mode of action because this is associated with the decreased potential of a negative impact on the environment, including non-target organisms (McGrath, 2004). It is well known that multi-site fungicides affect multiple target sites, interfering with numerous metabolic processes of the fungus, and consequently fungicide resistance is rare with this type of fungicide (Zambolim, 2005).

The high fungal adaptability in soybean fields makes it difficult to control soybean rust with fungicides, and as reported above, decreased effectiveness is becoming increasingly evident (Schmitz et al., 2013). Due to the high risk of fungicide resistance combined with the use of DMIs and QoIs applied alone or in combination, researchers are working to find new molecules to be incorporated into the control of ASR. For this reason, the objective of this study was to evaluate the new strategy of chemical control using multi-site, broad spectrum fungicides in different combinations with DMI and QoI fungicides to control ASR.

MATERIAL AND METHODS

Bioassay on detached leaves

A bioassay was performed using detached leaves to evaluate the translaminar effect of DMI and QoI fungicides, alone or in a mixture, on the control of ASR on the leaves of the cv. “Monarca”. Three expanded soybean leaves were placed in a gerbox (20 x 20 x 3 cm) on top of a stainless steel mesh with a humid nylon screen below. Then, the leaves were inoculated on the abaxial surface with a suspension of *P. pachyrhizi* urediniospores (10^8 urediniospores mL⁻¹) using a number 2 camel hair brush. Following the inoculation, the leaves were sprayed with the fungicides on the adaxial surface, using a hand knapsack mistblower. The experiment was setup in a randomized complete block design with nine treatments and four replications as follows: 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 33.2 + 10.2 g active ingredient (a.i.)/ha - 0.5 L/ha); 3. Cyproconazole + Trifloxystrobin (Sphere Max SC; 16.8 + 7.2 g a.i./ha - 0.3 L/ha); 4. Tebuconazole + Trifloxystrobin (Nativo SC; 100.0 + 50.0 g a.i./ha - 0.5 L/ha) + Aureo (0.5 L/ha); 5. Cyproconazole + Azoxystrobin (Priori Xtra; 18.0 + 7.2 g a.i./ha - 0.3 L/ha) + Nimbus (0.5% v/v); 6. Pyraclostrobin (Comet SC; 75.0 g a.i. /ha - 0.3 L/ha); 7. Flutriafol (Impact SC; 31.2 g a.i./ha - 0.5 L/ha); 8. Prothioconazole + Trifloxystrobin (Fox SC; 28.0 + 16.0 g a.i./ha - 0.4 L/ha); 9. Tebuconazole (Folicur SC; 50.0 g a.i./ha - 0.5 L/ha). Then, the detached leaves were incubated at 22°C, with a 12 h photoperiod under fluorescent light (7.35 Wm⁻²) for 20 days.

Greenhouse experiment

Soybean seeds from cv. “Monarca” were sown into 2 L plastic pots (Ecovaso, Jaguariúna, SP, Brazil) containing 2 kg of a mixture of soil, cow manure and sand (2:5:1 ratio). After the emergence of the cotyledons, plants were fertilized weekly with 15 mL of a nutrient solution, prepared using deionized water and containing 40 mM KNO₃, 10 mM NH₄H₂PO₄, 10 mM MgSO₄.7H₂O, 15 mM Ca(NO₃)₂.4H₂O, 2.4 mM ZnSO₄.7H₂O, 3 mM H₃BO₃, 10 mM K₂SO₄, 3.3 mM CH₄N₂O and 7.5 mM NH₄H₂SO₄ (Dallagnoll *et al.*, 2014). Plants were grown for 50 days (V10 growth stage) (Fehr *et al.*, 1971) and then sprayed with the following treatments to evaluate residual effects: 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha); 3. Cyproconazole + Trifloxystrobin (Sphere Max SC; 56.0 + 24.0 g a.i./ha - 0.3 L/ha); 4. Prothioconazole + Trifloxystrobin (Fox SC; 70.0 + 40.0 g a.i./ha - 0.4 L/ha); 5.

Cyproconazole + Azoxystrobin (Priori Xtra; 60.0 + 24.0 g a.i./ha - 0.3 L/ha) + Nimbus (0.5% v/v); 6. Pyraclostrobin (Comet SC; 250 g a.i./ha, 0.3 L/ha); 7. Mancozeb (Dithane M45 PM; 800 g a.i./ha - 3.0 kg/ha); 8. Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 9 Metiram + Piraclostrobin (Cabrio Top S; 550 + 50 g a.i./ha - 2.0 L/ha), 10. Tebuconazol + Trifloxistrobina (Nativo SC; 200.0 + 100.0 g a.i./ha - 0.5 L/ha). These fungicides were sprayed with a manual backpack sprayer (Jacto PJH, JD-12P spray nozzle) calibrated to apply a spray volume of 150 L/ha, on the adaxial surface of the expanded leaves of each plant. Three hours later the plants were inoculated with a urediniospore suspension (10^6 urediniospores/mL) using a manual backpack sprayer calibrated to apply a spray volume of 10 mL/plant, on the abaxial surface of the expanded leaves of each plant. The plants were maintained in a moist chamber for 48 h in the dark at relative humidity > 95 % and temperature $22 \pm 1^\circ\text{C}$, and then transferred to a greenhouse until the final evaluation (thirty days after the inoculation). The greenhouse conditions during the experiment had a maximum natural photon flux density at plant canopy height $\approx 700 \mu\text{mol m}^{-2} \text{s}^{-1}$, $25 \pm 2^\circ\text{C}$ (day) and $20 \pm 2^\circ\text{C}$ (night) and relative humidity at $80 \pm 3 \%$.

Field trials

All the three field trial experiments were conducted at the research field station at the Universidade Federal de Viçosa, in Viçosa, Minas Gerais, Brazil ($20^\circ 45' 14'' \text{ S } 42^\circ 52' 55''$; 648 m high) during the 2014/2015 growing season. In all experiments, soybean seeds of the cv. "Monarca" were sown in the experimental area in a soil with no cultivation till system, in four rows of 5 m each, spaced 0.5 m apart (500,000 plants/ha) per plot. Only the two of the four central rows were used for data collection. Trial number 1 was performed in an area previously cultivated with two years of crucifers, trial 2 with two years of potato, and trial 3 with two years of maize. Cropping practices were performed according to the extension instructions for soybean crop. Fertilization was performed with the commercial formula 05-25-20 (Nitrogen-Phosphorus-Potassium) at 250 kg per ha. The nutrient soybean foliar analyses were performed at the R1 stage of the field trials.

The inoculum of *P. pachyrhizi* was collected from symptomatic leaves of soybean plants grown under greenhouse conditions (temperature $25 \pm 2^\circ\text{C}$ day and $20 \pm 2^\circ\text{C}$ night; relative humidity $80 \pm 3 \%$). The viability of the spores was assessed before the inoculation by brushing a 50 μL aliquot of a 1.0 mg/mL urediniospores on the surface of Petri dishes containing 2 % water-agar; the Petri dishes were incubated at 22°C in the

dark for 16 hours. The urediniospore germination was then stopped with lactofenol and was assessed under a stereoscopic microscope (Carl Zeiss Axio Imager A1, Gottingen, Germany).

One hundred mL of urediniospore suspension with a minimum of 90 % germination at the concentration of 10^8 urediniospores/mL was sprayed in the plant canopy at night on the two outer soybean rows of each replication (10 m²) using a backpack mistblower, 25 days before the application of the fungicides, on the two center rows. All of the field trials were sprayed when the incidence of ASR reached 100 % in the experimental area. The application of fungicides was performed at different plant growth stages (R1, R2, and R5.3), using a manual backpack sprayer (Jacto PJH, JD-12P spray nozzle) calibrated to apply a spray volume of 150 L/ha, on the adaxial surface of the expanded leaves of each plant of the two center soybean rows.

Trial 1 - The effect on ASR control by varied doses of DMI with QoI combined in concert with multi-site fungicides.

The treatments used in trial were: 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 7. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 8. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 9. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 2.0 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 10. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha).

Trial 2 - The curative effect on ASR by varied doses of DMI with QoI combined with multi-site fungicide Mancozeb.

The treatments used in the trial were: 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.25 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.50 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha).

Trial 3 - The curative effect on ASR by varied doses of Cyproconazole and Pyraclostrobin combined with the multi-site fungicide Chlorothalonil.

Treatments used in this trial were: 1. Control; 2. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.15 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 3. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.30 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 4. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.45 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 5. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.60 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 6. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.75 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha).

Disease assessment

The disease severity in the laboratory bioassay was determined by counting the number of sporulated lesions (uredinia)/cm² in five random areas (1 cm²) of each leaf under a stereoscopic microscope (Carl Zeiss Axio Imager A1, Gottingen, Germany) at 20 × magnification.

In the greenhouse experiment, the disease severity was evaluated according to the diagrammatic scale proposed by Godoy et al. (2006) on the lower, medium and upper portion of the soybean plants.

In the field trial, three soybean plants from each replication were randomly removed at weekly intervals at R1 to R7 stages (Fehr et al., 1981) to evaluate the

incidence and severity of the disease. Data were taken from the lower, medium and upper portion of the canopy, evaluating all of the leaves of the plants in the replication.

Weather information regarding the daily rainfall and minimum and maximum temperatures were obtained from the weather station within 50 m of the experiment. Disease severity data were used to calculate the disease infection rate (r) and the area under the disease progress curve (AUDPC), as proposed by Shanner and Finney (1977). The efficacy of disease control was quantified following the equation:

$$E = \frac{(T - F)}{T} \times 100$$

Where:

E = control efficacy (%)

T = disease severity of the control treatment (%)

F = disease severity of the fungicides treatments (%)

For each replication, field assessment of defoliation was performed before harvesting, using an arbitrary scale ranging from 0 to 100 %. The evaluation occurred at the R8.2 stage, determined by the time that the control treatment had 85 to 90 % of complete leaf fall.

Yield determination

Soybean grain yield was harvested at the R8.2 stage. Harvest was manually performed and grains were threshed in a stationary threshing machine, cleaned, weighed and expressed as kg/ha. The results were corrected to 13 % humidity.

Experimental design and data analysis

A laboratory bioassay consisted of ten treatments arranged in a completely randomized design with 4 replications to evaluate the severity of ASR on detached leaves. The greenhouse experiment was organized in a randomized complete block design with nine treatments and four replications. Each experimental unit consisted of one pot with three plants at 55 days old. The field trials were organized in a randomized complete block design with ten treatments and four replications (trial 1) and with 10 treatments and 6 replications (trial 2 and 3).

Data from all variables were analyzed by analysis of variance (ANOVA) and means from the treatments were compared using the Tukey test ($P \leq 0.05$) in SAS (version 6.12; SAS Institute, Inc., Cary, NC).

RESULTS

Weather information regarding daily rainfall and daily minimum and maximum temperatures are shown in Figure 1. The environmental conditions favor the severity of ASR.

The results of the bioassay on detached leaves to evaluate the translaminar effect of DMI and QoI fungicides, either alone or in mixture, on soybean rust are shown in Figure 2.

All of the fungicide treatments translocated from the adaxial to the abaxial surface of the detached soybean leaves, based on the number of sporulated lesion/cm². There was a significant difference in ASR control by all of the fungicides relative to the control treatment. The efficiency of all fungicidal treatments on ASR control was above 92 %, but there were significant differences between the treatments. Cyproconazole + Azoxystrobin (18.0 + 7.2 g a.i./ha) was the most effective (100 % control), and Tebuconazol (50.0 g a.i./ha) (95 %) and Tebuconazole + Trifloxystrobin (100.0 + 50.0 g a.i./ha) (92 %) were the least effective for ASR control.

The resulting control of ASR by the residual effects of DMI and QoI alone or in mixture with the multi-site fungicides in the greenhouse are shown in Figure 3. All of the treatments differed from the control, but there were differences between the fungicidal treatments. Four treatments produced more than 88 % control of ASR: Epoxiconazole + Pyraclostrobin (33.0 + 10.2 g a.i./ha), Prothioconazole + Trifloxystrobin (28.0 + 16.0 g a.i./ha), Cyproconazole + Azoxystrobin (18.0 + 7.2 g a.i./ha) and Pyraclostrobin (75.0 g a.i./ha). The effectiveness of Mancozeb (2400 g a.i./ha) and Chlorothalonil (1500 g a.i./ha) were 86 and 82 % ASR control, respectively.

The dose effects of Epoxiconazole with Pyraclostrobin combined with multi-site fungicides (Mancozeb, Chlorothalonil and Metiram + Pyraclostrobin) on the control of ASR and on grain yield (trial 1) are presented in Figures 4 and 5, respectively. There was no difference between fungicidal treatments regarding soybean rust control (AUDPC) in the upper, medium and lower part of the plant canopy. However, the disease burden in the lower to the upper plant was significantly lower with all fungicidal treatments relative to the control treatment. The AUDPC decreased from the upper to lower part of the plant canopy (Figure 4). The treatments that differed significantly in soybean yield relative to the control were: Epoxiconazole + Pyraclostrobin (66.5 + 20.5 g a.i./ha) combined with Chlorothalonil (1500 g a.i./ha) and Epoxiconazole + Pyraclostrobin (66.5 + 20.5 g a.i./ha) combined with Metiram + Pyraclostrobin (1100 + 100 g a.i./ha). Although there were no differences between fungicidal doses, the best

treatment of Epoxiconazole + Pyraclostrobin (66.5 + 20.5 g a.i./ha) combined with Chlorothalonil (1500 g a.i./ha) yielded 34.0 % more than Epoxiconazole + Pyraclostrobin (66.5 + 20.5 g a.i./ha) combined with Metiram + Pyraclostrobin (1100 + 100 g a.i./ha) (Figure 5).

The curative effect of Epoxiconazole +Pyraclostrobin combined with Mancozeb on soybean rust control and on grain yield (trial 2), are shown in Figures 6 and 7, respectively. Except for the control, the severity of ASR did not vary across the lower, medium and upper part of the plant canopy in almost all of the fungicide treatment groups. All of the fungicidal treatments differed from the control but not relative to other fungicides (Figure 6). Considering leaf defoliation, except for the treatment of Epoxiconazole + Pyraclostrobin (16.6 + 5.1 g a.i./ha) combined with Mancozeb (2400 g a.i./ha) (72 %), all of the fungicidal treatments varied from 32 to 34 % defoliation. The control treatment exhibited 90 % defoliation (Figure 6). The treatments that differed from the control were: Epoxiconazole + Pyraclostrobin (16.6 + 5.1 g a.i./ha) combined with Mancozeb (2400 g a.i./ha); Epoxiconazole + Pyraclostrobin (33.2 + 10.2 g a.i./ha) combined with Mancozeb 2400 g a.i./ha); Epoxiconazole + Pyraclostrobin (66.5 + 20.5 g a.i./ha) combined with Mancozeb (2400 g a.i./ha) and Epoxiconazole + Pyraclostrobin (99.7 + 30.7 g a.i./ha) combined with Mancozeb (2400 g a.i./ha) (Figure 6).

The results of the dose effect of Cyproconazole with Azoxystrobin combined with the multi-site fungicide Chlorothalonil on soybean rust control and the grain yield are shown in Figures 8 and 9, respectively. Except for the control, the severity of ASR varied between the lower, medium and upper portions of the plant canopy with all of the fungicide treatments (Figure 8). The severity of ASR with all of the fungicidal treatments except Cyproconazole + Azoxystrobin (9.0 + 3.6 g a.i./ha) combined with Chlorothalonil (1500 g a.i./ha) differed from the control (Figure 8). Leaf defoliation for the control, and the fungicidal treatments were 85 and 38 to 45 %, respectively. All of the treatments differed from the control regarding soybean grain yield, except with the Cyproconazole + Azoxystrobin (45.0 + 18.0 g a.i./ha) combined with Chlorothalonil (1500 g a.i./ha) (Figure 9).

DISCUSSION

The present study provides novel information about the association of DMI and QoI with multi-site fungicides in different environmental conditions that can be used to improve the efficacy of ASR control. Considering the high fungal adaptability of *Phakopsora pachyrhizi* already described in the literature (Schmitz et al., 2013), and the consequent difficulty of chemically controlling this important pathogen, there is a need to search for new approaches like the association of systemic and multi-site fungicides.

Grain yield was higher than 100 % when potato was cultivated for the two years prior to soybean cultivation when compared with the other two areas (crucifers and maize). The result of the chemical analyses of the leaves was: N – 5.05; P – 0.30; K – 1.68; Ca – 1.64; Mg – 0.25 dag/kg, respectively, which are the optimum levels to promote a higher soybean grain yield. Therefore, the chemical control of ASR should also take into account the level of nutrients present in the soil (mainly K₂O) to achieve higher yields (Fixen et al. 2008; Doreto et al., 2012; Ballardín et al., 2012).

The selection pressure exerted by the DMI and QoI fungicides on soybean growing areas, especially in Midwest Brazil, might have played an important role in the decline in efficacy of these groups (Godoy and Palaver, 2011). The reasonable explanation lies in the fact that both groups act on a single specific site in the fungal cell (Brent, 1995; FRAC, 2015). The efficacy of ASR control by Tebuconazole from growing seasons 2003/05 (91 % ASR control) to 2009/10 (24 % ASR control) was significantly reduced by 67 % in soybean fields in Brazil (Godoy and Palaver, 2011). However, as demonstrated in the laboratory bioassay, the combination of DMI and QoI showed an ability to control 92 to 100 % of the disease. Additionally, the results obtained from the bioassay experiment agreed with the results of Schmitz et al. (2013), which showed a positive effect of QoI fungicides in a bioassay with detached leaves on the control of ASR.

Due the decline in efficacy of both DMI and QoI fungicides groups, the recommendation was the application of triazole with strobilurin to control ASR (FRAC, 2015). In the greenhouse, all the treatments with the systemic strobilurin combined with multi-site fungicides efficiently controlled ASR when applied before the ASR inoculation (protectant effect). On the other hand, the same triazole and strobilurin fungicides were not efficient in controlling ASR in some growing areas in Brazil (Godoy et al., 2009; Godoy et al., 2013). The anastomosis of germ tubes and the migration of nuclei in germ tube networks of the soybean rust pathogen *Phakopsora pachyrhizi* might explain how the fungi recombine their genetic material and develop

resistance to fungicides with a specific mode of action (Vittal et al., 2011). It is possible that this mechanism may occur in nature due to the sheer number of urediniospores that infect soybean leaves in the field.

Fungicides with a single-site mode of action are at relatively high risk for promoting the development of resistance compared to those with a multi-site mode of action (FRAC, 2015; McGrath, 2004). The great majority of the fungicides being developed today have a single-site mode of action because this is associated with a low potential for negative impact on the environment, including non-target organisms (McGrath, 2004). DMI and QoI fungicides act on a single specific site out of thousands of biochemical reactions in the fungal cell (FRAC, 2015); for this reason it is important to combine them with multi-site fungicides. Based on the results obtained in a bioassay (in the laboratory) and the greenhouse, three experiments were conducted in the field (doses of triazoles with strobilurin combined with multi-site fungicides Mancozeb, Chlorothalonil, and Metiram formulated with Piraclostrobin). The purpose of these experiments was to confirm that DMIs and QoIs can be mixed with the multi-site fungicides to improve ASR control and increase yield, while avoiding fungal resistance.

The introduction of a multi-site fungicide in the soybean rust control program may be an important tool for resistance management. Multi-site fungicides have the potential to preserve the lifetime of the site-specific (DMI and QoI) fungicides in soybean crop. The average level of ASR control with the application of triazole with strobilurin combined with protectant fungicides was 70.2 %. Except for in trial 1 (in which the disease severity was less than 1.0 %), the soybean plants of the other two trials were sprayed when the epidemics was over 2.0 %. This results shows that any one of the three protectant fungicides can be used in the mixture with Epoxiconazol and Piraclostrobin or Cyproconazole and Azoxystrobin.

Epoxiconazole (triazol) formulated with Pyraclostrobin (strobilurin) has been reported to cause phytotoxicity in beans and soybeans by reducing the photosynthetic capacity of the plants (Gomez et al. 2014). In our experiments, it was observed that Epoxiconazole + Pyraclostrobin over 33.0 + 10.2 g a.i./ha, respectively and Ciproconazole + Azoxystrobin higher than 36.0 + 14.4 g a.i./ha, respectively were phytotoxic to soybean (enrugation of the leaves and lower leaf area) independently of the protectant fungicide in the association, which reduced soybean grain yield.

The strobilurin fungicides act by inhibiting the biosynthesis of ethylene through the reduction of (ACC)-synthase activity (Grossmann and Retzlaff, 1997). Kresoxim-

methyl (strobilurin group) proved to inhibit the biosynthesis of ethylene through the reduction of (ACC)-synthase activity in wheat tissue (Grossmann and Retzlaff, 1997). Pyraclostrobin promoted the greening effect of the plants by influencing hormonal regulation and the assimilation of carbon and nitrogen by the plant Koehle et al. (1997). As demonstrated above, the strobilurin fungicides have positive effects to reduce leaf fall. In agreement with the reported information, in the field experiments the leaf fall were significantly reduced at high doses of triazole with strobilurin fungicides combined. For this reason strobilurin fungicides at higher doses maintained the leaves on the plants and defoliation on these treatments did not follow disease severity. However, the leaf fall on control plants followed disease severity, where increases in disease severity increased defoliation.

The efficiency of ASR control resulting from the application of triazole and strobilurin is dependent on several factors such as the application timing, size of the droplets, type of application equipment used, the uniformity and distribution of the droplets, and environmental conditions (Zambolim, 2006). In addition, the deposition of the droplets on the lower part of the plants is more difficult as the growth stage reaches the R2 (blooming stage) and R5 (pod formation) (Cunha et al., 2006; Zambolim, 2006). Our study showed that the fungicides applied at R1, R2 and R5-3 stages efficiently promoted ASR control even when the disease severity was over 2.0 %. The disease severity increased from the lower to the upper part of the plant canopy. However, the efficacy of ASR control in the lower, medium and upper part of the canopy, with the application of Epoxiconazole with Pyraclostrobin or Cyproconazole with Azoxystrobin combined with Mancozeb, Chlorothalonil and Metiram with Pyraclostrobin significantly reduced the AUDPC and leaf fall (%) in the plant canopy. Based on the information above, we suggest that the application of triazole and strobilurin combined with multi-site fungicides in the earliest stages of the soybean crop (V9) could promote better ASR control, especially on the lower part of the plant canopy, which is the main source of ASR inoculum in the field.

Overall, the DMIs with QoIs combined with multi-site fungicides had an efficacy higher than 68 % and yielded 70.2 % more than the control at a disease severity of approximately 2.0 %. The protectant, multi-site fungicides Mancozeb (2400 g a.i./ha) and Chlorothalonil (1500 g a.i./ha) combined with lower doses of Epoxiconazole + Pyraclostrobin (16.5 + 10.1 g a.i./ha) or Cyproconazole + Azoxystrobin (9.0 + 3.6 g a.i./ha) increased soybean grain yield by at least 45.0 %, and the same combination of fungicides at the dose ahead of the lowest increased yield by 89.5 % and 109.0 %.

This study illustrates that the application of DMI with QoI combined with multi-site fungicides promoted the effective reduction of ASR, increased yield and reduced leaf fall. The use of a commercial formulation of high risk (DMI with QoI) fungicides combined with low risk (multi-site) fungicides, may provide a new strategy for the management of ASR in the short and long term. Furthermore, due to their residual effect and multi-site action, fungicides such as Mancozeb, Chlorothalonil and Metiram can promote increased longevity of DMI and QoI molecules and decrease the number of required applications for systemic fungicides.

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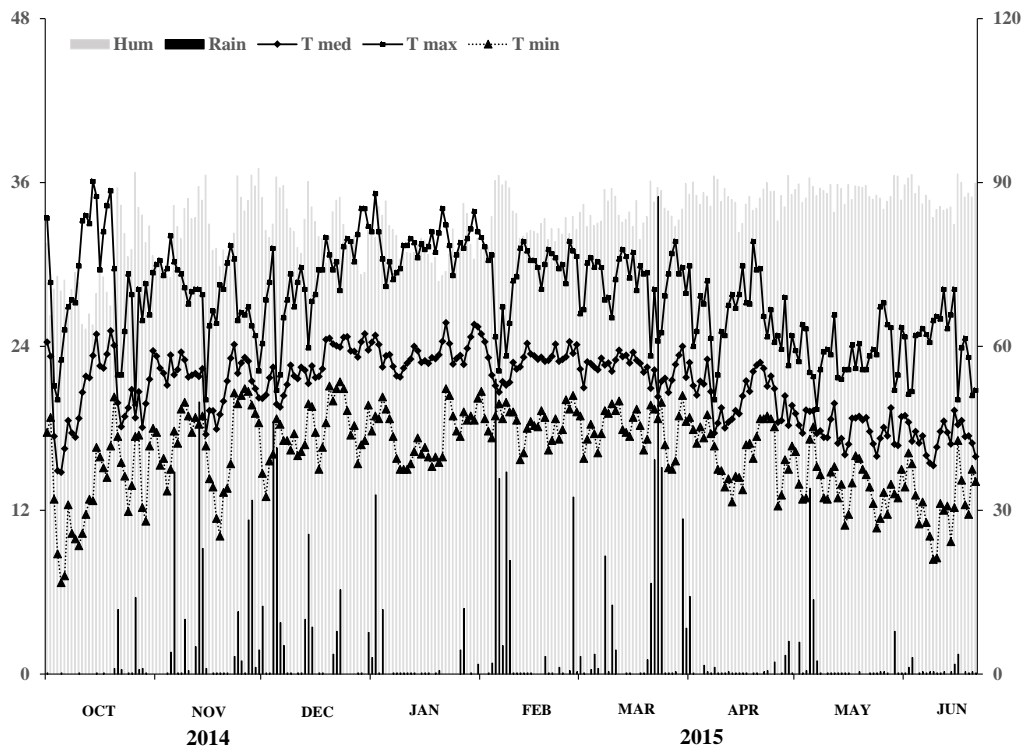


Fig. 1. Environmental conditions (rain, relative humidity, and maximum, minimum average temperature) during October 2014 to June 2015. Data were taken from the meteorological station of the UFV.

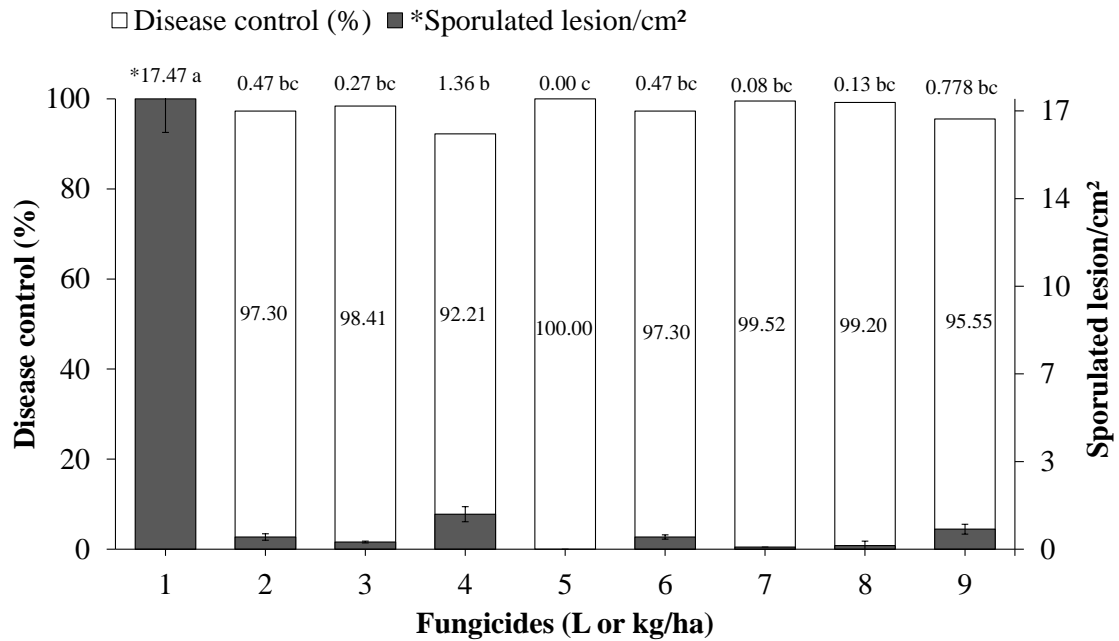


Fig. 2. Translaminal effect of DMI and QoI fungicides, alone or in mixture, on the control of Asian soybean rust (ASR) on detached leaves. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g active ingredient (a.i.)/ha - 0.5 L/ha); 3. Cyproconazole + Trifloxystrobin (Sphere Max SC; 56.0 + 24.0 g a.i./ha - 0.3 L/ha); 4. Tebuconazole + Trifloxystrobin (Nativo SC; 200.0 + 100.0 g a.i./ha - 0.5 L/ha) + Aureo (0.5 L/ha); 5. Cyproconazole + Azoxystrobin (Priori Xtra; 60.0 + 24.0 g a.i./ha - 0.3 L/ha) + Nimbus (0.5% v/v); 6. Pyraclostrobin (Comet SC; 250.0 g a.i./ha - 3 L/ha); 7. Flutriafol (Impact SC; 62.5 g a.i./ha - 0.5 L/ha); 8. Prothioconazole+ trifloxystrobin (Fox SC; 70.0 + 40.0 g a.i./ha - 0.4 L/ha); 9. Tebuconazole (Folicur SC; 100.0 g a.i./ha - 0.5 L/ha). Means followed by the same letter do not differ by the Tukey test ($P \leq 0.05$). CV = 2.35. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown.

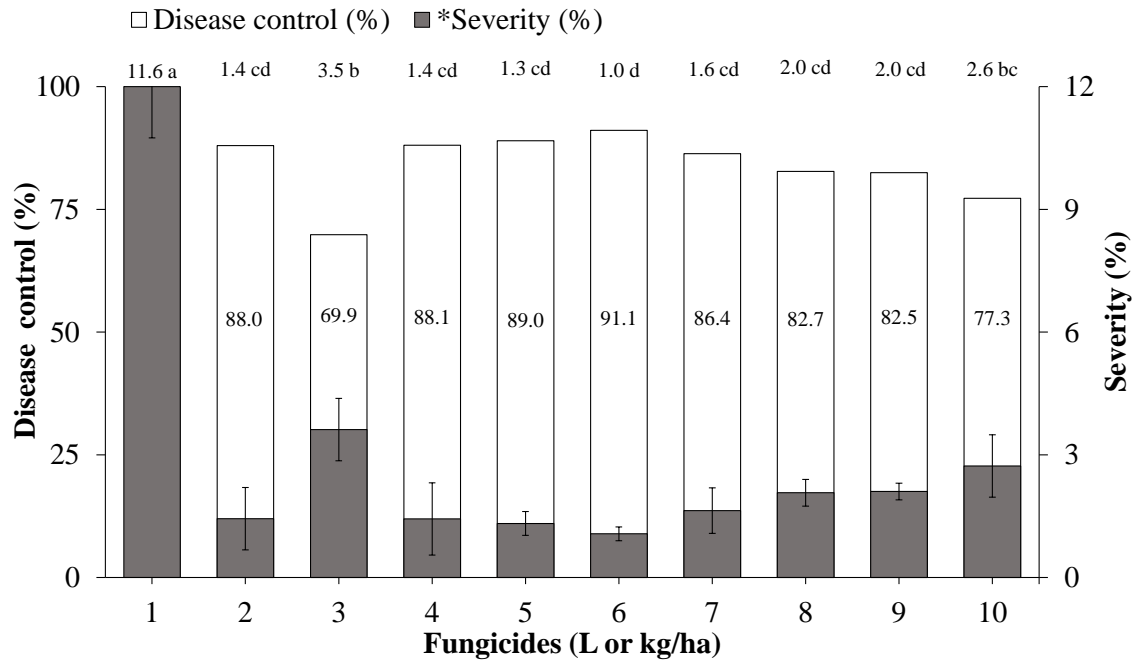


Fig. 3. Systemic, translaminar and residual effects of DMI and QoI, alone or in mixture, associated with multi-site fungicides on ASR in greenhouse. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha); 3. Cyproconazole + Trifloxystrobin (Sphere Max SC; 56.0 + 24.0 g a.i./ha - 0.3 L/ha); 4. Prothioconazole + Trifloxystrobin (Fox SC; 70.0 + 40.0 g a.i./ha - 0.4 L/ha); 5. Cyproconazole + Azoxystrobin (Priori Xtra; 60.0 + 24.0 g a.i./ha - 0.3 L/ha) + Nimbus (0.5% v/v); 6. Pyraclostrobin (Comet SC; 250 g a.i./ha - 0.3 L/ha); 7. Mancozeb (Dithane M45 PM; 800 g a.i./ha - 3.0 kg/ha); 8. Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 9. Metiram + Piraclostrobin (Cabrio Top S; 550 + 50 g a.i./ha - 2.0 L/ha), 10. Tebuconazol + Trifloxistrobina (Nativo SC; 200.0 + 100.0 g a.i./ha - 0.5 L/ha). The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown.

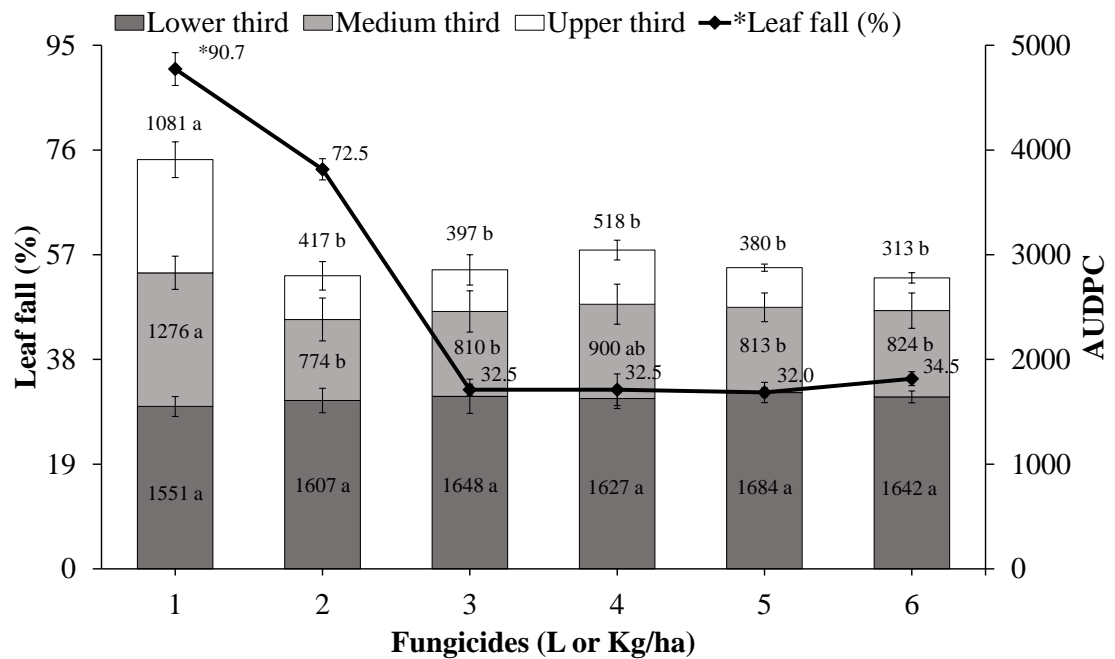


Fig. 4. Effect of dose rate of Epoxiconazole, Pyraclostrobin, and multi-site fungicides, alone or in mixture, on the ASB control. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 7. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 8. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 9. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 2.0 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 10. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha). The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown. AUDPC – area under disease progress curve; *leaf fall – (%).

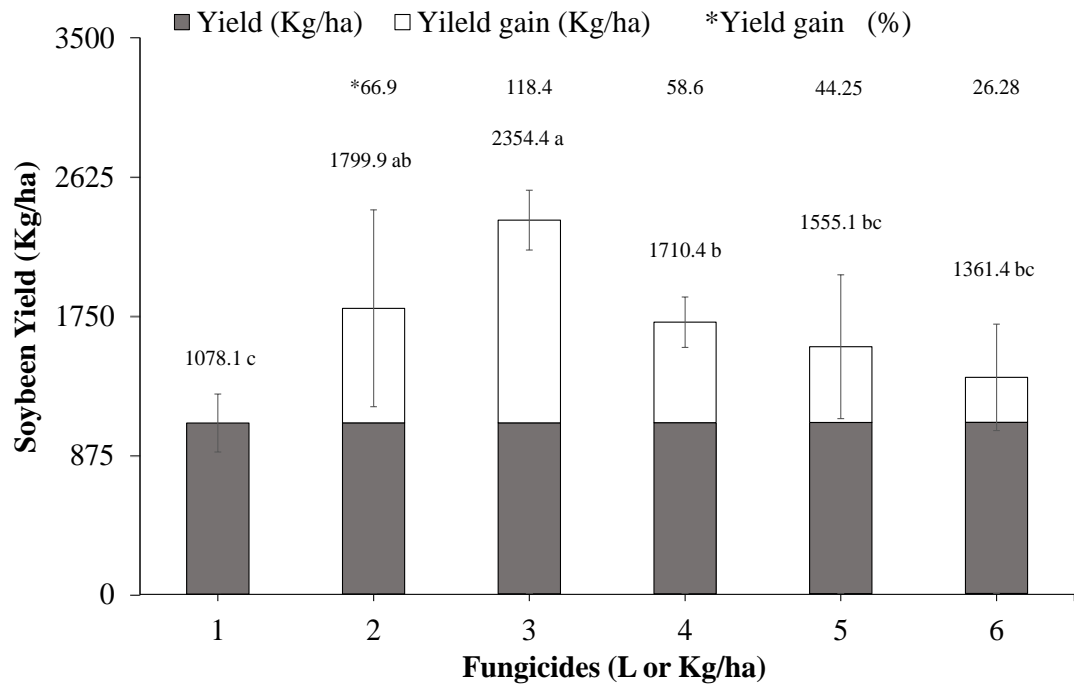


Fig. 5. Effect of dose rate of Epoxiconazole, Pyraclostrobin, and multi-site fungicides, alone or in mixture, on soybean grain yield. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.5 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 7. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha); 8. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 9. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 2.0 L/ha) + Chlorothalonil (Daconil 500 PM; 500 g a.i./ha - 3.0 L/ha); 10. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Metiram + Pyraclostrobin (Cabrio TOP SC; 550 + 50 g a.i./ha - 2.0 L/ha). Means followed by the same letter do not differ by the Tukey test ($P \leq 0.05$). CV = 18.3. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown. *Yield gain – was obtained in relation to the control treatment.

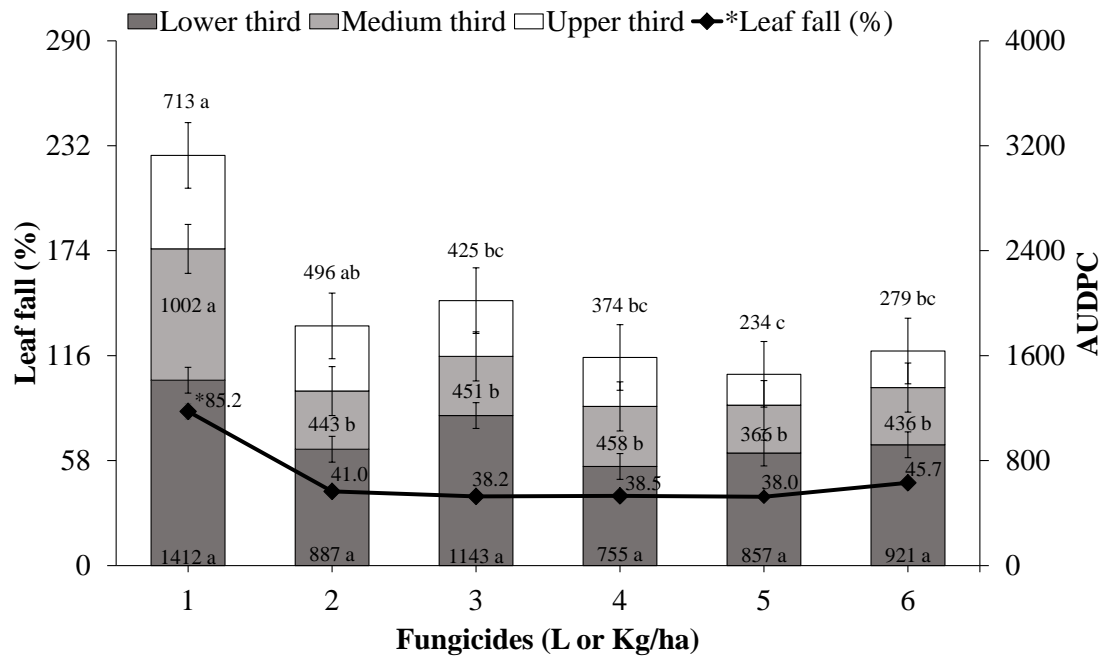


Fig. 6. Curative effect of dose rate of Epoxiconazole and Pyraclostrobin, in mixture, associated with Mancozeb on ASR control. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.25 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.50 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha). Two experiments were conducted with consistent results; results from one representative experiment are shown. AUDPC – area under disease progress curve.

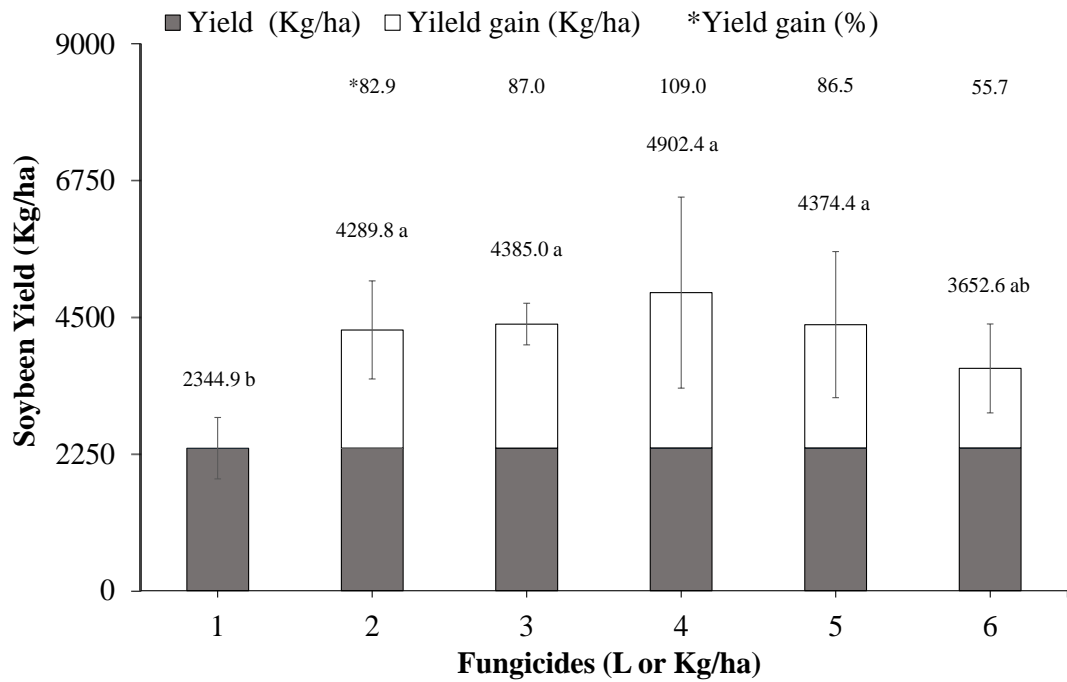


Fig. 7. Curative effect of dose rate of Epoxiconazole and Pyraclostrobin, in mixture, associated with Mancozeb on soybean grain yield. 1. Control; 2. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.25 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 3. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.50 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 4. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 0.75 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 5. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.0 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha); 6. Epoxiconazole + Pyraclostrobin (Opera SC; 66.5 + 20.5 g a.i./ha - 1.5 L/ha) + Mancozeb (Dithane M 45 PM; 800 g a.i./ha - 3.0 L/ha). Means followed by the same letter do not differ by the Tukey test ($P \leq 0.05$). CV = 20.8. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown.

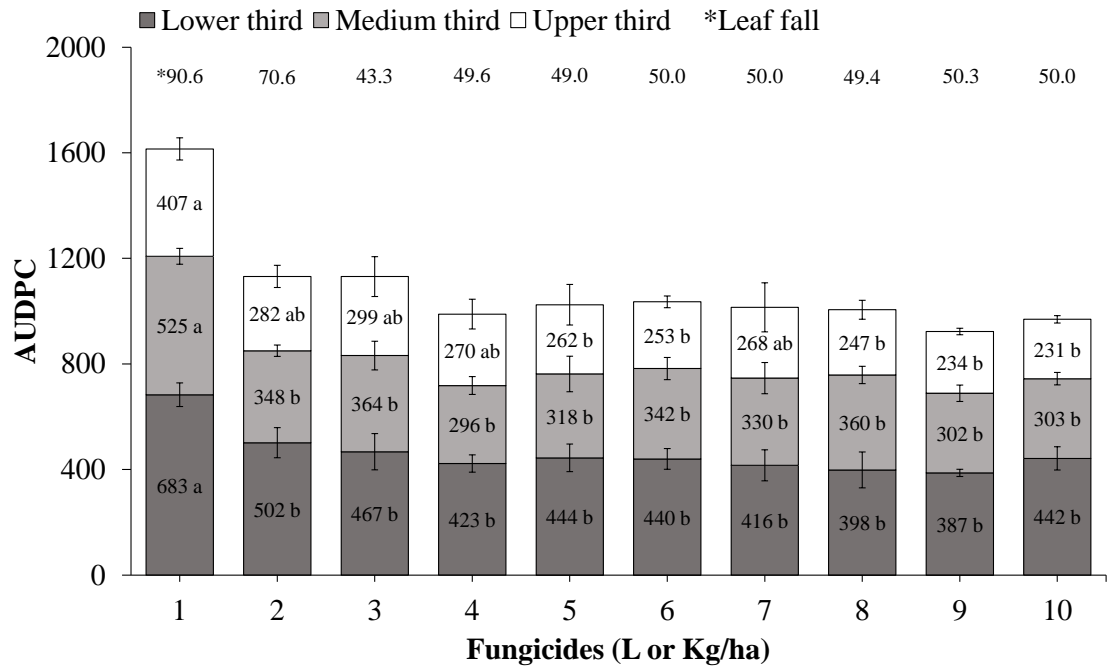


Fig. 8. Curative effect of dose rate of Cyproconazole and Azoxytrobilin associated with the multi-site fungicide Chlorothalonil on disease control. 1. Control; 2. Cyproconazole + Azoxytrobilin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.15 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 3. Cyproconazole + Azoxytrobilin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.30 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 4. Cyproconazole + Azoxytrobilin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.45 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 5. Cyproconazole + Azoxytrobilin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.60 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 6. Cyproconazole + Azoxytrobilin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.75 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha). The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown. AUDPC – area under disease progress curve.

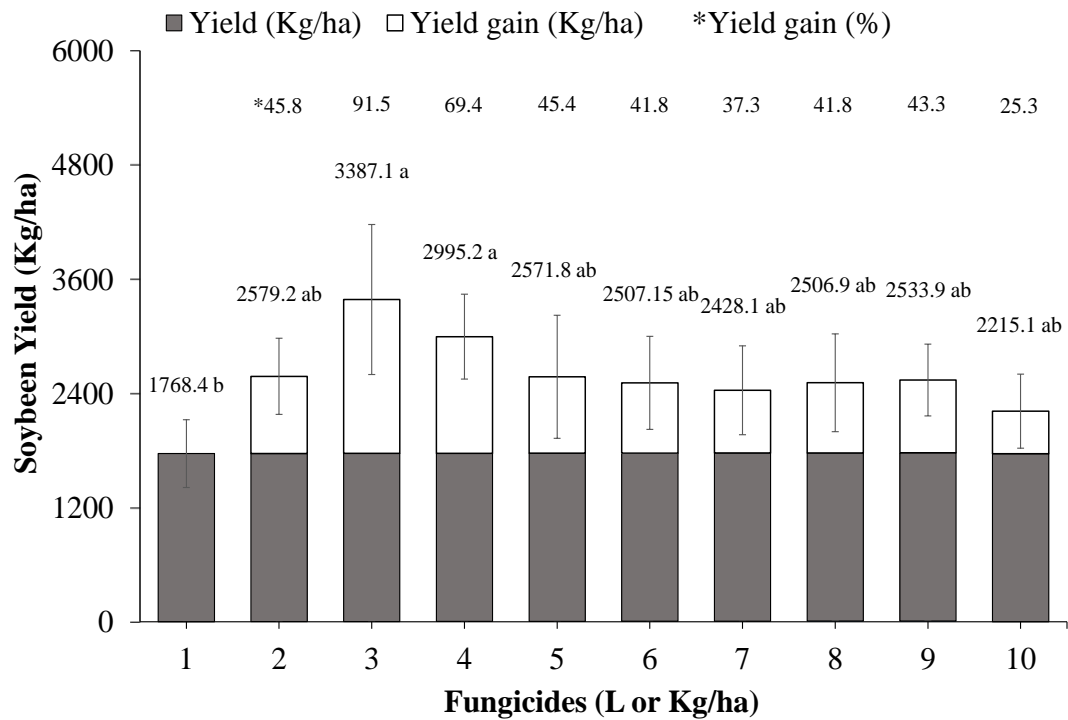


Fig. 9. Curative effect of dose rate of Cyproconazole and Azoxystrobin associated with the multi-site fungicide Chlorothalonil on soybean grain yield. 1. Control; 2. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.15 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 3. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.30 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 4. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.45 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 5. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.60 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha); 6. Cyproconazole + Azoxystrobin (Priori Xtra SC; 60.0 + 24.0 g a.i./ha - 0.75 L/ha) + Chlorothalonil (Daconil PM; 500 g a.i./ha - 3.0 kg/ha). Means followed by the same letter do not differ by the Tukey test ($P \leq 0.05$). CV = 16.31. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; results from one representative experiment are shown.

GENERAL CONCLUSIONS

1. The three field trials involving all the doses of DMI's and QoI's fungicides proportioned 70.1, 78.0 and 56.8 % minimum average disease control, respectively over the non-atomized treatment at the disease severity around 2.0 %.
2. The three field trials involving the DMI's and QoI's fungicides yielded at minimum 49.0, 77.4 and 84.2 % average, respectively over the control treatment.
3. DMI's and QoI's fungicides at higher doses showed symptoms of phytotoxicity (leaves crinkled and reduced leaf area).
4. Protectant fungicides Mancozeb (3.0 Kg/ha) and Chlorothalonil (3.0 Kg/ha) added to Epoxiconazole + Pyraclostrobin (0.25 L/ha) and Cyproconazole + Azoxystrobin (0.15 L/ha) soybean grain yield increased 45.0 %, respectively.
5. The triazole + strobilurin at lower dose did not control the rust efficaciously and the protectant fungicides were very important to control the disease and increase yield.
6. DMI's and QoI's fungicides at higher doses reduced soybean yield.