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Leandro Nascimento Marques

MANCOZEBE ASSOCIADO AO PATOSSISTEMA *Phakopsora pachyrhizi* × *Glycine max*: RESPOSTAS FISIOLÓGICAS DAS PLANTAS

Santa Maria, RS
2017

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

Orientador: Prof. PhD. Ricardo S. Balardin

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Aprovado em 17 de fevereiro de 2017:

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Santa Maria, RS
2017

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RESUMO

MANCOZEBE ASSOCIADO AO PATOSSISTEMA *Phakopsora pachyrhizi* × *Glycine max*: RESPOSTAS FISIOLÓGICAS DAS PLANTAS

AUTOR: LEANDRO NASCIMENTO MARQUES
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Mancozebe tem sido o fungicida protetor com mecanismo multi-sítio amplamente utilizado associado aos programas fungicidas na cultura da soja. Este composto tem proporcionado incrementos significativos no desempenho de fungicidas sistêmicos no controle da ferrugem da soja. Além do efeito evidente como fungicida, tem sido relatado efeitos sobre parâmetros fisiológicos nas plantas conferindo benefícios como redução de fitotoxicidade de outros fungicidas e incrementos no vigor de plantas. Nossos estudos objetivaram compreender a interação de mancozebe associado a fungicidas sistêmicos frente ao controle da ferrugem da soja, além de determinar respostas fisiológicas das plantas oriundas da relação produto *versus* planta. Foram conduzidos ensaios em casa de vegetação e a campo, aqui divididos em quatro capítulos no formato de manuscritos. Em um primeiro estudo o efeito da interação de mancozebe com misturas comerciais de IQo + IDM foi investigado com base em duas metodologias. A partir disso foi possível verificar que mancozebe agrega mais significativamente a eficácia de controle quando associado a misturas menos eficientes. Em tais interações obteve-se uma resposta sinérgica de controle. Quando associado a misturas com alta eficácia o incremento no controle foi menor, entretanto no mínimo uma resposta aditiva é esperada. Em um segundo estudo a atividade de enzimas ligadas à resposta antioxidante, a concentração de pigmentos fotossintetizantes e a peroxidação lipídica foi investigada em plantas expostas ao fungicida trifloxistrobina + protioconazol, com potencial de causar fitotoxidade, estando este associado ou não ao mancozebe. Aumento na atividade de enzimas antioxidantes, redução no conteúdo de pigmentos e danos celulares evidenciadas pela maior peroxidação de lipídios foram verificadas em plantas expostas ao fungicida sistêmico. Tais danos foram atenuados quando o fungicida sistêmico foi associado ao mancozebe. Nesse mesmo sentido, parâmetros de fotossíntese e fluorescência da clorofila *a* foram analisados em plantas expostas a mancozebe e trifloxistrobina + protioconazol, sendo este o terceiro estudo aqui apresentado. Decréscimo na eficiência fotossintética foi verificado em plantas tratadas com o fungicida sistêmico caracterizando um efeito fitotóxico significativo. Os parâmetros avaliados elucidam um efeito benéfico de mancozebe reduzindo os danos causados pelo fungicida sistêmico. Um quarto e último estudo visou avaliar alterações no estado nutricional de folhas de soja expostas a mancozebe. Concentração de macro e micronutrientes foram determinadas aos 4 e 8 dias após exposição. Alterações significativas na concentração de S, Mn e Zn foram observadas. Tais alterações foram relacionadas a incrementos na coloração verde da cultura após exposição à mancozebe. Os dados trazem informações novas em relação ao recente uso da molécula mancozebe em soja. A contribuição é original e apresenta dados que podem contribuir na definição de estratégias no manejo anti-resistência e aumento da vida efetiva dos fungicidas sistêmicos sítio-específico comumente utilizados em soja. Além disso, os resultados apresentam uma série de respostas fisiológicas importantes de mancozebe sobre as plantas que podem agregar em termos de desenvolvimento, vigor e produtividade.

Palavras-chave: Soja. Ferrugem Asiática da Soja. Resistência a fungicidas. Estresse oxidativo. Atividade fotossíntética.

ABSTRACT

MANCOZEB ASSOCIATED TO THE PATHOSYSTEM *Phakopsora pachyrhizi* × *Glycine max*: PLANTS PHYSIOLOGICAL RESPONSES

AUTHOR: LEANDRO NASCIMENTO MARQUES
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Mancozeb has been the protective fungicide with a multi-site mechanism widely used associated with fungicide programs in soybean cultivation. This compound has provided significant increases in the systemic fungicides performance in the soybean rust control. Besides the obvious fungicide effect, mancozeb has provided also effects on physiological parameters in plants promoting benefits such as fungicide phytotoxicity reduction and increases in plant vigor. The studies aimed to understand the interaction of mancozeb associated to systemic fungicides against the soybean rust control, in addition to determine the physiological plants responses from the product versus plant relation. The experiments were conducted in greenhouse and field, divided in four chapters in manuscript format. The first study investigates the effect of the mancozeb interaction with commercial mixtures of QoI + DMI based on two methodologies. From this, it was possible to verify that mancozeb increases significantly the control efficiency when associated with less efficient mixtures. In such interactions was obtained a control synergistic response. However it is expected at least an additive response, when associated to mixtures with high efficiency, the increase in control is lower. The second study consisted of the enzymes activity linked to the antioxidant response, the photosynthetic pigments concentration and lipid peroxidation investigation in plants exposed to the fungicide trifloxystrobin + prothioconazole, with potential to cause phytotoxicity, associated or not with mancozeb. Antioxidant enzymes activity increase, reduction in pigment concentration and cell damage due to increased lipid peroxidation were observed in plants exposed to systemic fungicide. These damages were attenuated when the systemic fungicide was associated with mancozeb. Moreover, photosynthesis and chlorophyll *a* fluorescence parameters were analyzed in plants exposed to mancozeb and trifloxystrobin + prothioconazole, constituting the third study. Decreased in the photosynthetic efficiency was verified in plants treated with systemic fungicide characterizing a significant phytotoxic effect. The parameters evaluated elucidate a beneficial effect of mancozeb reducing the damages caused by the systemic fungicide. The fourth study aimed to evaluate changes in the leaves soybean nutritional status exposed to mancozeb. Macro and micronutrients concentration were determined on 4th and 8th days exposure. Significant changes in concentration of S, Mn and Zn were observed. Such changes were related to “green effect” on plants after mancozeb exposure. All data provide new information regarding the recent use of the mancozeb molecule in soybean. The contribution is original and presents data that can contribute to the definition of strategies in anti-resistance management and increase site-specific systemic fungicides effective life commonly used in soybean. In addition, the results present important physiological responses on plants induced Figure 1by mancozeb that can aggregate on development, vigor and productivity.

Keywords: Soybean. Asian Soybean Rust. Resistance to fungicides. Oxidative stress. Photosynthetic rate.

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LISTA DE ABREVIAÇÕES

AACPD – Área abaixo da curva de progresso da doença
ASR – Asian Soybean Rust
AUDPC – Area under disease progress curve
Bo – Relative amount Q_B-non-reducing of PSII centers
BOD – Biochemical oxygen demand
Chl – Chlorophyll
Ci – Intercellular CO_2 concentration
CO_2 – Carbon dioxide
DAA – Dias após aplicação (days after application)
DAS – Days after spraying
DMI – Demethylation Inhibitor
DNA - Deoxyribonucleic acid
E – Transpiration rate
ED_{50} – 50% do efeito total possível na variável resposta
EDTA – Ethylenediaminetetraacetic
ETR – Electron transport rate
ETU – Ethylenethioureia
FAS – Ferrugem asiática da soja
F_m – Maximum fluorescence
F_o – Initial fluorescence
FRAC – Fungicide resistance action committee
F_v/F_m – Maximum quantum yield of PS_{II}
FW – Fresh weight
gs – Stomatal conductance
H_2O_2 – Hydrogen peroxide
MDA – Malondialdehyde
Mz – Mancozebe
NDAPP – Número de dias para aparecimento das primeiras pústulas
NPK – Nitrogênio, fósforo e potássio
$O_2^{\cdot-}$ - Superoxide anion
PAR – Photosynthetically active radiation
Pn – Photosynthetic rate
Pn/Ci – Instantaneous carboxylation efficiency
POX – Peroxidase
PS_{II} – Photosystem II (P680)
QoI – Quinone outside Inhibitor
*q*P – Coefficient photochemical quenching
RNA - Ribonucleic acid
ROS – Reactive oxygen species
RuBisCo - Ribulose-1,5-bisfosfato carboxilase oxigenasse
S, Fe, Mn and Zn – Sulphur, Iron, Manganese and Zinc
SOD – Superoxide dismutase
SPAD – Chlorophyll index
TBA – Thiobarbituric acid
WG – Grânulos dispersíveis
WUE – Water use efficiency
Φ_{PSII} – Quantum efficiency of PSII photochemistry

SUMÁRIO

INTRODUÇÃO

A soja destaca-se como a principal cultura de grãos semeada no Brasil, ocupando a maior área plantada e responsável pelo maior volume de grãos colhidos (CONAB, 2016). Um dos grandes desafios fitossanitários que ocorrem nas diversas regiões de cultivo no Brasil é a ferrugem da soja (FAS) causada por *Phakopsora pachyrhizi* (*P. pachyrhizi*). Até o ano de 2001, a América do Sul esteve livre do ataque desse patógeno (FREIRE et al., 2008). A partir de 2001 a doença foi encontrada no Paraguai e também no Brasil, tornando-se uma pandemia que trouxe grandes desafios a produção de soja no país (YORINORI et al., 2005). Dessa data em diante, o patógeno se tornou altamente adaptado às condições do ambiente e vem causando danos expressivos ininterruptamente ao longo dos anos (GODOY et al., 2016a).

Dentre os métodos mais utilizados no manejo de FAS está o método químico através do uso de fungicidas (LANGENBACH et al., 2016; GODOY et al., 2016a; BALARDIN et al., 2010; SCHERM et al., 2009; MILES et al., 2007). Os principais fungicidas utilizados para manejo químico da doença pertencem aos grupos químicos dos inibidores da respiração celular do fungo, agindo na quinona externa nas cristas mitocondriais (*QoIs – Quinone outside Inhibitors*) e os inibidores da desmetilação da cadeia carbônica na síntese de esteróis nas membranas celulares (*DMIs – DeMethylation Inhibitors*) (GODOY et al., 2016a; LAUGENBACH et al., 2016). Recentemente, o grupo de fungicidas conhecidos genericamente como carboxamidas também passou a fazer parte deste arsenal. As carboxamidas pertencem ao grupo químico dos inibidores da respiração mitocondrial, os quais se ligam ao complexo II da cadeia de transporte de elétrons, tendo como alvo a enzima succinato desidrogenase (*SDHI - Succinate DeHydrogenase Inhibitors*) (KEON et al., 1991). Todos os produtos pertencentes a esses grupos químicos agem em sítios específicos no metabolismo do patógeno e por isso são considerados de alto risco à perda de sensibilidade e resistência do patógeno (BRENT; HOLLOMON, 2007).

Em quinze anos de ferrugem no Brasil, passamos de menos de uma aplicação de fungicidas para próximo de quatro aplicações considerado em termos médios. Inicialmente foram utilizados ingredientes ativos isolados, evoluindo para misturas formuladas de diferentes ativos e mistura de tanque de dois ou mais ingredientes ativos considerando mecanismos de ação diferentes. Uma das principais razões para esta mudança na estratégia de controle deveu-se a uma progressiva perda de eficácia. Por longos anos, fungicidas inibidores de desmetilação (IDMs) e inibidores da quinona externa (IQo) forneceram os mais

consistentes resultados (GODOY, 2011; SCHERM et al., 2009; IVANCOVICH, 2005). Porém, por alguns anos adotou-se a prática de posicionamento de ingredientes ativos sítio-específico aplicados de forma isolada. Dados de ensaios conduzidos em rede, no período entre 2003/04 e 2006/07, foram analisados e mostraram que para controle eficiente da FAS, IDMs aplicados isoladamente apresentavam bom desempenho, superior ao desempenho de IQos isolados (SCHERM et al., 2009). Tal fato pode ter acelerado o surgimento de isolados menos sensíveis a esses mecanismos de ação. Nas safras seguintes, 2007/08 a 2009/10, começaram a ser notado problemas de eficácia a fungicidas do grupo IDMs no controle de FAS (GODOY, 2011) e mais tarde para IQos (GODOY et al., 2014). O uso intensivo e inadequado de fungicidas, como o uso repetido de moléculas com o mesmo mecanismo de ação, utilização de produtos isolados com mecanismo sítio-específico, alterações de doses recomendadas, cobertura insuficiente depositando um número de gotas inferior ao necessário para conter a dose letal do fungicida, uso de produtos sistêmicos de forma erradicativa, contribuem definitivamente para a seleção de isolados fúngicos menos sensíveis, e, por consequência, diminuir a eficiência dos fungicidas no controle da doença ao longo do tempo (BRENT; HOLLOMON, 2007).

A queda de eficácia dos fungicidas foi relacionada em algumas safras a problemas com tecnologia de aplicação, condições ambientais, pressão de inóculo, posicionamento errôneo de aplicações e cultivares. Porém, a partir de esforços da pesquisa e uso de ferramentas moleculares, adaptações de *P. pachyrhizi* foram caracterizadas e demonstradas recentemente (KLOSOWSKI et al., 2015; SCHIMITZ et al., 2014). Mecanismos de resistência contra IDMs são variáveis e complexos, envolvendo mutações pontuais, super expressão do gene alvo *CYP51* e a regulação de transportadores de efluxo (SCHMITZ et al., 2014). Em relação à sensibilidade a IQos, mutações no gene codificante para citocromo *b* (*cytB*) resultam em reduzida sensibilidade de *P. pachyrhizi* em função da alteração parcial do sítio alvo reduzindo a afinidade de ligação desses fungicidas (GRASSO et al., 2006). Apesar desta queda aparente, os IQos e IDMs ainda são utilizados com sucesso no controle da ferrugem, existindo uma variação de eficácia significativa entre os ingredientes ativos disponíveis pertencente a esses grupos. Alguns produtos que eram utilizados em menor proporção parecem estar apresentando uma eficácia maior comparado àqueles que eram e são utilizados em grande escala. Isso parece evidenciar uma variabilidade nas raças do patógeno ao longo do tempo e uma alteração na sensibilidade as moléculas utilizadas. Mesmo com a redução relativa na eficácia, produtos IDMs + IQos ainda mantém um nível de atividade que

os tornam indispensáveis como ferramenta no controle da ferrugem da soja (GODOY et al., 2016a).

Deve-se ressaltar que o uso de fungicidas SDHIs deve aumentar rapidamente em função da eficácia de controle que têm apresentado e em função das misturas IQo + IDM estarem com menor efetividade. Tal fato pode conduzir a uma forte pressão de seleção para resistência a este grupo de fungicidas (GODOY et al., 2016a). Nesse contexto, na busca de estratégias de manejo contra a resistência do patógeno aos fungicidas, surge no mercado à possibilidade de incorporação aos programas de controle fungicidas protetores com mecanismo de ação em múltiplos sítios conferindo amplo espectro de controle. Dentre estes produtos, aqueles com mancozebe na composição têm sido os mais amplamente utilizados. Os resultados de pesquisas são promissores e refletem um ganho significativo em termos de controle de doenças, não só em relação à ferrugem da soja, mas outras doenças que ficam em segundo plano. Acreditava-se que a fungitoxicidade de mancozebe frente a *P. pachyrhizi* era baixa ou inexistente. A fungitoxidade de mancozebe frente a *P. pachyrhizi* tem sido amplamente monitorada (GODOY et al., 2016b; SILVA et al., 2015). No entanto, o melhor desempenho e função desses produtos é a utilização em misturas em tanque com produtos sítio-específico para aumentar a espectro de ação, aumentar eficácia de controle e reduzir o risco de resistência (GODOY et al., 2016b).

As misturas de fungicidas podem levar a ocorrência de interações que podem se manifestar de forma aditiva, antagônica ou sinérgica. Dependendo da interação podem-se obter ganhos ou perdas no controle da doença (TREZZI et al., 2005). Segundo Maciel et al. (2009) pouco se conhece sobre a compatibilidade e o efeito da mistura de diferentes produtos na agricultura. A sinergia entre fungicidas é inteiramente desejada podendo resultar em um incremento significativo no controle da doença (EVENHUIS et al., 1996). Mas para minimizar ou evitar os riscos envolvidos, mais conhecimento sobre possíveis efeitos da associação entre fungicidas em misturas são necessários (EVENHUIS et al., 1996). O sinergismo é a ação de dois ou mais compostos em que a resposta total de controle de um organismo é maior do que a soma dos componentes individuais (WAARD, 1987). Hipóteses sobre os mecanismos fisiológicos e bioquímicos de sinergismo envolvem aumento da absorção e ligação dos fungicidas ao local de ação, ação em diferentes locais da célula fúngica e diminuição da biodegradação (GISI, 1991). Alguns estudos anteriores já demonstraram ação sinérgica de mancozebe associado a outros fungicidas no controle de doenças na batata (WANG et al., 2002; SAMOUCHA; COHEN, 1988; 1989).

A ação potencial de etilenobisditiocarbamatos como o mancozebe, quando aplicados em mistura com fungicidas sistêmicos na proteção de plantas, é conhecida por resultar em efeito sinérgico (GISI et al., 1985). Tal efeito foi visualizado por Gozzo et al. (1988) com associação de benalaxil com mancozebe. Uma redução significativa do metabolismo do fungicida benalaxil foi identificada quando este foi aplicado em mistura com mancozebe (GOZZO et al., 1988). O menor metabolismo do fungicida sistêmico foi acompanhado de uma redução na velocidade de absorção quando acompanhado do mancozebe. Ao reduzir a taxa de absorção do benalaxil, atribuiu-se a isso uma menor indução de oxidases responsáveis pela degradação oxidativa desse composto e também se sugeriu que o mancozebe presente na superfície da folha pode ter papel na inibição dessas enzimas oxidases (GOZZO et al., 1988).

O processo de degradação oxidativa que ocorre em plantas foi investigado utilizando técnicas com microssomos hepáticos (GOZZO et al., 1988). Tais estudos mostraram alta eficácia dos microssomos na hidroxilação de benalaxil. Quando os microssomos tinham sido pré-incubados com mancozebe por 20 min, a degradação de benalaxil foi substancialmente reduzida. Isto sugere que os subprodutos da degradação de mancozebe podem estar ligados a inibições das oxidases dos microssomos com função de ataque enzimático ao benalaxil (GOZZO et al., 1988). Frente a isso, supõe-se que a associação de mancozebe a fungicidas sistêmicos atualmente utilizados pode contribuir significativamente para que efeitos sinérgicos sejam alcançados gerando maior eficácia de controle da doença.

Além dos ganhos substanciais no controle do patógeno, benefícios de mancozebe em parâmetros fisiológicos nas plantas também têm sido relatados em situações de campo. A relação mancozebe × planta parece revelar efeitos benéficos no caso da cultura da soja. São inúmeros os relatos que indicam um efeito nutricional, efeito verde nas folhas e um efeito como redutor de fitotoxicidade de outros produtos sistêmicos. Fungicidas do grupo dos ditiocarbamato como o mancozebe, carregam em suas moléculas metais ligados, como zinco e manganês, sendo estes uns dos primeiros sítios de quebra no processo de degradação da molécula (HWANG et al., 2003). A dissociação da molécula do fungicida e a liberação dos íons sugerem que tais elementos podem ficar disponíveis para absorção pela planta e exercer uma função nutricional.

É bem documentado na literatura que fungicidas além de exercer um importante papel no controle de fitopatógenos, podem também exercer efeitos sobre parâmetros fisiológicos e bioquímicos nas plantas (PETIT et al., 2012, DIAS, 2012, SALADIN et al., 2003). Tem sido mostrado que a sensibilidade das espécies aos efeitos fitotóxicos diferem entre si, e que o estágio de desenvolvimento, idade de folhas e o produto em questão são fatores

determinantes. A maior parte dos trabalhos dedica-se a investigar o impacto dos fungicidas sobre a eficácia de controle de agentes patogênicos ou acúmulo de resíduos nas culturas (PETIT et al., 2012). No entanto, o impacto negativo dos fungicidas sobre a fotossíntese e o estresse oxidativo tem sido menos explorado e a maioria dos estudos foram conduzidos apenas com fungicidas sistêmicos (DIAS et al., 2014).

Alguns relatos indicam sérias implicações de fungicidas causando reduções na taxa de assimilação líquida de CO_2 e eficiência fotossintética (PETIT et al. 2008; XIA et al., 2006). Os fungicidas parecem inibir a biossíntese de clorofilas (Chl) e retardar a integração de Chl nos fotossistemas (PETIT et al., 2012). Além disso, tem sido mostrado um efeito sobre o aumento da atividade de enzimas antioxidantes em plantas expostas a alguns fungicidas (GOPI et al., 2007; JALEEL et al., 2007;; WU; TIEDEMANN, 2002; CALATAYUD; BARRENO, 2001).

Os estudos que procuram elucidar os efeitos da aplicação de mancozebe sobre as plantas tem sido escassos (DIAS et al., 2014; PEREIRA et al., 2014). A taxa fotossintética e o conteúdo de clorofilas não sofreram alterações em plantas de trigo expostas a mancozebe (LORENZ; COTHREN, 1989). Já em alface, mancozebe induziu sérios danos incluindo reduções da taxa fotossintética e pigmentos fotossintetizantes (DIAS et al., 2014). Também em alface, mancozebe induziu alterações no metabolismo de aminoácidos das plantas com ligação na alteração dos níveis de α-tocoferol (vitamina E), lignina e pigmentos vegetais em folhas expandidas (PEREIRA et al., 2014). Outras mudanças envolveram a ativação de antioxidantes possivelmente ligados a compostos polifenólicos. Todas essas observações parecem indicativas de uma maior procura por mecanismos de defesa após a exposição das plantas ao fungicida, sugerindo um efeito fitotóxico (PEREIRA et al., 2014). Em soja a utilização desse composto é recente e por isso não há relatos científicos da resposta fisiológica das plantas a este composto. No entanto, percebe-se que o uso dessa molécula em soja parece ser duradouro em função dos benefícios gerados, e a relação produto × planta merece ser investigada.

Com base no exposto, foram desenvolvidos trabalhos com a finalidade de elucidar os efeitos de mancozebe associado a outros fungicidas sistêmicos no controle de *P. pachyrhizi* e quanto às respostas fisiológicas das plantas expostas a este composto.

Manuscrito I

Efeito da interação entre fungicidas IQo + IDM com mancozebe no controle da ferrugem da soja

Resumo

O controle eficaz da ferrugem da soja tem sido amplamente alcançado utilizando misturas de fungicidas sistêmicos associados ao multissítio mancozebe. Este trabalho objetivou determinar o efeito da interação entre fungicidas IQo+IDM com mancozebe. No ensaio em casa de vegetação foram utilizadas as doses comerciais recomendadas para a cultura da soja dos produtos piraclostrobina + epoxiconazol, trifloxistrobina + protioconazol e mancozebe, avaliado o desempenho de controle com base na Área Abaixo da Curva de Progresso da Doença (AACPD) e determinado a interação entre os fungicidas. No ensaio a campo foi estudado apenas a associação de piraclostrobina + epoxiconazol com mancozebe sendo desenhadas curvas de dose-resposta dos produtos isolados e em associação, e definido o efeito das interações. Com base nos testes em casa de vegetação obteve-se que mancozebe associado à piraclostrobina + epoxiconazol promoveu um efeito sinérgico e quando associado a trifloxistrobina + protioconazol um efeito aditivo. Já nos estudos de campo, mostrou que as dose que provoca 50% do efeito total possível na variável resposta analisada (ED50) das curvas de dose-resposta foram significativamente reduzidas em função da interação entre os fungicidas quando comparada a ED_{50} dos fungicidas isolados. Ficou evidente a contribuição do mancozebe associado ao fungicida piraclostrobina + epoxiconazol apresentando um efeito sinérgico com base no isobolograma das ED_{50}.

Palavras-chave: *Pachyrhizi pachyrhizi*, *Glycine max*, sinergismo, curvas de dose-resposta

Effect of the interaction between fungicides QoI + DMI with mancozeb in the soybean rust control

Abstract

The efficient control of soybean rust has been widely achieved using mixtures of systemic fungicides in mixture with multi-site mancozeb. This work aimed to determine the effect of the interaction among fungicides QoI + DMI and mancozeb on soybean rust control. In the greenhouse, the dose recommended by the manufacturer of the products pyraclostrobin + epoxiconazole, trifloxystrobin + prothioconazole and mancozeb were used, and control performance was assessment based on the area under the Disease Progression Curve (AUDPC) and determined the interaction between fungicides. In the field trial, only the combination of pyraclostrobin + epoxiconazole with mancozeb was studied, and dose-response curves of the isolated and associated products were designed and defined the resulting interaction. Based on greenhouse tests, it was observed that mancozeb associated with pyraclostrobin + epoxiconazole had a synergic effect and when associated with trifloxystrobin + prothioconazole there was an additive effect. Already in the field tests, it was shown that the ED_{50} of the dose-response curves were significantly reduced as a function of the interaction between the fungicides when compared to the ED_{50} of fungicides isolated. It was evident the contribution of mancozeb associated with the fungicide pyraclostrobin + epoxiconazole presenting a synergic effect based on the isobologram of ED_{50}.

Key words: *Phakopsora pachyrhizi*, *Glycine max*, synergism, dose-response curves

1. Introdução

Fungicidas do grupo químico das estrobilurinas inibidores da quinona oxidase (IQo) e dos triazóis inibidores da desmetilação (IDM) atuam em apenas um sítio específico entre milhares de processos bioquímicas na célula fúngica. Portanto, eles são mais vulneráveis a redução ou perda de sensibilidade pela seleção de isolados resistentes do patógeno. Tais fungicidas IDMIs e IQos são classificados como de alto risco para o desenvolvimento da resistência e, portanto, não são recomendados para utilização isoladamente (FRAC, 2012). A dificuldade em controlar a ferrugem asiática da soja (FAS) com fungicidas está se tornando cada vez mais evidente, comprovada a alta capacidade de adaptação e variabilidade do fungo (Schmitz et al., 2014). Como exemplo, a eficiência de tebuconazol foi gradualmente reduzida de 90% para 24% em dez safras de cultivo de soja no Brasil (Godoy et al., 2013).

O uso de produtos multi-sítio é uma estratégia importante que pode contribuir para reverter a diminuição da sensibilidade a fungicidas IDMs e IQos. Fungicidas com maior espectro de ação, como mancozebe, podem ser aliados no controle da ferrugem da soja (Gullino et al., 2010). São encontrados relatos do uso desse ativo para aumentar o espectro de ação de programas de controle contendo fungicidas sítio-específico (Godoy et al., 2016). Além disso, o uso de mancozebe constitui estratégia importante no manejo de raças do patógeno com eventos de adaptação conferindo menor sensibilidade aos fungicidas IQo e IDM. A eficácia de mancozebe já tinha sido relatada antigamente no controle de FAS (Torres; Quebral, 1976). No entanto, em função de formulações inadequadas eram necessárias aplicações sequenciais semanais (Torres; Quebral, 1976; Sangawongse et al., 1977; El-Gantiry et al., 1990). Recentemente a ação fungicida de mancozebe no controle de FAS tem sido amplamente revisada e novas formulações (ex. WG) estão disponíveis no mercado propiciando associações com outros fungicidas de maneira mais segura.

A associação de dois ou mais fungicidas para o controle de doenças em plantas tem sido prática bastante usual. A partir dessas interações são esperados efeitos, sendo a aditividade, o sinergismo e o antagonismo os mais aceitos e descritos. Dentre as metodologias para descrever tais efeitos, o uso de curvas de dose-resposta e isobologramas tem permitido definir eficientemente o tipo de interação (Streibig et al., 1998; Streibig et al., 1999). As curvas de dose-resposta consistem em descrever o controle da doença em relação ao aumento crescente das doses de um fungicida isolado ou em associação, seguindo um fator constante de diluição, de forma que se obtenham doses equidistantes em escala logarítmica. Normalmente obtém-se uma curva simétrica em formato sigmoidal, que pode ser ajustada pelo modelo logístico. Deste ajuste obtém-se a estimativa da dose que provoca 50% do efeito total possível na variável resposta analisada (ED_{50}) (Seefeldt et al., 1995; Blackshaw et al., 1996).

A representação gráfica das ED_{50} das curvas dos produtos isolados e em associação é chamada de isobolograma. O isobolograma foi adaptado à área de interesse da agronomia inicialmente por Tammes (1964). Segundo este autor, no isobolograma, os ED_{50} oriundos da aplicação isolada de cada um dos produtos são unidos, gerando a isobole de aditividade ou linha teórica de aditividade. Os demais ED_{50} obtidos da associação em diferentes proporções dos produtos podem então ser analisados em relação a sua posição frente à isobole de aditividade. Se estes pontos posicionarem-se em torno da isobole de aditividade, a ação é de aditividade, se posicionar-se abaixo, a ação é de sinergismo e se posicionarem-se acima, a ação é de antagonismo (Kruse et al., 2006).

As metodologias acima descritas podem ser de grande utilidade na definição do efeito da interação resultante da associação de fungicidas. Entre os diferentes efeitos, sem dúvida a existência de sinergismo é vantajosa, porque amplia o espectro de ação dos fungicidas, contribuindo na prevenção do surgimento de resistência e aumenta-se a eficiência de controle

(Wrubel; Gressel, 1994). Nesse sentido, acredita-se que a associação de mancozebe a misturas comerciais de fungicidas sistêmicos possa resultar em sinergismo, isso porque pelo seu mecanismo de ação em múltiplos sítios possa combater possíveis isolados não sensíveis aos IQo e IDM.

Dessa forma, o objetivo desse trabalho foi avaliar o resultado da interação entre fungicidas IQo + IDM com mancozebe no controle da ferrugem da soja.

2. Material e métodos

2.1 Experimento I

O experimento I foi realizado em casa de vegetação, localizada na Estação Experimental do Instituto Phytus, em Itaara, RS, Brasil. Sementes da cultivar de soja DM 6563 RR Ipro foram semeadas em vasos com capacidade de 5 L, utilizando substrato a base de solo + casca de arroz (3:2). Foi utilizada uma adubação de base de 250 kg ha^{-1} (4-23-18 NPK) e pré-inoculação das sementes de soja com estirpes de bactérias do gênero *Bradyrhizobium* spp. A casa-de-vegetação continha condições parcialmente controladas através de exaustores e nebulizadores. Temperaturas na faixa de 30±4 e umidades na faixa de 70±10 eram mantidas durante o período diurno. A semeadura foi realizada em 15/12/2014 e a emergência das plântulas ocorreu seis dias após, sendo mantidas duas plantas por vaso. Aplicações de clorfenapir (Pirate®, BASF) na dose de 240 g i.a. ha^{-1} foram utilizadas para controle de ácaros.

O delineamento experimental utilizado foi inteiramente casualizado, em esquema bifatorial (3x2) totalizando seis tratamentos e quatro repetições. O fator A foi composto por fungicidas QoI + DMI, sendo eles: 1. sem fungicida (Controle); 2. piraclostrobina + epoxiconazol; e 3. trifloxistrobina + protioconazol. O fator B foi composto pela presença

(+Mz) ou ausência (-Mz) da associação com mancozebe. A aplicação dos tratamentos foi realizada de maneira preventiva em plantas sadias aos 30 dias após a emergência, no estágio fenológico V_5 (Fehr; Caviness, 1977). Os dois últimos trifólios totalmente expandidos (V_3 e V_4) foram marcados e totalmente expostos no plano horizontal utilizando um suporte plástico, para garantir um padrão homogêneo de deposição de gotas. Piraclostrobina + epoxiconazol (Opera®, BASF) foi utilizado na dose de 66.5 + 25 g i.a. ha^{-1}, respectivamente, com adição de óleo mineral Assist® (0.5% v/v). Trifloxistrobina + protioconazol (Fox®, Bayer Crop Science) utilizado na dose de 60 + 70 g i.a. ha^{-1}, respectivamente, com adição de óleo mineral Aureo® (0.25% v/v). Mancozebe (Unizeb Gold, UPL) foi utilizado na dose de 1125 g i.a. ha^{-1}, com adição de óleo mineral Aureo® (0.25% v/v) quando utilizado isolado.

Os tratamentos foram aplicados utilizando pulverizador pressurizado a CO_2, com barra provida de quatro pontas de pulverização (*Teejet* XR 11002) reguladas a uma pressão de 30 Psi, velocidade de caminhamento de 1.5 m s^{-1} gerando uma vazão de 150 L ha^{-1}. O processo de inoculação foi realizado 24 h após a aplicação dos tratamentos com deposição de inóculo por toda a planta em ambas as faces dos trifólios durante o período noturno seguindo metodologia utilizada por Lenz et al. (2011). Doze horas após a inoculação iniciaram-se nebulizações sequenciais a fim de proporcionar molhamento foliar, indispensável a infecção do patógeno.

Amostras de esporos de *P. pachyrhizi* foram coletadas das folhas das plantas controle sem fungicida para avaliação do efeito fungicida de mancozebe sobre esporos de *P. pachyrhizi* através de teste *in vitro*. Os esporos foram coletados por sucção a vácuo das folhas e uma solução de esporos foi preparada em água destilada e Tween 20 (0,1 mL L^{-1}). Para a quantificação do percentual de esporos germinados, 1 mL de suspensão de esporos foi colocada em placas de Petri (9 cm de diâmetro), contendo ágar-água e o fungicida diluído no meio nas concentrações de 0.0, 12.5, 25, 50 e 100 ppm. Após as placas foram incubadas em

BOD a 23±2ºC no escuro e a contagem de esporos germinados realizada seis horas após. Foram montadas duas placas por concentração, sendo contados 100 esporos ao acaso por ponto, 2 pontos por placa, utilizando microscópio ótico (aumento de 10X). Os esporos foram considerados germinados quando o comprimento do tubo germinativo ultrapassou o diâmetro do esporo.

Nas plantas, inicialmente foi determinado o número de dias para aparecimento das primeiras pústulas (NDAPP) através de avaliações diárias nos trifólios marcados três dias após a inoculação. O NDAPP na testemunha indica o período latente necessário para o processo de infecção e início da reprodução. A diferença de dias entre o NDAPP nos tratamentos em relação a testemunha indica o numero de dias de residual efetivo. Avaliações de severidade da doença foram realizadas aos 7, 14, 21 e 28 dias após o aparecimento das primeiras pústulas na testemunha (período latente). A avaliação de severidade considerou percentual de tecido lesionado pelo patógeno, atribuindo-se notas visuais com auxílio de uma escala diagramática (Godoy et al., 2006). A partir dos dados de severidade foi calculada a área abaixo da curva de progresso da doença (AACPD) segundo Campbell e Madden (1990) e a eficiência de controle dos tratamentos com base na fórmula de Abbot (1925). O efeito da interação entre os fungicidas foi calculado segundo metodologia proposta por Colby (1969) Esse método calcula o controle esperado da associação, o qual em comparação com o controle observado possibilita fazer inferências sobre o tipo de interação. O controle Esperado (E) para a combinação entre dois fungicidas pode ser calculado em acordo com Gowing (1960), da seguinte maneira:

$$E = X + \frac{[Y\ (100-X)]}{100}$$

Onde:

X = percentual de controle do fungicida X isolado (QoI + DMI)

Y = percentual de controle do fungicida Y isolado (Mz)

Quando o controle observado é maior do que o esperado, a combinação é sinérgica, quando observado menor do que o esperado, é antagônico e quando observado e esperado são iguais, a combinação é aditiva.

A significância do efeito dos tratamentos foi determinada pela análise de variância (ANOVA), por meio do programa estatístico Assistat 7.5 Beta. As médias foram comparadas pelo teste de Tukey (P<0.05%).

2.2 Experimento II

O experimento II foi realizado a campo em áreas da estação experimental do Instituto Phytus, em Itaara, RS, Brasil. Foi utilizado a cultivar de soja DM 6563 RSF IPRO cultivada através do sistema de semeadura direta com semeadora mecanizada, em espaçamento de 0.5 m entre linhas, sendo estabelecida uma densidade populacional de 14 plantas m^{-2} e 280.000 plantas ha^{-1}. Foi utilizada adubação de base de 250 kg ha^{-1} NPK (fórmula 4-23-18). O manejo de plantas daninhas e insetos-praga foram realizados de acordo com as recomendações técnicas para a cultura (Indicações, 2014).

O experimento considerou apenas a interação entre os fungicidas mancozebe com piraclostrobina + epoxiconazol (Pirac+epoxi) em função desse ultimo apresentar menor eficácia de controle. As combinações necessárias para a obtenção das curvas de dose resposta totalizaram 27 tratamentos, em quatro repetições, seguindo metodologia utilizada por Kruse et al. (2006). Foram utilizadas seis doses do produto comercial Opera® (BASF) contendo piraclostrobina + epoxiconazol e seis doses do produto comercial Unizeb Gold® (UPL) contendo mancozebe. Para Pirac+epoxi empregou-se as doses de 0, 125, 250, 500, 1000 e 2000 mL ha^{-1}. Para o mancozebe empregou-se as doses de 0, 375, 750, 1500, 3000 e 6000 g ha^{-1}. Para estabelecer curvas de resposta do efeito da associação, as doses de 375 e 750 g ha^{-1} de mancozebe foram associadas com todas as doses de Pirac+epoxi. Da mesma forma, as

doses de 125 e 250 mL ha^{-1} de Pirac+epoxi foram associadas com todas doses de mancozebe. Tais doses foram utilizadas a fim de gerar patamares de controle mínimo (doses pequenas) e patamares de controle máximo (doses maiores) e assim criar um intervalo de alta resposta a alteração de doses (Kruse et al., 2006).

O delineamento experimental foi inteiramente casualizado com quatro repetições. As unidades experimentais foram compostas por 5 linhas de semeadura (2.5 m) e 5 m de comprimento, totalizando 12.5 m^2 de área total. Inicialmente aos 35 dias após emergência no estágio V_7 da cultura foi realizado uma aplicação preventiva padrão em todo o ensaio utilizando o fungicida azoxistrobina + ciproconazol [(Priori Xtra, Syngenta) na dose de 60 + 24 g i.a. ha^{-1}]. Após isso foram realizadas três aplicações dos tratamentos anteriormente descritos nesse estudo, sendo a segunda no estágio R_2 (floração plena), a terceira 15 dias após a segunda e a quarta e última aplicação 13 dias após a terceira. Os tratamentos foram aplicados utilizando pulverizador pressurizado a CO_2, com barra provida de cinco pontas de pulverização (*Teejet* XR 11002) reguladas a uma pressão de 30 Psi, velocidade de caminhamento de 1.5 m s^{-1} gerando uma vazão de 150 L ha^{-1}.

A partir do aparecimento de doença no experimento que ocorreu entre a segunda e terceira aplicação, iniciaram-se avaliações quinzenais de severidade considerando o percentual de tecido lesionado pelo patógeno, atribuindo-se notas visuais com auxílio de escala diagramática (Godoy et al., 2006). A partir dos dados de severidade foi calculada a área abaixo da curva de progresso da doença (AACPD) segundo Campbell e Madden (1990).

Os dados obtidos para cada variável foram organizados de maneira a formar três grupos de tratamentos ou curvas de resposta às doses para cada fungicida. Uma curva foi formada pelas seis doses de Pirac+epoxi aplicado isoladamente, outra com as mesmas seis doses associadas com 375 g ha^{-1} de mancozebe e a terceira com as seis doses associadas com 750 g ha^{-1} de mancozebe. Para o mancozebe uma curva com seis doses do produto isolado,

outra com as seis doses em associação com 125 mL ha^{-1} de Pirac+epoxi e a terceira com as seis doses em associação com 250 mL ha^{-1} de Pirac+epoxi. Cada grupo de tratamentos assim organizado sofreu análise da variância e após, regressão não linear, ajustando-se o modelo log-logístico com quatro parâmetros utilizando o pacote estatístico R (Ritz et al., 2015; Ritz; Streibig, 2005). Com o ajuste deste modelo obteve-se para cada curva o valor de ED_{50}, em um total de seis valores para cada variável. Estas doses de ED_{50} foram representadas graficamente, formando um isobologramas com Pirac+epoxi *versus* mancozebe (Tammes, 1964). Os ED_{50} de cada fungicida aplicado isoladamente foram unidas, gerando a isobole de aditividade. Os quatro valores de ED_{50} resultantes das curvas com associação de Pirac+epoxi e mancozebe foram também plotados no gráfico e a partir da posição em relação à isobole de aditividade é possível inferirmos quanto ao efeito da interação entre os fungicidas.

3. Resultados e Discussão

3.1 Experimento I

Inicialmente ficou evidente o efeito fungicida de mancozebe sobre o processo de germinação de esporos de *P. pachyrhizi* (Figura 1). Dentre as concentrações de Mz testadas *in vitro*, todas causaram inibição de 100% da germinação dos esporos não sendo possível calcular a ED_{50}. Através deste teste ficou evidente o efeito de Mz sob esse processo da patogênese de *P. pachyrhizi*. Quanto aos testes de eficácia na planta, os dados elucidam efeito significativo da interação entre os fatores fungicidas IQo+IDM e –Mz ou +Mz para as variáveis analisadas. O efeito fungicida de Mz frente a *P. pachyrhizi* ficou evidenciado através do efeito residual de controle de 5.4 dias em média quando aplicado isoladamente (Figura 2). Para os fungicidas sistêmicos, Pirac+epoxi apresentou um residual similar a mancozebe isolado próximo a cinco dias e Trifl+proti mostrou-se altamente eficaz com

residual superior a 20 dias. Tais efeitos sobre o residual de controle definem influências diretas no momento de início das infecções e refletem diretamente sobre a severidade da doença e os dados de AACPD (Figura 3).

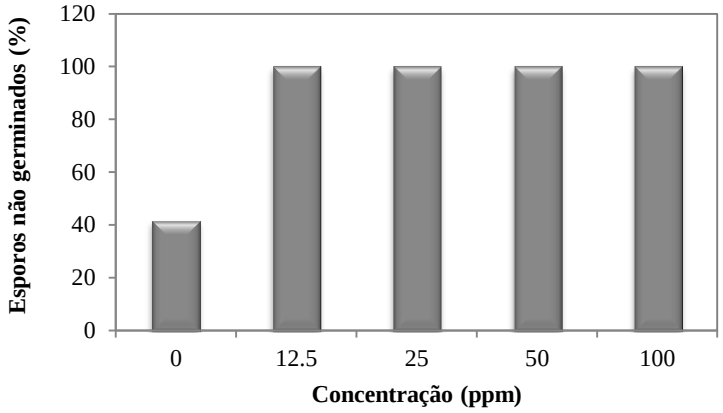

Figura 1. Eficácia de diferentes concentrações de mancozebe sobre a germinação de esporos de *P. pachyrhizi* em ensaio *in vitro*.

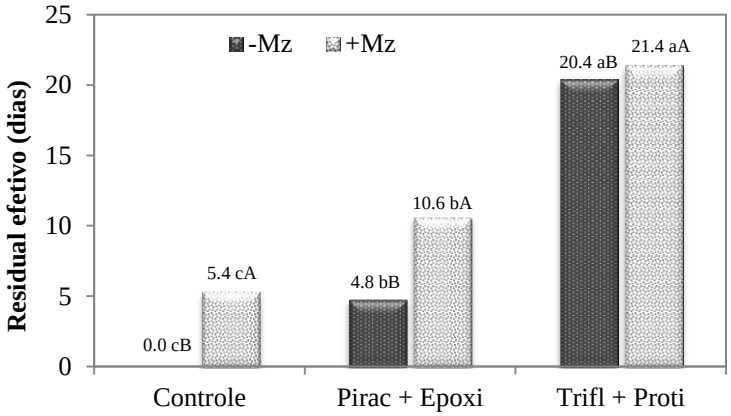

Figura 2. Efeito residual em dias dos tratamentos fungicidas aplicados no controle de P. pachyrhizi em casa de vegetação. Médias seguidas pela mesma letra não diferem pelo teste de Tukey ($p<0.05$). Letras minúsculas comparam os fungicidas IQo+IDM; letras maiúsculas comparam –Mz com +Mz.

Todos os fungicidas tiveram efeito significativo na redução da AACPD da doença. Mancozebe isolado teve uma eficácia de controle de 54% comparado a 41% do fungicida Pirac+epoxi. Já Trifl+proti teve uma alta eficácia de controle próximo a 99% nas condições

desse ensaio. Interessante de considerar que nesse estudo, os trifólios foram expostos a pulverização, ou seja, foi obtida uma cobertura completa e homogênea do trifólio para todos os tratamentos. Nesse caso, tem-se que tais eficácias observadas são desempenhos potenciais dos produtos, as quais em condições de campo são menores, em função de dificuldades em atingir o alvo.

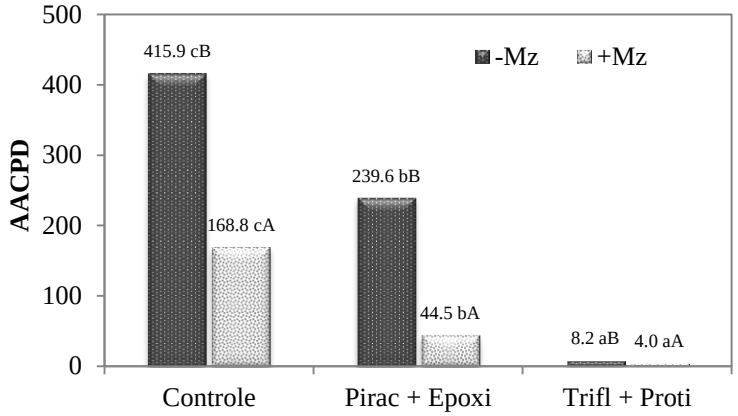

Figura 3. Área abaixo da curva de progresso da ferrugem da soja em função dos tratamentos fungicidas em experimento em casa de vegetação. Médias seguidas pela mesma letra não diferem pelo teste de Tukey ($p<0.05$). Letras minúsculas comparam os fungicidas IQo+IDM; letras maiúsculas comparam –Mz com +Mz.

A associação de mancozebe (+Mz) aos fungicidas sistêmicos IQo+IDM foi positivo no controle da doença, porém com variação em relação ao fungicida acompanhante (Figura 2 e 3). Quando mancozebe foi associado à Pirac+epoxi foi notado um incremento expressivo no residual de controle e redução da AACPD. A mistura dos dois fungicidas (Pirac+epoxi + Mz) proporcionou eficácia de controle de 83.3% comparado a 41% de Pirac+epoxi isolado. Tais incrementos também ocorreram na associação de mancozebe com Trifl+proti, porém em função de Trifl+proti apresentar alta eficácia isoladamente, os incrementos se tonam menos expressivos. Notou-se um ganho médio de aproximadamente 1 dia de residual e a eficácia da

mistura foi de 99% comparado com 98% do fungicida Trifl+proti isolado. Em termos práticos isso tem pouca expressividade.

A análise do efeito da interação entre fungicidas segundo metodologia proposta por Colby (1967) ajuda a descrever o que foi discutido anteriormente (Tabela 1). Nota-se que a associação do fungicida Pirac+epoxi ao mancozebe resultou em uma interação sinérgica, ou seja, a eficácia observada foi maior que a esperada. Por outro lado, a interação de Trifl+proti com mancozebe evidencia um efeito aditivo, ou seja, a eficácia observada foi igual a esperada. Sugere-se dessa forma que mancozebe não atrapalha o desempenho do fungicida em mistura e que pelo menos um efeito aditivo é esperado. Já, quando associado a fungicidas menos eficazes ficou evidente que os incrementos foram maiores e um efeito sinérgico é esperado. Quando o percentual de controle dos fungicidas aplicados isoladamente é elevado (>70%) fica comprometida a determinação dos fatores de sinergia entre eles (Gisi et al., 1985). Lindner et al. (1994) afirmam que a interação entre os fungicidas pode ser melhor comparada quando a as eficácias individuais não são muito altas e que quando um ou ambos são superiores a 70%, a análise pode não definir eficientemente o efeito sinérgico.

Tabela 1. Resultado da interação entre fungicidas segundo metodologia proposta por Colby (1967).

Colby (1967)	**Pirac+Epox + Mz**	**Trifl+Proti + Mz**
Eficácia Esperada (Esp%)	76.6 ^{*b}	99.0 ^{ns}
Eficácia Observada (Obs%)	88.0 ^{a}	99.2
Obs% / Esp%[1]	1.16	1.00
CV%	1.32	0.61

* Significativo pelo teste de médias de Tukey ($p<0.05$). ^{ns} não significativo. [1] Relação Obs/Esp > 1 (sinérgico); Obs/Esp = 1 (aditivo); e Obs/Esp < 1 (antagônico). CV% - coeficiente de variação.

3.2 Experimento II

As curvas resposta para AACPD da ferrugem da soja às doses de Pirac+epoxi aplicado isoladamente e em associação com 0.375 e 0.750 kg ha^{-1} de Mz encontram-se na Figura 4A.

As curvas obtidas nessas três combinações se ajustaram adequadamente ao modelo log-logístico. Os três ED_{50} obtidos pelo ajuste do modelo, de 0.59, 0.34 e 0.24 L ha^{-1}, respectivamente para Pirac+epoxi isolado, Pirac+epoxi + 0.375 de Mz e Pirac+epoxi + 0.750 de Mz, demonstram claramente a significativa contribuição proporcionada pela associação das duas doses de Mz às doses de Pirac+epoxi. Pequenas doses de Mz incrementaram o desempenho de controle associado ao fungicida sistêmico.

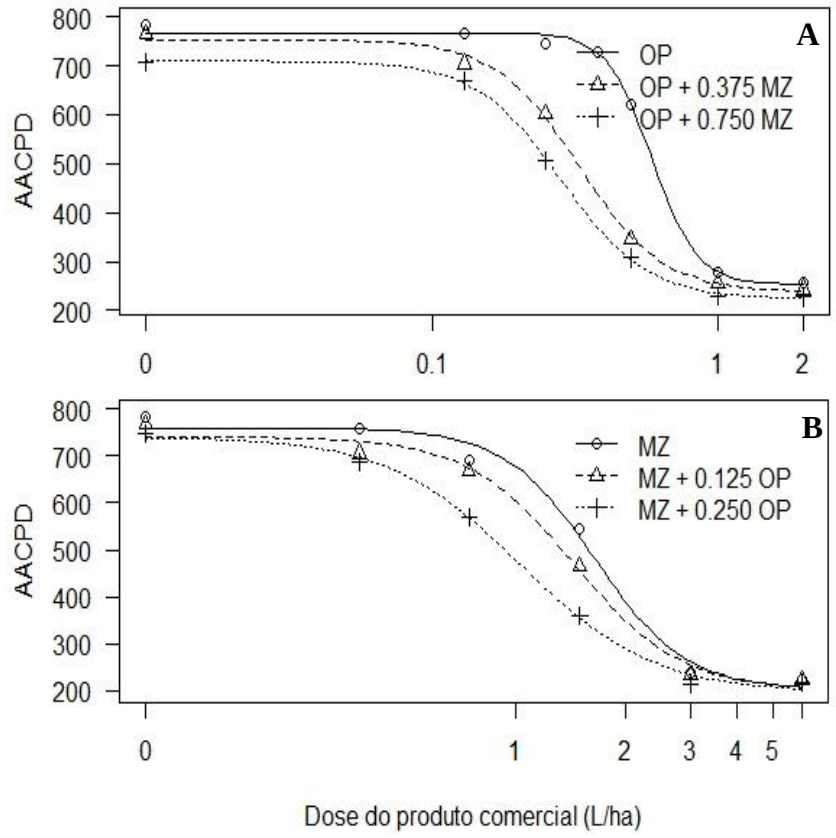

Figura 4. Curvas de dose-resposta para variável AACPD da ferrugem da soja às doses dos fungicidas Pirac+epoxi – OP (A) e mancozebe (B) aplicados isoladamente e em associação.

Da mesma forma, as curvas de resposta para AACPD às doses de Mz aplicado isolado e em associação com 0.125 e 0.250 de Pirac+epoxi (Figura 4B) também se ajustaram ao modelo log-logístico. Os ED_{50} obtidos pelo ajuste do modelo aos resultados foram, de 1.64, 1.44 e 1.03 kg ha^{-1}, respectivamente para Mz isolado, Mz + 0.125 de Pirac+epoxi e Mz +

0.250 de Pirac+epoxi também refletem a contribuição da associação das duas doses de Pirac+epoxi ao desempenho do Mz.

A observação das curvas deixa evidente que houve resposta ao aumento de dose para ambos os fungicidas. No entanto a resposta é expressiva em uma faixa intermediária de dose e tende a estabilizar. Nessa mesma faixa intermediária é que se notam também os maiores incrementos no controle em função da associação entre os fungicidas. Comparando os pontos das curvas nos patamares de maior AACPD (menores doses) e menor AACPD (maiores doses) nota-se que as curvas tendem a estabilizar e os incrementos pela associação com o outro fungicida são menores comparados a região intermediária da curva. Justifica-se assim o porquê de se trabalhar com as ED_{50} das curvas no estudo das respostas de interação, em função de ser essa faixa a mais sensível e responsiva ao aumento de dose.

A distância entre as curvas na região intermediária evidencia que a contribuição das doses de Mz a curva do OP foi maior comparada à contribuição das doses de OP na curva do Mz. Tal fato também pode ser visualizado no teste de comparação entre as curvas mostradas na Tabela 2. Em todas as associações dos fungicidas em comparação a curva deles isolados houve uma redução da ED_{50} (dose necessária para provocar 50% do efeito avaliado). Tais diferenças foram significativas ao nível de 5% de probabilidade de erro ($p>0.05$). Os valores da estimativa contidos na Tabela são expressos em potência relativa, ou seja, a razão entre o ED_{50} da curva do produto isolado com o ED_{50} dele associado. Nesse caso, pode-se perceber que seria necessário um aumento de 80% na dose de Pirac+epoxi para gerar o mesmo efeito que Pirac+epoxi+0.375Mz e de 107% a mais de Pirac+epoxi para produzir o mesmo controle que Pirac+epoxi+0.750Mz. Isso mostra que a contribuição do Mz foi significativa e que compensa mais associar este produto ao Pirac+epoxi do que somente aumentar a dose de Pirac+epoxi.

Tabela 2. Análise da relação entre as curvas de dose-resposta usando a ferramenta SI do pacote *drc* do software R.

Comparação das curvas	Estimativa	Erro padrão	p-valor (5%)
OP isolado e associado ao Mz			
OP[1] isolado / OP + 0.375 Mz[2]	1.80	0.07	0.000
OP isolado / OP + 0.750 Mz	2.07	0.08	0.000
OP+0.375Mz / OP+0.750Mz	1.15	0.04	0.001
Mz isolado e associado ao OP			
Mz isolado / Mz + 0.125 OP	1.14	0.05	0.012
Mz isolado / Mz + 0.250 OP	1.59	0.09	0.000
Mz+0.125OP / Mz+0.250OP	1.39	0.08	0.000

[1] OP – Pirac+epoxi; [2] Mz - mancozebe

Da mesma forma, verificamos que a contribuição da associação de Pirac+epoxi na curva de Mz foi menor. A estimativa mostra que é necessário aumentar apenas 14% a dose de Mz para gerar o mesmo efeito que Mz + 0.125 Pirac+epoxi e de 59% a mais de Mz para gerar o mesmo efeito que Mz + 0.250 Pirac+epoxi. Isso ajuda a evidenciar o grande desgaste da molécula Pirac+epoxi no controle de *P. pachyrhizi* e o grande impacto da redução de doses desse produto comparado à redução de dose do fungicida Mz. Isso coincide com os dois cenários distintos desses produtos quanto à sensibilidade do patógeno, em que Mz por ser um produto multissítio para o qual não há eventos de adaptação do patógeno, foi menos prejudicado pela redução de dose comparada ao Pirac+epoxi e mostrou incrementos significativos no controle. O cenário se inverte para Pirac+epoxi, pois existem estudos comprovando a existência de isolados com adaptações que conferem menor sensibilidade para os grupos químicos IQo e IDM (Schmitz et al., 2014; Klosowski et al., 2015) e isso ajuda a explicar os menores percentuais de incremento quando associado ao Mz.

O isobolograma formado para a variável AACP da ferrugem da soja com a isobole de aditividade, traçada entre os ED_{50} de Pirac+epoxi e Mz aplicados isoladamente são apresentados na Figura 5. Os ED_{50} das curvas das doses de Pirac+epoxi associado ao Mz se encontram abaixo da isobole, estando na região considerada sinérgica. Isso vai ao encontro

com o que já foi discutido anteriormente na interpretação das curvas. A plotagem das ED_{50} em relação à isobole ajuda a confirmar que o aumento de dose do fungicida Pirac+epoxi reflete em ganho de controle, porém se a quantidade de Pirac+epoxi aumentada for substituída pela quantidade equivalente de Mz, o ganho no controle é muito maior, sendo esse o cenário da interação sinérgica.

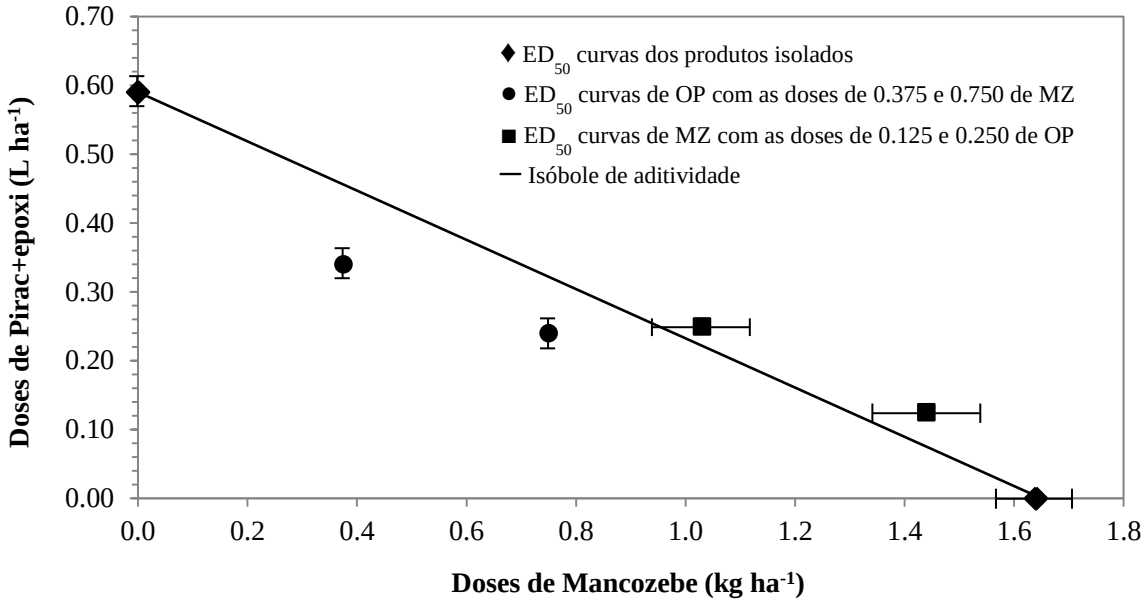

Figura 5. Isobolograma da variável AACPD com os valores da dose necessária para obter 50% de controle efetivo do fungo (ED_{50}), obtidos de curvas de dose-resposta dos fungicidas Pirac+epoxi (OP) e mancozebe (Mz), aplicados isolados e em associação. Pontos indicam ED_{50} das curvas ± erro padrão.

Por outro lado, os ED_{50} das curvas das doses de Mz associado ao Pirac+epoxi ficaram plotadas tocando a isobole de aditividade, considerando o erro padrão, com tendência a estarem acima dessa linha. O efeito dessa interação indica aditividade (pontos localizados sobre a isobole) ou antagonismo (pontos acima da isobole). Dessa forma, sugere-se que ao aumentarmos a dose de Mz a resposta de controle é maior do que acrescentar Pirac+epoxi em doses equivalentes. Em um raciocínio mais aplicado a prática, sugere-se que ao utilizar Pirac+epoxi na dose recomendada, a associação de Mz se torna indispensável e o aumento de

dose do próprio Pirac+epoxi agrega menos do que o que será incrementado pela associação com Mz. Por outro lado, se pensarmos no Mz como fungicida principal utilizado na dose comercial recomendada, seria melhor aumentar a dose dele próprio do que acrescentar quantidades equivalentes de Pirac+epoxi. Vale ressaltar que do ponto de vista técnico Mz não tem sido recomendado em aplicações isolado e sim em mistura com fungicidas sistêmicos.

4. Conclusão

Mancozebe isolado apresentou significativo efeito fungicida. A associação de mancozebe a mistura comercial de piraclostrobina + epoxiconazol incrementa o residual e o controle da ferrugem da soja. Quando mancozebe é associado a fungicidas menos eficientes espera-se um incremento de controle mais expressivo e uma interação sinérgica é esperada. A associação de mancozebe a fungicidas IQo+IDM menos efetivos compensa mais do que aumentar a dose do IQo+IDM. Quando mancozebe é associado à fungicidas com maior eficácia de controle, como nesse trabalho trifloxistrobina + protioconazol, os incrementos no controle da doença são menores, porém espera-se no mínimo uma interação aditiva.

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Manuscript II (Submitted to Crop Protection)

Mancozeb relieves trifloxystrobin + prothioconazole – induced oxidative stress in soybean

Abstract

Trifloxystrobin + prothioconazole and mancozeb are extensively used in Brazil for soybean diseases control. Phytotoxicity symptoms have been related in plants exposed to trifloxystrobin + prothioconazole and the mancozeb association seems to reduce these symptoms. Phytotoxicity in plants can cause unwanted physiological changes and needs to be investigated. Thus, this study aimed to investigate physiological responses of soybean plants exposed to trifloxystrobin + prothioconazole and mancozeb. Doses recommended by the manufacturer were used. The assessments were focused on the antioxidative enzymes activity superoxide dismutase (SOD) and peroxidase (POX), the accumulation of H_2O_2, the lipid peroxidation and photosynthetic pigments concentration. The results revealed that the systemic fungicide trifloxystrobin + prothioconazole induced oxidative stress on plants verified by increased on SOD and POX activity, in H_2O_2 content, lipid peroxidation and decrease in photosynthetic pigments. Plants exposed to systemic fungicide presented symptoms on leaves confirming the damage occurrence. Mancozeb singly not induce significantly physiological changes on soybean in comparison to control plants. Furthermore, when associated with systemic fungicide played an important role relieving the damages. This effect was not linked to an increase in antioxidant capacity. Mancozeb appears to provide prior protection reducing the formation of reactive oxygen species. It is suggested that changes in the uptake rate or in metabolism of the systemic fungicide in the plant might be linked with this effect.

Keywords: Fungicide, phytotoxicity, lipid peroxidation, superoxide dismutase.

1. Introduction

The soybean [*Glycine max* (L.) Merr] have shown to be the main crop in Brazil with 102.4 million tons of grain harvested in an area of 33.9 million hectares at 2015/2016 season crop (Conab, 2016). Disease incidence continues to be a barrier to grain production due to favorable weather conditions. Asian soybean rust (*ASR*), caused by the fungus *Phakopsora pachyrhizi*, is the most severe disease of the crop and can cause yield losses of up to 90% (Godoy et al., 2016a). The disease management has obtained mainly by chemical control, but a lower sensibility of the fungus to fungicides has been reported in Brazil (Schmitz et al., 2014; Klosowski et al., 2015; Godoy et al., 2016a).

There are not many fungicide options with high efficacy for *ASR* management in Brazil. Trifloxystrobin + prothioconazole (T+P) are a commercial mixture widely used because has been shown a good *ASR* control efficacy (Godoy et al., 2016b). More than one application of this fungicide per crop cycle is commonly performed. Prothioconazole belongs to the class of triazolinthione fungicides acting in the De-Methylation Inhibition (DMI) (sterol biosynthesis inhibition) to kill the fungus (Dutzmann and Suty-Heinze, 2004). Trifloxystrobin is in the class of strobilurins fungicides, acting in the Quinone outside Inhibition (QoI) blocking electron transport in the mitochondrial respiration (Bartlett et al., 2002). Several cases of phytotoxicity in soybean induced by the fungicide T+P have recently been reported. However, the physiological response of soybean plants to exposure to this fungicide is unknown.

Since effective fungicides with a new mode of action are not readily available, old multi-site fungicides, e.g. mancozeb, which have low resistance risk, have been recently retested for *ASR* control in mixtures to increase control efficiency and reduce the risk of resistance (Godoy et al., 2016b). Mancozeb is included in the dithiocarbamates class of fungicides, one of the most extensively used non-systemic fungicides in the world with multi-

site mechanism of action (Gullino et al., 2010). There are some reports of mancozeb inducing phytotoxicity in some crops such as lettuce (Dias et al., 2014; Pereira et al., 2014) and *Cassia angustifolia* (Majid et al., 2014). However, in soybean, mancozeb have been used with a new formulation and the evidence indicates a beneficial physiological effect in this crop. Relieving the phytotoxicity of other fungicides is one of the most relevant assumptions.

Most of the work dealing with the impact of fungicides in agriculture is focused on their efficiency against fungal pathogens or their residual accumulation in crops (Dias, 2012; Petit et al., 2012). However, fungicides might induce oxidative stress and active antioxidants responses in plants, and this is less explored (Dias, 2012; Dias et al., 2014). The molecular oxygen (O_2) is a free radical involved in all aerobic organisms, as it has two impaired electrons that have the same spin quantum number. This spin restriction makes O_2 prefer to accept its electrons one at a time, leading to the generation of the so called Reactive Oxygen Species (ROS), which can damage the cells (Gill and Tuteja, 2010). The phytotoxicity reported by trifloxystrobin + prothioconazole in soybean might be involved with ROS accumulation and oxidative stress. ROS are also produced continuously as byproducts of various metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes (Del Rio et al., 2006; Navrot et al., 2007). Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (Foyer and Noctor, 2005). The equilibrium between ROS production and the antioxidant response can be perturbed by fungicides (Dias, 2012).

Some effects of fungicides altering the antioxidant response of plants have been well related to a protective role against various types of stress. Triazoles protect *Hordeum vulgare* and *Arachis hypogaea* against ozone exposure or salt stress by stimulating antioxidative enzymes (Wu and Tiedemann, 2002). Moreover, azoxystrobin and epoxiconazole induced a delay of senescence of *Triticum aestivum* mainly due to an enhancement of the antioxidative

potential protecting the plants from harmful ROS (Wu and Tiedemann, 2001). The potential protector was associated a crucial role of fungicides in activating H_2O_2-sensing transcription factors, on active oxygen species and antioxidant enzymes in which induce the antioxidant gene expression (Wu and Tiedemann, 2001). However, changes in antioxidant response of plants induced by fungicides are interest if they are not followed by cell damage. Thus, in the present study, were investigated physiological responses of soybean plants exposed to trifloxystrobin + prothioconazole and mancozeb with emphasis on the oxidative/antioxidative status and photosynthetic pigments contents.

2. Materials and methods

2.1 Plant growth and experimental design

Seeds of soybean cultivar BMX Ponta GM (Brasmax genetics) pretreated with *Bradyrhizobium* strains (Masterfix®, Stoller) at 0.2 L 100 kg^{-1} of seed were used. The experiment were carried out in greenhouse at Itaara city (29°35'8"S; 53°48'28"W), Rio Grande do Sul state, Brazil. Plants were grown in 5L pots filled with soil mixed with rice husk (3:1). It was carried out soil fertilization using 300 kg ha^{-1} of formula 8-28-18 (nitrogen, phosphorus and potassium, respectively). Two plants were grown in each pot, which were considered a sampling unit. The plants were kept disease-free, grown at 30±5 °C, with a 13/11h (day/night) photoperiod and 65±10% air humidity. Irrigations were performed daily. The experimental design consisted of completely randomized with four treatments and fifteen replicates (pots with two plants). The fungicides treatments were applied two times in different growth stages and was performed four leaf harvests after each spraying to laboratorial analyses.

2.2 Fungicides application

The treatments were: (1) Control – plants without fungicide and water spraying; (2) T+P - trifloxystrobin + prothioconazole [(Fox®, Bayer Crop Science) 60 + 70 g a.i. ha^{-1}] singly; (3) T+P + Mz - trifloxystrobin + prothioconazole [(Fox®, Bayer Crop Science) 60 + 70 g a.i. ha^{-1}] + mancozeb [(Unizeb Gold®, UPL) 1,125 g a.i. ha^{-1}] in mixture; and (4) Mz – mancozeb [(Unizeb Gold®, UPL) 1,125 g a.i. ha^{-1}] isolated. The solution was prepared on distilled water and was added mineral oil Aureo (Bayer) at 0.25% v/v. The solution was applied by using a compressed-air spraying system equipped with four nozzles (Teejet XR 110.02) on a movable bar, at controlled speed (1.5 m s^{-1}), pressure (30 psi) for to create a 150 L ha^{-1} application rate. The first application was carried out at stage 51/16 (Munger et al. 1997) and second application 14 days after the first.

2.3 Leaf blade sampling for biochemical assays

Samples were obtained at 12, 24, 48 e 96 hours after fungicide application. The sampling was repeated after second application. The samples consisted of third, fourth and fifth trefoil (down-up) of each plant and three replicates per treatment was used. The samples were collected, packed on aluminum foil, immediately frozen in liquid nitrogen and stored in ultra-freezer at -80 °C for later biochemical evaluations.

2.4 SOD and POX activities

Frozen leaf samples were used for enzyme analysis. One gram (1 g) of tissue was homogenized in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) including 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% Triton X-100. The homogenate was centrifuged at 13,000 rotations per minute (RPM) for 20 min at 4 ºC. Supernatant was used for enzyme activity and protein content assays (Zhu et al., 2004). Peroxidase (POX –

1.11.1.7) was measured according to Zeraik et al. (2008). The reaction mixture contained 1.0 mL potassium phosphate buffer [100 mM (pH 6.5), 1.0 mL of guaiacol (15 mM) and 1.0 mL de H_2O_2 (3 mM)]. After the homogenization this solution was added 50 uL of plant extract. The guaicol oxidation to tetraguaiacol was measured through of increase in the absorbance at 470 nm. The activity of superoxide dismutase (SOD - E.C.1.15.1.1) was assessed according to the spectrophotometric method described by Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme that inhibits nitroblue tetrazolium (NBT) by a photo reduction of 50% (Beauchamp and Fridovich, 1971).

2.5 Determination of hydrogen peroxide

The H_2O_2 contents of both control and treated seedlings were determined according to Loreto and Velikova (2001). Approximately 100 mg of fresh shoot samples were homogenized at 4 °C in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 RPM for 15 min and 0.5 mL of 10 mM KH_2PO_4/K_2HPO_4 (pH 7.0) and 1 mL of 1 M KI. The H_2O_2 content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The H_2O_2 content was expressed as µmol/g fresh weight.

2.6 Estimation of lipid peroxides

The degree of lipid peroxidation was estimated following the method of El-Moshaty et al. (1993). Fresh shoot samples of 1 g were homogenized in 4 mL of 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100, using mortar and pestle. The homogenate was filtered with two paper layers and centrifuged for 15 min at 20,000 RPM. One milliliter of the supernatant fraction was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) of thiobarbituric acid (TBA). The mixture was heated at 95ºC for 40 min and then quickly

cooled in an ice bath for 15 min, and centrifuged at 10,000 RPM for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value of the absorbance at 600 nm. The lipid peroxides were expressed as nmol MDA mg^{-1} protein, by using an extinction coefficient of 155 L $mmol^{-1}$ cm^{-1}.

2.7 Chlorophyll and carotenoid determination

Chlorophyll (a+b) and carotenoids were extracted following the method of Hiscox and Israeslstam (1979) and estimated with the Lichtenthaler's formulae (Lichtenthaler, 1987). Fresh leaves (0.05 g) were incubated at 65 ºC in dimethylsulfoxide (DMSO) until tissues were completely bleached. Absorbance of the solution was then measured at 663 and 645 nm for chlorophyll and 470 nm for carotenoids on a spectrophotometer. Chlorophyll and carotenoid concentrations were expressed as mg g^{-1} fresh weight (FW).

2.8 Phytotoxicity symptoms on leaves

At 3 days after first application (DAA), the fifth and sixth leaves of four replicates were individually digitalized at a resolution of 300 dpi, with the aid of a scanner. For each leaf, the proportion of phytotoxicity symptoms area was determined using the Quant® software (Vale et al., 2003). The data were show in percentage of leaf surface injured.

2.9 Statistical analysis

The statistical tests were conducted using the software PlotIT version 3.2 at 5% significance level. Quantitative changes of different parameters were analyzed through variance analysis (ANOVA), with Duncan's honestly significant difference multiple comparison test being used to determine significant differences among treatments.

3. Results

3.1 SOD and POX activities

The SOD activity data are shown in the Fig 1. In plants exposed to T+P fungicide singly had an increase in SOD activity in comparison to control in all the times of assessment after first application. At 48 and 96 h after spray the increase was the 51 and 47 % of control, respectively. This effect was maintained on the plants after the second application, with 65 and 39 % compared to control, at 48 and 96 h after spraying, respectively. However, when the T+P fungicide was associated with Mz (T+P+Mz) the activity of the SOD enzyme was lower compared to T+P singly. Furthermore, in plants exposed to Mz singly there were no significant changes in SOD activity compared to control plants, even after the second application.

Similar results can be verified for POX enzyme activity (Fig 2). Higher POX activity was observed in plants exposed to the T+P fungicide. This effect was maintained at all assessments times after the first and second applications. The fungicide Mz when associated to the fungicide T+P reflected in a reduction in POX activity compared to T+P. Mz isolated induced an increase in POX activity more pronounced after the second application.

3.2 Hydrogen peroxide levels

Significant changes in H_2O_2 concentration were only observed in plants exposed to T+P singly at 48 and 96 h after the first application (Table 1). Increases of 43 and 31% in relation to control plants were observed, 48 and 96 h after first application, respectively. The increase in H_2O_2 concentration in plants treated with the T + P + Mz was lower or non-existent compared to control plants. Based on H_2O_2 production, Mz reduced the phytotoxic effect of T+P on plants. In addition, isolated Mz did not cause increases in H_2O_2

concentration compared to control plants in any assessment. After the second application of the fungicide the data maintained the same trend. Higher levels of H_2O_2 were observed in plants exposed to T+P singly. At 96 h after the second application, a 20% increase in H_2O_2 was visualized by the T+P application and only 10% by the T+P+Mz.

3.3 Estimation of lipid peroxides

Changes in MDA concentration in the leaves started to be noted at 24 h after first application and have maintained in all assessments (Table 2). These changes indicated increase in the lipid peroxidation in plants exposed to T+P singly. Increases of up to 34 and 36% were observed 96 h after the first and second application, respectively, compared to control. In plants treated with T+P+Mz had a lower increase, 18 and 10 % were observed after first and second application, respectively. Mz singly did not reflect significant changes in MDA concentration in leaves compared to control plants.

3.4 Chlorophyll and carotenoid determination

In plants treated only with Mz, no significant change was observed in the concentration of total chlorophylls, chlorophyll *a* (Chl *a*) and carotenoids (Fig 3, 4 and 5). However, the concentration of chlorophylls and carotenoids were reduced in plants that received application of the fungicide T+P, with different magnitude in relation to the presence or absence of Mz. In plants exposed to T+P singly the reductions were more accentuated compared to plants exposed to T+P+Mz. Based on concentration of chlorophyll and carotenoids, Mz relieved the phytotoxic effects of the systemic fungicide.

Chl *a* was the most affected pigment on leaves by fungicide T+P (Fig 4). Severe reductions in Chl *a* concentration were seen 24 and 96 h after the first application and at all times of evaluation after the second application. In these times, reductions of 27 and 13%

were observed in plants treated with T+P after the first and after the second application, respectively, compared to control. These reductions were lower in the plants treated with T+P+Mz, 11 and 5% respectively. As a result, the *Chl a* content in plants treated with T+P+Mz did not differ from control plants.

The same trend of Chl *a* data was observed for the carotenoid content (Fig 5). Severe reductions in carotenoid levels were observed 96 h after application of the isolated T+P fungicide. Reductions of 29 and 21 % were detected after first and second application, respectively, in plants exposed to T+P in comparison to control plants. These reductions were lower in T+P treatment than T+P+Mz, only 7 and 10 % in relation to control plants.

3.5 Phytotoxicity symptoms on leaves

In plants treated with Mz singly there was no manifestation of visual symptoms of phytotoxicity (Table 3 and Fig 6). Severe symptoms were seen on leaves exposed to fungicide T+P with different magnitude in relation to the presence or absence of Mz fungicide in mixture. The presence of Mz caused a significant reduction of severity of symptoms (Fig. 6).

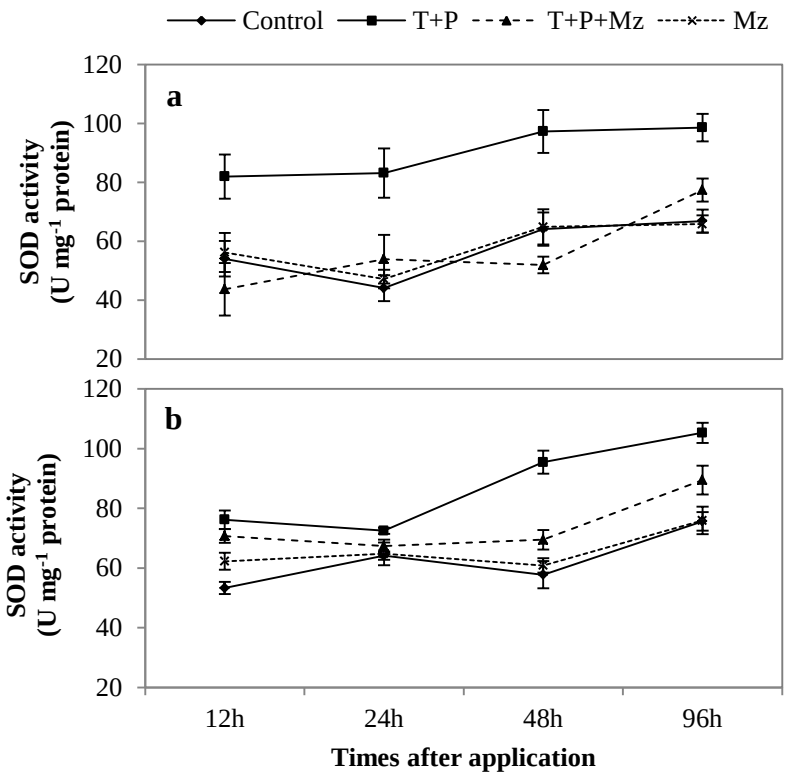

Fig 1. Superoxide dismutase (SOD) activity on soybean leaves as recorded at different times after first (a) and second (b) application of fungicides. Bar diagrams show the means ± SE (n = 3). T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

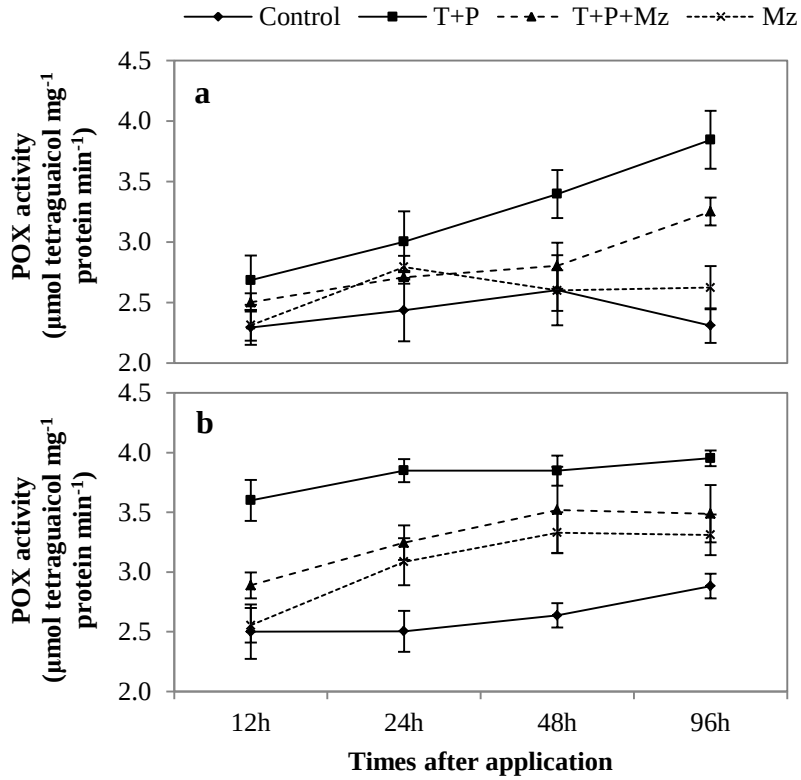

Fig 2. Peroxidase (POX) activity on soybean leaves as recorded at different times after first (a) and second (b) application of fungicides. Bar diagrams show the means ± SE (n = 3). T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

Table 1 Hydrogen peroxide concentration [(H_2O_2) µmol g^{-1} FW] in soybean leaves recorded at different times after first and second application of fungicides.

Treatments	**12h**	**24h**	**48h**	**96h**
		After first application		
Control	2.97 ± 0.15 ^{ns}	2.70 ± 0.24 ^{ns}	2.71 ± 0.12 ^{a*}	2.74 ± 0.17 ^{a}
T+P	3.01 ± 0.09	3.19 ± 0.16	3.87 ± 0.26 ^{b}	3.60 ± 0.16 ^{b}
T+P + Mz	3.01 ± 0.22	2.79 ± 0.10	2.95 ± 0.08 ^{a}	2.72 ± 0.11 ^{a}
Mz	2.91 ± 0.12	2.76 ± 0.25	2.84 ± 0.22 ^{a}	2.71 ± 0.05 ^{a}
		After second application		
Control	3.53 ± 0.15 ^{b}	3.51 ± 0.25 ^{ns}	3.61 ± 0.08 ^{ns}	3.31 ± 0.11 ^{b}
T+P	4.05 ± 0.01 ^{a}	3.81 ± 0.22	3.88 ± 0.09	3.97 ± 0.08 ^{a}
T+P + Mz	3.65 ± 0.10 ^{ab}	3.45 ± 0.19	3.74 ± 0.19	3.67 ± 0.10 ^{ab}
Mz	3.43 ± 0.11 ^{b}	3.38 ± 0.08	3.62 ± 0.20	3.34 ± 0.16 ^{b}

Values are means ± SE (n = 3). *Values with different letters in the column are significantly different from each other (Duncan's multiple range test ($p < 0.05$)). ^{ns} Not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb

Table 2 Malondialdehyde concentration (nmol MDA mg^{-1} protein) of soybean leaves as recorded at different times after first and second application of fungicides.

Treatments	12h	24h	48h	96h
		After first application		
Control	0.77 ± 0.06^{ns}	0.71 ± 0.02 ^{b*}	0.64 ± 0.02 ^{b}	0.65 ± 0.01 ^{b}
T+P	0.83 ± 0.06	0.81 ± 0.03 ^{a}	0.80 ± 0.03 ^{a}	0.87 ± 0.01 ^{a}
T+P + Mz	0.76 ± 0.03	0.79 ± 0.02 ^{ab}	0.68 ± 0.04 ^{ab}	0.77 ± 0.02 ^{ab}
Mz	0.69 ± 0.02	0.71 ± 0.02 ^{b}	0.64 ± 0.03 ^{b}	0.67 ± 0.03 ^{b}
		After second application		
Control	0.77 ± 0.02 ^{b}	0.82 ± 0.03 ^{b}	0.65 ± 0.02 ^{c}	0.67 ± 0.05 ^{b}
T+P	0.98 ± 0.08 ^{a}	1.07 ± 0.04 ^{a}	0.78 ± 0.02 ^{a}	0.91 ± 0.07 ^{a}
T+P + Mz	0.72 ± 0.06 ^{b}	0.76 ± 0.07 ^{b}	0.74 ± 0.01 ^{ab}	0.74 ± 0.06 ^{ab}
Mz	0.70 ± 0.04 ^{b}	0.78 ± 0.06 ^{b}	0.68 ± 0.02 ^{bc}	0.67 ± 0.03 ^{b}

Values are means ± SE (n = 3). *Values with different letters in the column are significantly different from each other (Duncan's multiple range test ($p < 0.05$)). ^{ns} Not significant. T+P – trifloxystrobin+ prothioconazole; Mz – mancozeb.

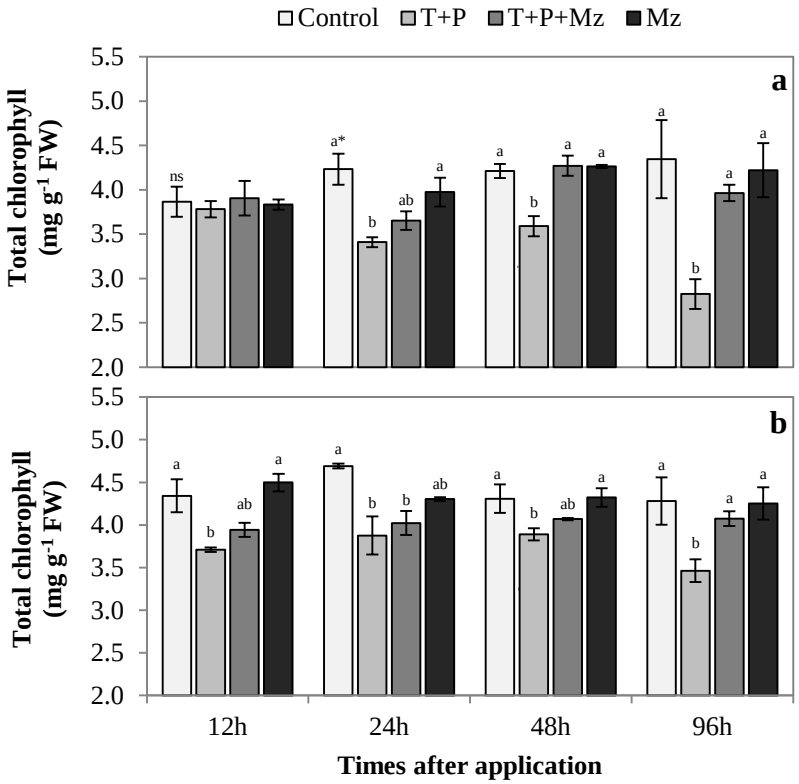

Fig 3. Total chlorophyll concentration on soybean leaves recorded at different times after first (a) and second (b) application of fungicides. Bar diagrams show the means ± SE (n = 3). * Different letters between columns indicate significantly differences inside each harvest time (Duncan's multiple range test ($p < 0.05$)). ^{ns} Not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

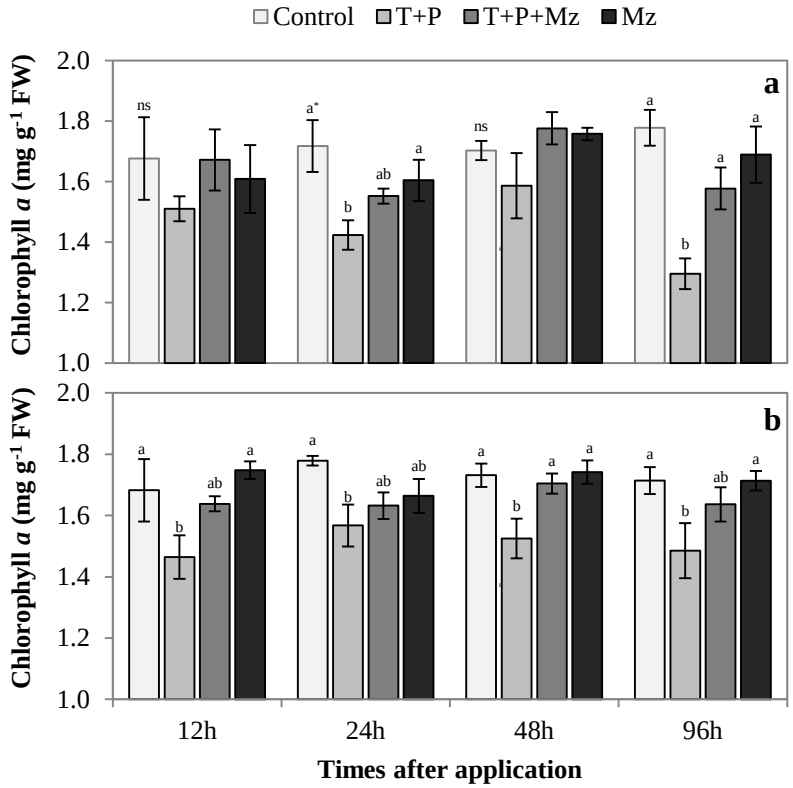

Fig 4. Chlorophyll *a* concentration of soybean leaves recorded at different times after first (a) and second (b) application of fungicides. Bar diagrams show the means ± SE (n = 3). * Different letters between columns indicate significantly differences inside each harvest time (Duncan's multiple range test ($p < 0.05$)). ^{ns} Not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

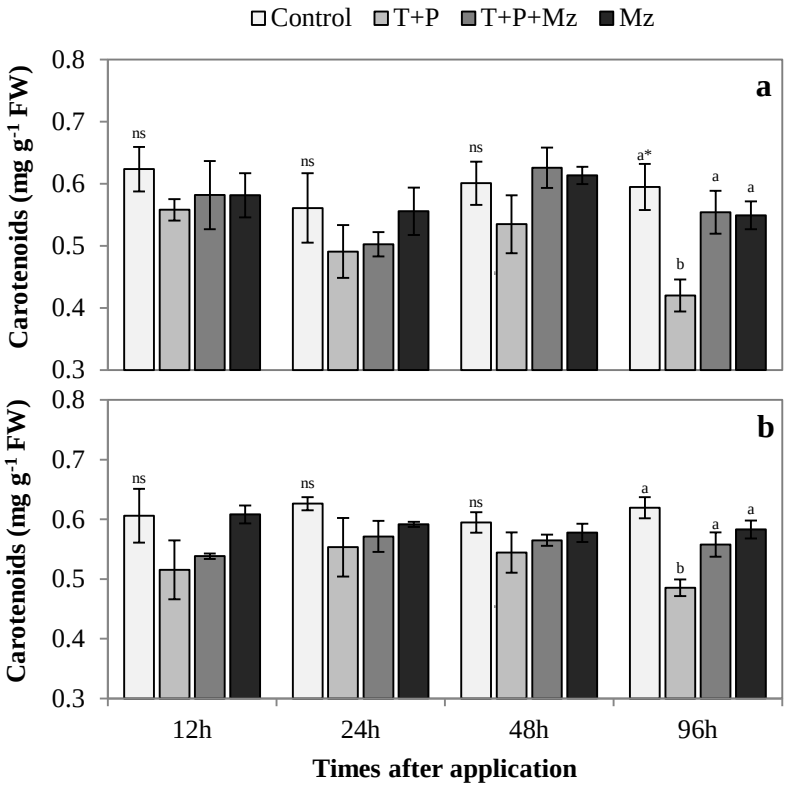

Fig 5. Carotenoids concentration on soybean leaves recorded at different times after first (a) and second (b) application of fungicides. Bar diagrams show the means ± SE (n = 3). * Different letters between columns indicate significantly differences inside each harvest time (Duncan's multiple range test ($p < 0.05$)). ns Not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

Table 3 Fungicide phytotoxicity in soybean measured by percentage of leaf surface injured.

Treatment	Phytotoxicity (%)
Control	0.0 ± 0.0 a*
T+P	7.0 ± 0.9 c
T+P + Mz	1.6 ± 0.2 b
Mz	0.0 ± 0.0 a

Values are means ± SE (n = 4). *Values with different letters in the column are significantly different from each other (Duncan's multiple range test ($p < 0.05$)). T+P – trifloxystrobin+ prothioconazole; Mz – mancozeb

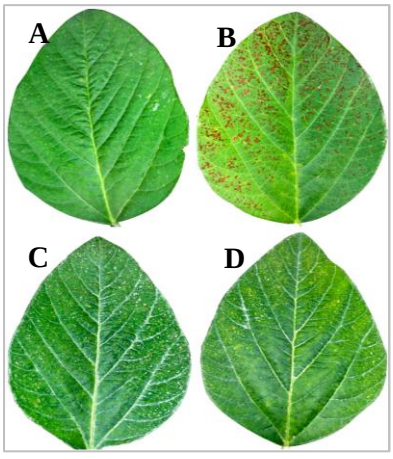

Fig 6. Fungicide phytotoxicity symptoms detailed in soybean leaves. A – control leaf; B – trifloxystrobin + prothioconazole; C – mancozeb; D – trifloxystrobin + prothioconazole + mancozeb.

4. Discussion

Studying the physiological response of plants to exposure to fungicides can help to define potential risks with phytotoxicity. It has been shown that many fungicides, especially azoles, *e.g*. prothioconazole, may play an important role in increasing resistance to abiotic stresses in plants (Wu and Tiedemann, 2002; Saito et al., 2006; Dittgen et al., 2013; Horn et al., 2013). Increase in enzymes activity from the ROS-scavenging system in plants exposed to fungicides followed by reduction on lipid peroxidation, electrolyte leakage and H_2O_2 accumulation have been shown as tolerance markers of abiotic stress (Calatayud and Barreno, 2001; Wu and Tiedemann, 2002; Gopi et al., 2007; Jaleel et al., 2007). However, the induction changes in the antioxidant response are acceptable when not followed by physiological damage.

Especially in soybean, investigations on physiological responses have gained more importance in the last years due to the occurrence of trifloxystrobin + prothioconazole – induced phytotoxicity at field conditions. To our understanding, in this study, we would expect improvements in the antioxidant response of plants exposed to T+P fungicide followed by less damage compared to control plants. However, data this study shows that there are negative effects on plants exposed to T+P. Phytotoxicity might induce damage, affect the harmonious working of the plant and may reflect of productive potential losses. To our knowledge, this work is the first reports of the physiological response of soybean plants to the fungicides trifloxystrobin + prothioconazole and mancozeb.

SOD is an essential component of a plant's anti-oxidative defense system. An important role is played by SOD in dismutation of free radicals by the formation of H_2O_2. Increase activity of SOD was recovered in plants exposed to T+P fungicide singly at different times after application. It is suggested that SOD was stimulated by scavenging $O_2^{\cdot -}$ to protect

soybean plants from T+P toxicity. Beyond SOD activity, POX is involved in the elimination of ROS and is another indicator of oxidative damage to plants (Parween et al., 2012). The breakdown of H_2O_2 can be driven by POX activity (Bowler et al. 1992). A higher POX activity in plants exposed to the T+P fungicide suggests again an oxidative stress induced by T+P. The increase in enzymes activities induced by fungicides can reflect the degree of toxicity this compound (Wu and Tiedemann 2002; Song et al. 2007). The lower SOD and POX activity when Mz was associated with T+P suggests an important role in relieving the toxicity of systemic fungicide. This may be linked to an unknown effect causing less ROS formation. Furthermore, plants exposed to Mz singly had null or minimal change in SOD and POX activity compared to control plants proving to be a safe product to be used in soybean.

A slight decrease in uptake rate of systemic fungicide pyraclostrobin and epoxiconazole was observed when mancozeb was mixed (Stefanello, 2017). Similar result was verified for the fungicide benalaxyl when associated with mancozeb (Gozzo et al., 1988). The latter linked the reduction of benalaxyl uptake rate with reduced metabolism of systemic compound and higher accumulation on leaves. It is suggested that the reduced metabolism might be a consequence of the reduced uptake. If plant oxidases are induced by benalaxyl concentration dependent, a reduced uptake might involve less induction and consequently less metabolic transformation (Gozzo et al., 1988). Another hypothesis considers that plant oxidases might be partially inhibited when mancozeb is present on the leaf surface (Gozzo et al., 1988). Mancozeb is very instable and release ions and various organic products. It is possible that these compounds act as an oxidase-inhibitor inside the leaves. It is suggested that the reduction of uptake rate and the possible play in oxidase-inhibition may be related to relieve of oxidative stress induced by systemic fungicide on leaves. Such suggestions can be confirmed by further experiments.

The increase in enzymes activities induced by fungicides can reflect also the ability to tolerate the stress as well (Wu and Tiedemann 2002; Song et al. 2007). Overproduction of H_2O_2 to eliminate the toxicity of $O_2^{\cdot -}$ can be related to upper SOD activity in plants exposed to T+P. Plants have multiple strategies to confer their tolerance to chemicals – induced toxicity and prevention of oxidative damage to cells (Prasad et al., 2005). Such enzymatic based anti-oxidative system has been one of the important strategies for plants to respond to fungicide phytotoxicity. However, the increased lipid peroxidation in plants exposed to T+P in the present study suggest that ROS induced damage. Furthermore, the increase in anti-oxidative enzymatic activities was not able to prevent such damages. The reduction on visual symptoms of phytotoxicity on leaves caused by mancozeb association could be correlated with the reduction on lipid peroxidation.

Chlorophyll is the principal compound responsible for photosynthesis and can be a good indicator of stress conditions in plants. It is suggested that Chl degradation occurs after ROS formation due to photosynthetic pigments are susceptible to ROS attack (Merzlyak and Hendry, 1994). The MDA accumulation caused by ROS excess may induce changes in integrity of the thylakoid membranes. Thus, the membrane disintegration and the resultant loosening of pigment binding to the thylakoids membranes may be the major causes of massive pigment destruction due to free pigments in organic solvents are labile and more sensitive to oxidative degradation (Sakaki et al., 1983). Therefore, chlorophyll degradation does not appear to be caused by direct action of the chemical compounds, but to the attack of ROS as anion superoxide, singlet oxygen or hydroxyl radical (Merzlyak and Hendry, 1994; Jakhar and Mukherjee, 2014). The decrease in Chl concentration in plants exposed to T+P might be involved with ROS increase and lipid peroxidation. In this way, the impact of Mz association to relieve the ROS levels and decrease of lipid peroxidation was reflected on higher Chl concentration.

The present work encompasses the responses of *Glycine max* L. against exposure to the systemic fungicide trifloxystrobin + prothioconazole and the multi-site fungicide mancozeb. The systemic fungicide induced alterations in the plant reflecting in oxidative stress. This could be visualized by the greater accumulation of ROS and lipid peroxidation. Increase in the lipid peroxidation may be one of the main toxic effects of T+P and might explain the visual lesions of phytotoxicity on leaves. In response to such changes an increased activity of anti-oxidant enzymes was observed. The data suggest that plant anti-oxidant strategies were not sufficient to prevent damage to cells that relied on chlorophyll and carotenoid degradation. The fungicide mancozeb singly did not induce relevant alterations that evidenced damage. When Mz was mixed to trifloxystrobin + prothioconazole, it relieved the production of ROS and reduced the damage to the cells. The lowest damage could be visualized by reducing the symptoms of phytotoxicity in leaves. These biochemical results do not allow us to define how mancozeb reduces the phytotoxic effects of the systemic fungicide. Investigations of these results using 'omics' tools are targets for further studies.

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Manuscript III (Submitted to Pesticide Biochemistry and Physiology)

Changes in leaf gas exchange and chlorophyll *a* fluorescence as an indicator of fungicide-induced phytotoxicity in soybean

Abstract

It has been efficiently demonstrated that some fungicides can cause changes in plant photosynthesis. In soybean this has been worrying due to the intense use of fungicides and some with phytotoxic potential. This work aimed to evaluate photosynthetic and Chl *a* fluorescence parameters in soybean plants exposed to fungicides trifloxystrobin + prothioconazole and mancozeb singly and in association. The experiments were carried out on greenhouse with manufacture recommended doses of fungicides. The effects on photosynthesis were investigated by gas exchange and chlorophyll fluorescent measurements. Plants treated with trifloxystrobin + prothioconazole (T+P) showed severe phytotoxic symptom with the reduction in net photosynthetic rate (*P*n). The inhibition of *P*n was accompanied by declines in stomatal conductance (G_s) and intercellular CO_2 concentration (C_i). The Chl *a* fluorescence parameters also were altered by T+P exposition. The changes were more significantly on areas more affected by fungicide on leaves. The phytotoxicity reflected in decrease on leaf area and plant height. Inhibitions of photosynthesis and Chl *a* fluorescence parameters were relieves by mancozeb association with systemic fungicide. Mancozeb singly promoted a slight decrease on *P*n 1 day after application (DAA). However, this decrease was not observed at 7 DAA.

Keywords: Photosynthetic activity; mancozeb; trifloxystrobin + prothioconazole; CO_2 assimilation

1. Introduction

The soybean crop (*Glycine max*) has a prominent place in the world's agriculture scenario. One of the major obstacles to the soybean production is the occurrence of diseases. The protection against foliar diseases currently requires sequential fungicide application. One of the main diseases to be managed is the Asian Soybean Rust (ASR) (*Phakopsora pachyrhizi*), which confers huge risks due to its potential of damaging the crop.

Until recently it was believed that the fungicide did not offer great risks of causing phytotoxicity to the plant, nor affecting its photosynthetic processes [1]. The majority of the studies had focused on the impact of the fungicides efficiency to control plant pathogen fungi and the residual accumulation in crops [1,2]. Nonetheless, more recent works at the cellular level have reported damages to the photosynthetic apparatus due to the plant exposition to some fungicides [3,4]. The investigations indicate a reduction in the net CO_2 assimilation rate (P_N) and on the photosynthesis efficiency by the fungicide application [2,5]. Some fungicides seem to inhibit the chlorophyll biosynthesis (Chl) and to delay the Chl integration into the photosynthetic apparatus [2]. Since the total biomass yield depends largely on photosynthetic performance of the plant, it is important to study effects of fungicides toxicity in plant photosynthetic apparatus. Plants are sensitive and respond rapidly to the presence of fungicides, as they affect primary and secondary processes in photosynthesis.

Trifloxystrobin + prothioconazole (T+P) and mancozeb (Mz) are recently introduced products for the soybean crop in Brazil. T+P is a mixture of commercial fungicides with mode of action inhibiting the demethylation (DMI) in the sterol biosynthesis and in the inhibition of the Quinone outside (QoI) in the mitochondrial respiration of the fungus. Nowadays, it is one of most used mixtures to manage ASR on soybean crop in Brazil, because of its high efficacy. Furthermore, the Mz is a multi-site fungicide with proved activity against ASR, and this way,

it has been added to management programs as a strategy to increase efficiency and as an important anti-resistance strategy [6].

It has been largely reported that the fungicide T+P contains high potential of causing toxicity symptoms in soybean. On the other hand, some results indicate a mitigating effect of Mz, reducing the phytotoxicity caused by other fungicides. So far, however, there are no studies elucidating the effects of such products in the physiological and biochemical parameters of soybean. Some investigations have not observed any relationship between the use of Mz and photosynthesis alterations [7], yet other studies have reported its effects, reducing the CO_2 assimilation rate and increase in nocturnal respiration [8]. Nevertheless, such researches did not study its effects on soybean crop, further the used formulation of the products were different from the current formulations.

In response to stresses, plants may mobilize nutrients and metabolites to develop defense mechanisms in detriment to growth plant [9]. The resistance towards stress implicates in energy costs, energy that could be used for growth, and this impact is bound to the intensity of the stress induced by the fungicide. The observed reductions in growth after exposition to pesticides suggest a metabolic reorientation. Therefore, the primary carbon metabolism which regulates the plant growth rate may provide important information to understand possible damages in plants and yield losses [10]. Concurrently, the parameters of chlorophyll *a* fluorescence (Chl *a*) is a useful tool to describe the photochemical activity in plant as a response to stress situations [11]. The fluorescence of Chl *a* efficiently expresses changes in electron flow in the photochemistry apparatus [12]. Furthermore, such parameters provide information about the structure and functionality of the photosystems, especially the photosystem II (PS_{II}). The performance of PS_{II} has been used to describe the plant response to several stresses, as those caused by high temperature, salinity, drought and environmental pollutant [11,13,14].

In this perspective, our work aimed to evaluate the photosynthetic parameters and Chl *a* fluorescence in soybean plants exposed to fungicides trifloxystrobin + prothioconazole and mancozeb and relate it to some growth parameters.

2. Materials and methods

2.1 Plant growth and experimental design

Two experiments were carried out in greenhouse in Itaara, (29°35'8"S; 53°48'28"W), Rio Grande do Sul, Brazil. The first assay was conducted in February/March 2015 and was assessed the photosynthetic and growth parameters. The second essay took place in February/March 2016, and was assessed the parameters of Chl *a* fluorescence. In both experiments, seeds of soybean cultivar BMX Ponta GM (Brasmax genetics) were pre-treated with *Bradyrhizobium* strains (Masterfix®, Stoller) with 0.2 L 100 kg^{-1}. Plants were grown in 5L pots filled with a mix of soil and rice husk, at 3:1 ratio. It was performed soil fertilization using 300 kg ha^{-1} of formula 8-28-18 (nitrogen, phosphorus and potassium, respectively). Two plants were grown in each pot, which were considered a sampling unit. The plants were kept disease-free, grown at 30 ± 5°C, with a 13/11h (day/night) photoperiod and 65 ± 10% air humidity. Irrigations were performed daily. The experimental design consisted of a completely randomized, in a unifactorial arrangement with four treatments (three fungicides and one control fungicide free) and five replications (each pot with two plants considered one replication).

2.2 Fungicides application

Four fungicides treatments were used: C – control without fungicide; T+P - trifloxystrobin + prothioconazole [(Fox®, Bayer Crop Science) 60 + 70 g a.i. ha^{-1}] isolated; T+

P+M - trifloxystrobin + prothioconazole [(Fox®, Bayer Crop Science) 60 + 70 g a.i. ha^{-1}] + mancozeb [(Unizeb Gold®, UPL) 1,125 g a.i. ha^{-1}] in mixture; and T4 – mancozeb [(Unizeb Gold®, UPL) 1,125 g a.i. ha^{-1}] isolated. The solution was prepared with distilled water and added mineral oil Aureo® (Bayer) at 0.25% v/v in the fungicides treatments. The solution was applied by using a compressed-air spraying system equipped with four nozzles (*Teejet* XR 110.02) on a movable bar, at controlled speed (1.5 m s^{-1}) and pressure (30 psi) to create a 150 L ha^{-1} application rate. The application was carried out at stage 105 of soybean [15] 32 days after emergence in both experiments.

2.3 Photosynthetic parameters

The physiological evaluations were performed using measured Li-Cor 6200 portable infrared gas analyzer (LI-COR - Model LI-6400XT). In this occasion were determined the stomatal conductance (g_s), photosynthetic rate (P_N), transpiration rate (E) internal CO_2 concentration (C_i), water use efficiency (WUE) and instantaneous carboxylation efficiency (P_N/C_i) obtained by the ratio between the amount of CO_2 fixed in photosynthesis and amount of water transpired. The conditions in the measuring chamber were controlled at a flow rate of 500 mol s^{-1}, a saturating PAR at 1000 µmol m^{-2} s^{-1} and 400 µmol mol^{-1} of CO_2, relative humidity of 50–60%. The measurements were always made on the trifoliate from the fifth node the plant, in the middle region of the central leaflet, leaves were totally exposed to solar radiation, starting at 09:00 am. The forth trifoliate (down – up) from eight plants per treatment was analyzed. The assessment was measured 1 and 7 days after fungicides application (DAA).

2.4 Individual leaflet area and height of plants

The individual leaflet area was determined using a non-destructive method proposed previously [16]. The measurements were taken on 6^{th} and 7^{th} leaflet at 8 DAA. The leaflet

area (LA) corresponded to the product of linear dimensions (length x width) of central leaflet multiplied by a correction factor (2.0185), as follow the equation: *LA=a.(L.W)*. Plant height was measured with a metric tape, the distance between the soil and the shoot. Both assessments were considered eight repetitions (plants).

2.5 Chl a fluorescence measurements

The fluorescence data was obtained through the experiment II, running a modulated pulse fluorimeter Junior-Pam (Walz, Germany). The measures were performed in the central foliole at the forth fully expanded trifoliate from six plants per treatment, between 8:00 and 10:00 am. The magnetic clip was held at the same position in all leaflets, on the right side of the central nervure and at the boundary between the apex and base. It was performed two measurements, firstly at 1 DAA and second at 7 DAA. The detached leaves for the measurements were initially pre-adapted to darkness for 30 minutes. Running the modulated pulse at (<0.05 µmol m^{-2} s^{-1} per 1.8 µs), which is insufficient to cause significant physiological alterations in the plant, it was determined the minimal fluorescence (F_o). The maximum fluorescence (F_m) was obtained after the leaf receive a saturating light pulse (10000 µmol m^{-2} s^{-1}) for 0.6 seconds. Using the values of F_o and F_m, it was determined the values of fluorescence ($F_v=F_m-F_o$) and the fluorescence-based maximum quantum yield for photosystem II (F_v/F_m). The fluorescence data, obtained in conditions of dark and light, made it possible to the determine the coefficient of photochemical quenching (*q*P) [$qP=(F_m' - F)/(F_m'- F_o')$], which provides an estimation of the number of open reaction centers in the photosystem II (PS_{II}) [17]. The quantum efficiency of PS_{II} photochemistry (Φ_{PSII}), closely associated with the quantum yield of non-cyclic electron transport, was estimated by $\Phi_{PSII}=(F_m' - F)/F_m'$) [18].

The maximum electron transport rate (ETR_{max}) was measured between 4:00 and 6:00 am, creating curves of light (electron transport rate versus photosynthetically active radiation (PAR) through the application of nine levels of radiation (0, 125, 190, 285, 420, 625, 820, 1150, 1500 µmol m^2 s^1) for 10 seconds and adjusting the values by the equation $ETR=ETR_{max}$ [1-e-kQ], wherein k is a fitting constant and Q is the value for PAR [19,20]. The determination of reducing side heterogeneity was calculated according to [11] through the following equation: Bo = [(Fv/Fm) – (Fv'/Fm')] / (Fv/Fm)*100. Wherein, Bo = Relative amount of Q_B-non-reducing PS_{II} centers.

2.6 Statistical analysis

The statistical analysis was obtained running the software Assistat version 7.7, at 5% of significance level. Quantitative changes of different parameters were analyzed through analysis of variance (ANOVA), with Tukey's honestly significant difference multiple comparison test being used to determine significant differences among treatments.

3. Results

3.1 Effect of fungicides on photosynthetic parameters

The analyzed photosynthetic parameters in the plants suggested significant influence of the fungicide exposition. Either P_N (Fig 1a) and g_s (Fig 1b) were more significantly reduced in plants sprayed with fungicides T+P, on both evaluation dates, this fungicide association had the greatest impact on photosynthetic activity. Reductions of 29 and 18% were observed in P_N and 54 and 25% in g_s in plants sprayed with T+P, at 1 and 7 DAA, respectively. The fungicide Mz induced reduction in P_N and g_s only in the first assessment, at 1 DAA. However, such reductions were smaller than those found in the treatment T+P. In this treatment, isolated

Mz, P_N and g_s were not reduced when compared to non-treated plants at 7 DAA. For the association, T+P+Mz, there was an intermediate response, with higher P_N and g_s in comparison to T+P, but lower than Mz alone. The damage attenuation by adding Mz to T+P increased the P_N at 10 - 12% and g_s at 23 - 16% on 1 and 7 DAA, respectively.

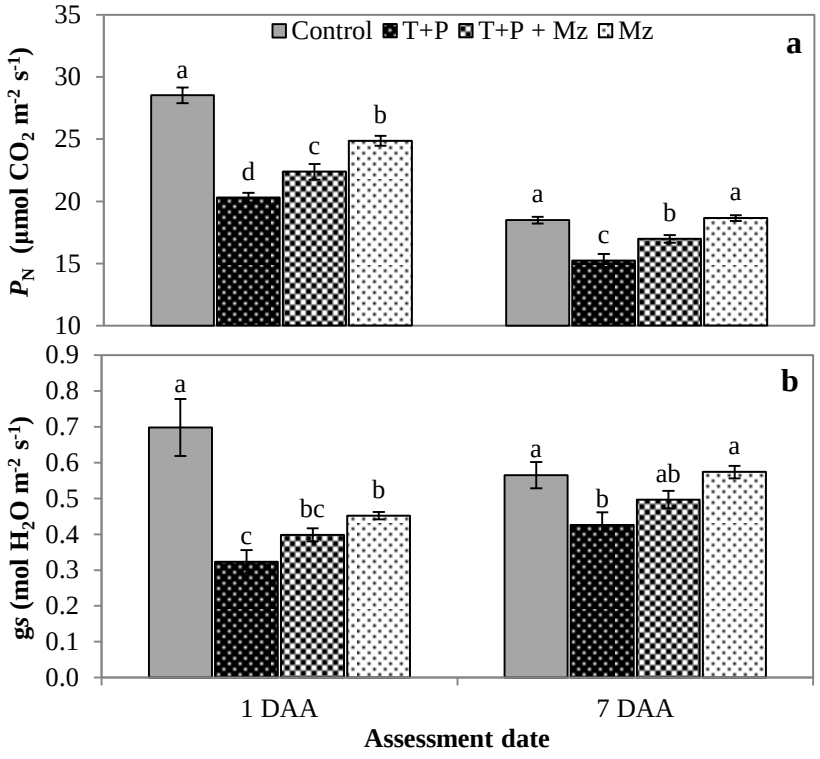

Fig 6. The photosynthetic rate (P_N, a) and stomatal conductance (g_s, b) in the leaves of soybean plants after fungicide application. The means ± SE of eight replications are shown. The different letters on the column indicate significant differences for Tukey test ($P < 0.05$) among different treatments: T+P – trifloxystrobin + prothioconazole; Mz – mancozeb

The C_i was influenced by the fungicide application only in the first assessment, 1 DAA (Fig 2a). On this date, T+P caused significant reductions in C_i. On the same date, Mz isolated, the impact on C_i was smaller, showing no difference from the non-treated check. The same happened when Mz was mixed to T+P (T+P+Mz), implicating in a smaller impact on C_i compared to T+P. At 7 DAA no alteration was found for C_i in treated plants. The P_N/C_i ratio

which reflects instantaneous carboxylation efficiency confirmed the damages observed on other parameters (Fig 2b). The fungicide T+P showed to be the most damaging in this study. Confirming the beneficial effects of Mz associated to the fungicide T+P, implicating on a greater P_N/C_i ratio when compared to plants which were exposed to T+P isolated.

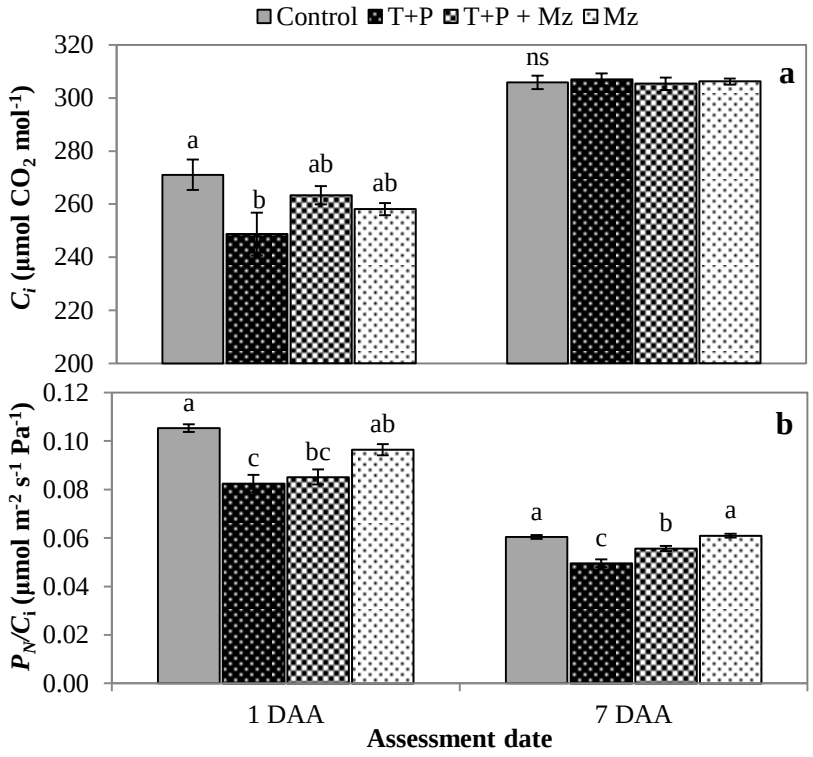

Fig 2. The intercellular CO_2 concentration (C_i, a) and instantaneous carboxylation efficiency (P_N/C_i, b) in the leaves of soybean plants after fungicide application. The means ± SE of eight replications are shown. The different letters on the column indicate significant differences for Tukey test ($P < 0.05$) among different treatments: T+P – trifloxystrobin + prothioconazole; Mz - mancozeb

In the first evaluation, 1 DAA, the treated plants presented a remarkable reduction in E (Table 1). On this date, the reductions reached up to 44, 33 and 22% for the treatments T+P, T+P+Mz and Mz, respectively. At 7 DAA, plants treated only with Mz did not differ from the non-treated plants. Nevertheless, plants that were sprayed with T+P kept the lowest E, with a reduction of 27% in comparison to the control. For the treatment, T+P+Mz the reduction was

as low as 10%. The change on E has a direct impact on the increase of WUE in plants exposed to fungicides, mainly at the assessment of 1 DAA (Table 1). The fungicide T+P induced a bigger increase of WUE, at 7 DAA the plants treated with Mz did not show any difference for this variable in comparison to non-treated plants. The same occurred to T+P+Mz which did not differ from the check at 7 DAA.

Table 1 The transpiration rate (E) and the water use efficiency (WUE) in the leaves of soybean plants after being treated with fungicides

Treatments	E (mmol H_2O m^{-2} s^{-1})		WUE (mol CO_2 mol H_2O^{-1})	
	1 DAA	7 DAA	1 DAA	7 DAA
Control	6.37 ± 0.35 [a1]	6.24 ± 0.17 [a]	4.55 ± 0.20 [c]	2.97 ± 0.05 [b]
T + P	3.53 ± 0.28 [d]	4.54 ± 0.29 [c]	5.94 ± 0.35 [a]	3.43 ± 0.13 [a]
T + P + Mz	4.25 ± 0.15 [bc]	5.63 ± 0.11 [b]	5.30 ± 0.19 [ab]	3.02 ± 0.05 [b]
Mz	4.94 ± 0.09 [ab]	6.49 ± 0.11 [a]	5.04 ± 0.09 [b]	2.88 ± 0.04 [b]

The means ± SE of eight replicates are shown. [1] The different letters present on the column indicate significant differences at Tukey test ($P < 0.05$). T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

The fungicide impact on the photosynthesis processes caused reductions in the growth parameters (Table 2). The most notable impact was observed in the isolated T+P. The plants exposed to these systemic fungicides presented a smaller foliar expansion of the trifoliate after spraying fungicide. This was evident when analyzed the smaller foliar area in the sixth and seventh trifoliate, evaluated 8 DAA. The same way, there was an internode shortening what resounded in shorter plants when they were exposed to T+P. Plants treated only with Mz did not have reductions in foliar area and plant height when compared to non-sprayed plants. Yet, the bigger foliar area and higher plants when treated with the mixture T+P+Mz reflects the beneficial effect of Mz in reducing the damages caused by systemic fungicides.

3.2 Chl a fluorescence measurements

The results showed that changes in the Chl *a* fluorescence transient were dependent on fungicide exposition of plants with variation between fungicide type on both assessment date

(Table 3 and 4). When isolated, Mz did not implicate in any alteration in Chl *a* fluorescence on both assessment dates. Significant variation in Chl *a* fluorescence was observed in response to the application of the fungicide T+P in isolation on both evaluations. The mixture T+P+Mz reflected in improvements on the parameters of Chl *a*. When compared to the check, the F_m was reduced in 21 and 17% on plants exposed to T+P at 1 and 7 DAA, respectively (Tables 3 and 4). In the treatment, T+P+Mz this reduction was smaller, in a magnitude of 17 and 9% at 1 and 7 DAA, respectively. There was no significant alteration in F_o on plants treated with fungicides. The maximum quantum yield (F_v/F_m) was reduced only when plants were treated with T+P on both dates. Such reductions were at 7 and 5% in comparison to the untreated check. The association T+P+Mz presented greater F_v/F_m ratio, compared to isolated T+P and did not differ from non-treated plants. The decline on F_v/F_m ratio might be due to higher decrease in F_m.

Table 2 Individual leaflet area measured by linear dimensions (length and maximum width) of the central leaflet and height of soybean plants after being treated with fungicides

Treatments	Calculated individual leaflet area (cm^2)		Height of plants (cm)
	6^{th}	7^{th}	
Control	117.5 ± 3.5 [a1]	78.3 ± 2.7 [a]	52.9 ± 1.1 [a]
T + P	92.1 ± 2.6 [b]	58.2 ± 1.6 [b]	47.0 ± 0.3 [b]
T + P + Mz	111.3 ± 2.8 [a]	67.8 ± 1.8 [ab]	51.1 ± 1.6 [ab]
Mz	115.8 ± 0.9 [a]	77.2 ± 4.6 [a]	52.4 ± 0.4 [a]

The means ± SE (n=8) are shown. [1] The different letters present on the column indicate significant differences at Tukey test ($P < 0.05$). T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

The actual photochemical efficiency (Φ_{PSII}) and the electron transport rate (ETR) experienced a gradual decrease in plants exposed to T+P fungicide in both assessment dates (Table 3 and 4). At 1 DAA, reductions of 14% in Φ_{PSII} and 13% in ETR were observed. At 7 DAA the reductions were in smaller degree, in order of 5% in Φ_{PSII} and 1% in ETR, not enough for significant difference compared to control plants. At 7 DAA important increases were verified in Φ_{PSII} and ETR parameters in plants exposed to Mz, both isolate or associated

with T+P, in comparison to control plants. The Mz induced an improvement in these parameters reducing the damage caused by T+P isolated. The variations in the fraction of open PS_{II} centers (*q*P) between leaves exposed to fungicides and control leaves was not significant. However, reductions in 13% and 5%, 1 and 7 DAA, respectively, were verified on leaves exposed to the fungicide T+P and cannot be neglected. This reduction was lower (1 DAA) and did not exist (7 DAA) in leaves exposed to T+P + Mz.

Table 3 Chl *a* fluorescence parameters assessment at 1 DA fungicides exposition. Minimum fluorescence (F_o), maximum fluorescence (F_m); maximum quantum yield of PS_{II} (Fv/Fm), photochemical efficiency of photosystem II (Φ_{PSII}), electron transport rate (ETR), coefficient photochemical quenching (*q*P)

Treatments	**F_o**	**F_m**	**F_v/F_m**	**Φ_{PSII}**	**ETR**	***q*P**
Control	155 ± 6.4 ^{ns}	757 ± 13 ^{a1}	0.79 ± 0.01 ^{a}	0.38 ± 0.01 ^{a}	45.4 ± 0.8 ^{a}	0.58 ± 0.02 ^{ns}
T + P	157 ± 4.5	596 ± 7 ^{b}	0.74 ± 0.01 ^{b}	0.32 ± 0.01 ^{b}	39.4 ± 0.9 ^{b}	0.50 ± 0.03
T + P + Mz	143 ± 4.4	628 ± 21 ^{b}	0.77 ± 0.01 ^{a}	0.35 ± 0.01 ^{ab}	42.1 ± 0.5 ^{ab}	0.55 ± 0.01
Mz	165 ± 3.6	749 ± 22 ^{a}	0.78 ± 0.01 ^{a}	0.37 ± 0.01 ^{a}	44.3 ± 0.9 ^{a}	0.57 ± 0.01

The means ± SE (n=4) are shown. [1] The different letters present on the column indicate significant differences at Tukey test ($P < 0.05$). ^{ns} not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

Table 4 Chl *a* fluorescence parameters assessment at 7 DA fungicides exposition. Minimum fluorescence (F_o), maximum fluorescence (F_m); maximum quantum yield of PS_{II} (Fv/Fm), photochemical efficiency of photosystem II (Φ_{PSII}), electron transport rate (ETR), coefficient photochemical quenching (*q*P)

Treatments	**F_o**	**F_m**	**F_v/F_m**	**Φ_{PSII}**	**ETR**	***q*P**
Control	181 ± 5 ^{ns}	850 ± 22 ^{ns}	0.79 ± 0.01 ^{a}	0.34 ± 0.01 ^{ab}	39 ± 3.9 ^{ab}	0.55 ± 0.04 ^{ns}
T + P	175 ± 11	706 ± 38	0.75 ± 0.01 ^{b}	0.32 ± 0.01 ^{b}	39 ± 0.4 ^{b}	0.52 ± 0.04
T + P + Mz	161 ± 12	768 ± 61	0.79 ± 0.01 ^{a}	0.36 ± 0.01 ^{a}	42 ± 0.8 ^{a}	0.56 ± 0.01
Mz	158 ± 6	773 ± 15	0.80 ± 0.01 ^{a}	0.37 ± 0.01 ^{a}	44 ± 0.7 ^{a}	0.58 ± 0.01

The means ± SE (n=4) are shown. [1] The different letters present on the column indicate significant differences at Tukey test ($P < 0.05$). ^{ns} not significant. T+P – trifloxystrobin + prothioconazole; Mz – mancozeb.

Reducing side heterogeneity was estimated by measuring relative amounts of Q_B-reducing and Q_B-non-reducing centers and the data are shown on Table 5. In the leaves of control, 18 and 22% of total PS_{II} was Q_B-non-reducing, while 82 and 78% of PS_{II} centers

were Q_B-reducing centers, at 1 and 7 DAA, respectively. Exposure of plants to T+P fungicide led to a gradual increase in the fraction of Q_B-non-reducing PSII centers (Fig 3a). Mz isolate and the combination T+P+Mz did not induce significant changes in the Bo compared to control plants.

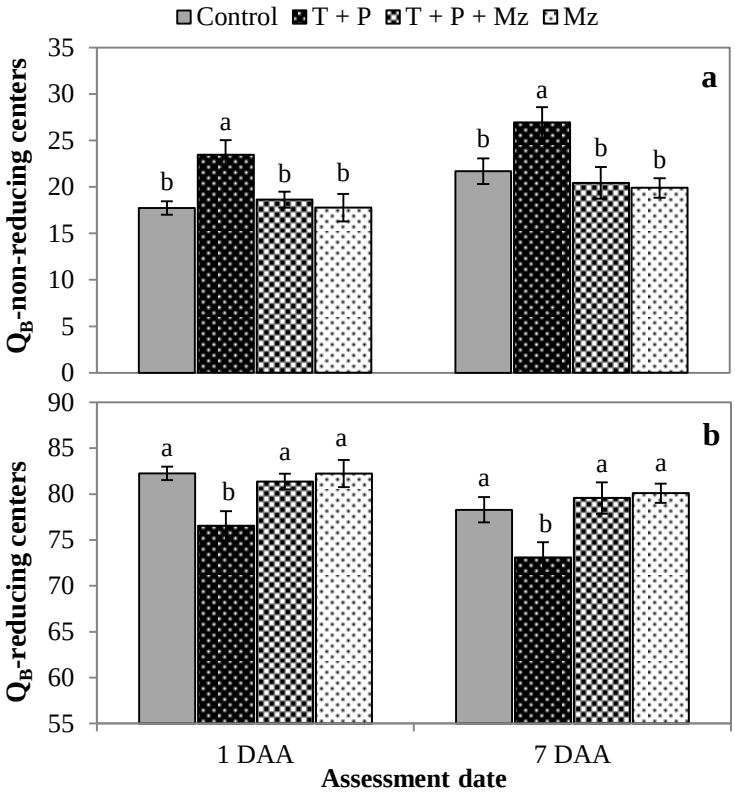

Fig 3. Reducing side heterogeneity on leaves exposed to fungicides, Q_B-non-reducing centers (a) and Q_B-reducing centers (b). The means ± SE of four replicates are shown. The different letters indicate significant differences at Tukey test ($P<0.05$) among different treatments. T+P – trifloxystrobin + prothioconazole; Mz - mancozeb

All the measures accounted for the magnetic clip fixation in a pattern position previously described. Nonetheless, the area of magnetic clip is little and the damaged regions in the leaves were not uniform, generating then, variations in evaluated parameters. For this reason, specific measurements were taken considering the different positions on the leaflet (base and apex) and different degrees of damages of phytotoxicity (non-treated leaves and leaves exposed to T+P), according to the Fig 3. The data are shown on Table 5. One important

relationship between the areas with lesions and the decreases in the parameters of Chl *a* fluorescence was evident. Same leaflet exposed to the fungicide T+P presented areas with very little effects and the photosynthesis efficiency was minimally affected, but in areas with more damage, it can be noticed significant reductions in the parameters of fluorescence. In these more harmed areas, it was initially noticed a decrease in F_m, what implicates in a lower F_v/F_m ratio. Further reductions of 24, 16 and 16% were observed in the variables Φ_{PSII}, ETR and *q*P, proving a lower photosynthetic efficiency in more damaged areas.

Table 5 Chl *a* fluorescence parameters assessment at 7 DA fungicides exposition considering different positions on leaf. Minimum fluorescence (F_o), maximum fluorescence (F_m); maximum quantum yield of PS_{II} (Fv/Fm), photochemical efficiency of PS_{II} (Φ_{PSII}), electron transport rate (ETR), coefficient photochemical quenching (*q*P)

Treat.	**Damage**	**F_o**	**F_m**	**F_v/F_m**	**Φ_{PSII}**	**ETR**	***q*P**
Control	center	169 ± 14 ^{ns}	825 ± 63 ^{a1}	0.79 ± 0.00 ^{a}	0.35 ± 0.01 ^{a}	40.7 ± 0.8 ^{a}	0.53 ± 0.01 ^{a}
	margin	162 ± 6	791 ± 20 ^{a}	0.79 ± 0.01 ^{a}	0.34 ± 0.01 ^{a}	42.1 ± 1.1 ^{a}	0.55 ± 0.01 ^{a}
T + P	- (center)	178 ± 8	777 ± 18 ^{a}	0.77 ± 0.01 ^{a}	0.32 ± 0.00 ^{a}	40.0 ± 1.4 ^{a}	0.52 ± 0.03 ^{a}
	+ (margin)	177 ± 13	532 ± 63 ^{b}	0.66 ± 0.02 ^{b}	0.27 ± 0.01 ^{b}	34.0 ± 0.8 ^{b}	0.44 ± 0.01 ^{b}

The means ± SE (n=4) are shown. [1] The different letters present on the column indicate significant differences at Tukey test ($P < 0.05$). ^{ns} not significant. T+P – trifloxystrobin + prothioconazole.

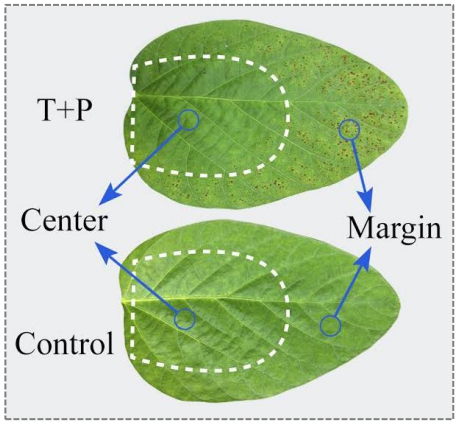

Fig 4. Soybean leaves with and without fungicide T+P exposition. The geometric dash lines divide leaf into two parts, - damage (center) and + damage (margin). Blue circles indicate the chlorophyll *a* fluorescence measuring positions.

4. Discussion

Alterations in carbon metabolism and/or nitrogen may reflect on significant losses in terms of plant growth. Physiological studies in several species of plants treated with fungicides have revealed modifications either on photosynthetic activity and fluorescence of chlorophyll *a* [3,5,8,21,22]. These reports are in accordance to the findings in this work, for the applications of fungicides T+P.

The fungicides T+P induced significant reductions in P_N, evidencing its damaging potential to CO_2 assimilation in soybean plants. The reduction in P_N can be attributed to stomatal factors, due to its closure visualized by the reduction in g_s and E. However, it may also be associated with non-stomatal factors, because of an interruption in the carboxylation capacity of RuBisCO, reductions in the content of RuBisCO or even reducing the regeneration of ribulose 1,5 bisphosphate [4], considering the lower P_N/Ci ratio in this study. Reductions in P_N accompanied by alterations in g_s and C_i have been reported on *Malus domestica* and *Cucumis sativus* after fungicide application [5,8]. Fludioxonil, a non-systemic fungicide, when sprayed on *Vitis vinifera* induced a reduction in P_N and in C_i, but, not reducing g_s [4]. In another study the fungicides fludioxonil and pirimetanil caused decreases in the P_N , E, g_s and also in the C_i on plants of *Vitis vinifera* grown *in vitro* [3]. These studies are in agreement with our findings, which showed significant reductions in the photosynthetic parameters assessed of plants treated with T+P.

The application of Mz induced small changes in the parameters of photosynthesis at 1 DAA, but at 7 DAA such changes were not very discernible, and these parameters remained similar to the non-treated plants. It is remarkable that the formulation and the dosage of Mz used in this investigation did not offer risks of phytotoxicity for soybean plants. This is one of the first studies aiming to determine the response of soybean plants to the exposition of Mz. Some other studies have recorded evidences of toxic effects of Mz in plants of lettuce

(*Lactuca sativa*) [23,24] and in *Cassia angustifolia* [25]. The parallel among these researches provides evidences of a differential response of plant species to Mz, and also a differential effect for different Mz formulations, which was different between our study and others. About the fungicide mixture, an attenuation of the systemic fungicide harm was perceived, clearly observed by the improvements attained in photosynthetic parameters, mainly with a greater P_N in comparison to T+P alone on both dates. The mechanism of how Mz reduces the phytotoxicity caused by other fungicides in the photosynthetic apparatus still remains unknown. We suggest that the effects of Mz might be related to improvements in the antioxidant system of the plant, preventing the harm of oxygen reactive species. Increases in proline concentration (96%), in total glutathione (144%), enzymatic activity for ascorbate peroxidase (63%) and glutathione reductase (154%) were reported in plants of *Cassia angustifolia* exposed to mancozeb [25].

It has been shown that some fungicides may improve water use efficiency (WUE) and the management stresses in plants under water deficit [26,27]. Our data provided evidences that fungicide isolated T+P reflected in a greater WUE. This gain in WUE may be a consequence of the significant reduction in E. The increase in WUE may be interpreted as a positive response mechanism of the plant under water deficiency [28]. Nonetheless, there are some doubts about these benefits, because of crop yield is strictly bound to the respiration process, which, when is reduced, works as the main factor to increase the WUE. In this case, the improvements in WUE is desirable only if not followed by a simultaneous reduction in the photosynthesis rate and the production of biomass, what is not the case in this research, which showed that plants treated with T+P had a considerable decrease in P_N.

Because of plants depend on photosynthesis to assimilate carbon and promote its growth, damages in the photosynthesis processes have negative consequences in terms of plant biomass and crop production [1]. Various reports support a decrease of biomass

production in plants treated with benomil, a systemic fungicide, reducing the growth of *Gossypium hirsutum*, *Helianthus annuus*, *Cucumis sativus*, *Lactuca sativa* and *Pinus taeda* [29,30]. Carbendazim, a systemic fungicide of the group of benzimidazole, negatively affected the plant biomass in *Nicotiana tabacum* [29]. Our research demonstrated that phytotoxicity induced by the fungicide T+P may reflect the reductions of plant growth explained by the reductions on P_N. The impact on plant growth was visualized mainly by the reduction in foliar area and the shortening of the internodes (lower plants). The intensity of damages in the plant growth will depend on the degree of severity. Application on plants in reproductive stages, in periods of low availability of water in the soil, low air humidity, high temperatures and high availability of solar radiation, accompanied by low volume of spray and no correction of adjuvant dosage for the volume, may potentialize the severity [31].

The analysis of several parameters of chlorophyll *a* fluorescence showed that the photosynthesis light reactions are also sensible to fungicide exposition and this was also reported in other researches [5,8,21]. A decrease in F_m and F_v/F_m was observed with more intensity after T+P treatment. A fall in these parameters might be involved with a large quantity of inactive PS_{II} centers due to oxidation or degradation of D1 proteins [32]. The quantification of protein D1 content might be a target for future investigations to better understanding the interaction plant *vs* fungicide. The decrease in F_v/F_m caused by fungicide T+P is a reliable sign of photoinhibition [33]. Evidently, there was no significant change in F_o values, but a significant change in F_m. The decline in values observed in the F_m and the F_v/F_m indicates that the photochemistry of PS_{II} and its ability to reduce the primary acceptor Q_A was affected by fungicide T+P. Such negative effect was attenuated when this fungicide was mixed with Mz, with a higher F_v/F_m ratio.

In the T+P-treated plants there was a significant decrease in *q*P, suggesting that active PS_{II} centers were converted into inactive centers, which are associated with down-regulation

of photochemistry and damage to the photosynthetic apparatus. This suggests that some reaction centers became inactive, incapable of reducing Q_A, and they were converted into heat dissipaters after the extinguishment of photosynthetic pigments [34]. The applications of benzimidazoles, triazoles and one contact fungicide dithiocarbamate reduced the efficacy of quantum yield of PS_{II} (Φ_{PSII}), as well as the maximum quantum efficiency of PS_{II} (F_v/F_m), and this reduction was attributed to the decrease in the coefficient of photochemistry quenching (*q*P) [5,8]. A reduction in Φ_{PSII} led to a decrease in primary photochemistry of photosynthesis. This fall was largely due to a lower F_m and unchanged F_o [34] and might be correlated with our data. This indicates that T+P inhibited trapping of the absorbed energy. The quantum yield of PS_{II} is very sensitive to stress and reflects lower efficiency in regulation of energy excitation, use and dissipation by photosynthetic membranes [34].

There also was a profound change in electron transport rate (ETR) in treated plants with T+P. This may be related to lower energy absorption by antenna pigments and lower energy trapping by reaction centers induced by fungicide. These results suggest that some fungicides, for example T+P, might alter absorption capacity, electron transport of antenna and overall primary chemistry in plants. The heterogeneity of reduced reaction centers in PS_{II} presents a good indicator of phytotoxicity (stress) which affect the photochemistry pathway (light-harvesting apparatus). Plants treated with T+P had and increment in the number of Q_B-non-reducing concurrently to a decrease in the number of Q_B-reducing centers in comparison to control plants. This indicates that the fungicide T+P may induce a conversion of Q_B-reducing centers into Q_B-non-reducing centers, which are incapable of reducing the Q_A to plastoquinone (PQ) pool, thus the energy production is affected.

The study of photosynthesis performance in soybean due to fungicide exposition provided important information about the mechanisms of phytotoxicity of these compounds. There was visual confirmation of the phytotoxicity induced by T+P on soybean, however, it

had not yet been shown which were its impacts on photochemistry parameters. The impact of fungicides (T+P) on photosynthesis depends on the degree of severity of phytotoxicity in the leaves and may vary in function of the position in the leaflet. Further, this study showed that undamaged areas may not have its processes affected when compared to non-treated leaves. Nonetheless, in more harmed areas these processes were affected.

In conclusion, the data from this study demonstrated that fungicide T+P application debilitates the photosynthesis of soybean leaves. A sharp reduction in photosynthesis rate (P_N) accompanied by down performance of the PS_{II} were induced by the exposure to T+P. The fungicide mancozeb promoted mitigation in the harms caused by the fungicide T+P, and future researches may provide a better understanding of how it occurs. Mz isolated, in the formulation used in this study can be safely sprayed on soybean without any risk of phytotoxicity.

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Manuscript IV (Will be submitted to Summa Phytopathologica)

Nutritional status on leaves for the assessment of the “green effect” induced by mancozeb fungicide on soybean

ABSTRACT

Mancozeb has been extensively used in Brazil as chemical management programs to control fungal diseases in soybean. In addition to its fungicide treats, a “green effect” on plants has been reported in areas sprayed with the fungicide. The aim of this work was to assessment the nutritional status on soybean leaves influenced by mancozeb exposition. The experimental design consisted of completely randomized, arranged in a 2 × 2 factorial, plants sprayed and non-sprayed with protector fungicide mancozeb and plants sprayed and non-sprayed with systemic fungicide trifloxystrobin + prothioconazole. All the macronutrients and micronutrients Fe, Mn, Zn and Cu were quantified on leaves at 4 and 8 days after fungicide application. Increases in S, Zn and Mn concentrations on leaves were observed by exposition to multi-site fungicide mancozeb. This finds might be correlated with the increase in SPAD index and explains in parts the visual “green effect” on plants after fungicide application.

Key words: *Glycine max*, trifloxystrobin + prothioconazole, dithiocarbamates, phytotoxicity

Estado nutricional das folhas para avaliação do "efeito verde" induzido pelo fungicida mancozebe na soja

RESUMO

Mancozebe tem sido amplamente utilizado no Brasil em programas de manejo químico para controle de doenças fúngicas na soja. Além de seu efeito fungicida, um "efeito verde" nas plantas tem sido relatado em áreas pulverizadas com esse composto. O objetivo deste trabalho foi avaliar o status nutricional em folhas de soja influenciadas pela exposição ao mancozebe.

O delineamento experimental foi inteiramente casualizado, em esquema fatorial 2 × 2, plantas pulverizadas e não pulverizadas com fungicida protetor mancozebe e plantas pulverizadas e não pulverizadas com fungicida sistêmico trifloxistrobina + protioconazol. Todos os macronutrientes e micronutrientes Fe, Mn, Zn e Cu foram quantificados nas folhas aos 4 e 8 dias após a aplicação do fungicida. Incrementos nas concentrações de S, Zn e Mn nas folhas foram observados pela exposição ao fungicida protetor mancozebe. Este achado pôde ser correlacionado com o incremento no índice SPAD e explicar em partes o "efeito verde" visual em plantas após a aplicação do fungicida mancozebe.

Palavras-chave: *Glycine max*, trifloxistrobina + protioconazol, ditiocarbamatos, fitotoxidade

INTRODUCTION

Introduced in 1962, the mancozeb [ethylene-bis-dithiocarbamate (EBDC)] is a sulfur organic compound widely used as a broad-spectrum fungicide to protect fruits, vegetables and grain crops from a range of fungal diseases. The EBDCs have a common organic skeleton ($C_4H_6N_2S_4$) differing only on a metal ion present in the molecule. Created from zinc ion complex of maneb (EBDC-manganese), the mancozeb (EBDC-Mn-Zn) become the most important and commercially significant of the EBDCs (5). In Brazil, the use of mancozeb on field crops is recent. Unizeb Gold® (mancozeb 75%) is a recently registered fungicide to use on soybean and others crops. This product has been sprayed associated to demethylation inhibitors (DMI) and Quinone outside inhibitors (QoI) fungicides to increase the control efficiency of Asian soybean rust (ASR) (*Phakopsora pachyrhizi*). This pathogen has developed adaptations towards both DMIs and QoIs (9,20) and the difficulties of controlling ASR have been becoming evident. Therefore, the presence of a multi-site fungicide has been an important strategy to improve control and resistance management (23).

Besides fungicide effect, a "green effect" has been reported in areas sprayed with mancozeb. The green effect might be associated with possible increases on nutrient concentrations and reduction on plant stresses. The EBDCs are generally unstable and the oxidation or hydrolysis turn the byproducts into high polarity and hydrophilic compounds, such as ethylenethioureia (ETU) and other. Analysis of the aqueous ozonation of mancozeb and its degradation products demonstrated that metal groups, such as manganese and zinc, are the first site of attack, and then the CS_2 or CS group were removed (7). The gains on nutritional status might be small, nevertheless, the contributions of micronutrients from fungicides should not be ignored (18). Micronutrients such as Mn and Zn are required in small quantities by the plants, and the fraction provided by fungicide may promote the "green effect", physiological improvements and productivity. The nutritional status of plants can be efficiently determined during the growing season with leaf analysis.

There are few studies dealing with the relationship between dithiocarbamates and nutritional status of plants. The repeated use of fungicide propineb increased the Zn contents in shoots of banana plants (13). Spraying fertilizers and Dithane® formulations (D-22, D-45 and D-78) increased the concentration of micronutrients ferro (Fe), manganese (Mn) and zinz (Zn) in mango leaves (21). To our knowledge this is the first study on soybean crop aiming to investigate the effect of mancozeb on plants nutritional status by foliar analysis.

MATERIALS AND METHODS

Plant growth and experimental design. Seeds of soybean cultivar Ponta GM (Brasmax genetics) previously treated with *Bradyrhizobium* strains (Masterfix®, Stoller) at 0.2 L 100 kg^{-1} of seed were used. The experiment was carried out in greenhouse in Itaara (29°35'8"S; 53°48'28"W), Rio Grande do Sul, Brazil. Plants were grown in 5L pots filled with soil mixed with rice husk (3:1). The soil fertilization was done applying 300 kg ha^{-1} of the

formulation 8-28-18 (nitrogen, phosphorus and potassium, respectively). Two plants were grown in each pot, which were considered a sampling unit. The plants were kept disease-free, grown at 28 ± 5°C, with a 13/11h (day/night) photoperiod and 65 ± 10% air humidity. Irrigations were daily performed.

The experimental design consisted of completely randomized, arranged in a 2 × 2 factorial. The first factor consisting of plants sprayed or non-sprayed with mancozeb, hereafter referred as –mancozeb and +mancozeb. The second factor consisting plants sprayed or non-sprayed with trifloxystrobin + prothioconazole (TRIF+PROT), hereafter referred as control and TRIF+PROT, respectively. Were used twelve replications per treatment, four replications to each destructive sample and four replications to non-destructive assessments. In the combination of TRIF+PROT + mancozeb, the products were sprayed mixed in the same solution. In the combination control –mancozeb, was sprayed distilled water. Trifloxystrobin + prothioconazole [(Fox, Bayer Crop Science) 60 + 70 g a.i. ha^{-1}] and mancozeb [(Unizeb Gold, UPL) 1,125 g a.i. ha^{-1}] were used to prepare the solution and added mineral oil [Aureo (Bayer Crop Science) at 0.25% v/v]. The solution was applied using a compressed-air spraying system equipped with four nozzles (Teejet XR 110.02) on a movable bar, at controlled speed (1.5 m s^{-1}), pressure (30 psi) to perform 150 L ha^{-1} application rate. The application was carried out at stage 17/36 of BBCH scale of soybean (15), 40 days after emergence. Before spraying, the pots were covered with plastic at the bottom to avoid losing the solution. At the moment of spraying (11h00 am) the weather conditions were temperature 28°C and air humidity 60%. During the afternoon of the day the temperature was higher (maximum of 32°C) and the humidity lower (minimum of 45%).

Leaf blade sampling and assessments. For the nutrients analysis, leaves from the fifth and the sixth nodes were collected and mixed to compose the sample. This procedure was done twice, 4 and 8 days after spraying (DAS) and the both leaf sides were washed by

following the procedures: (i) first with distilled water; (ii) washing with 0.1% detergent solution scrubbing with cotton; (iii) washing with distilled water; (iv) immersion in HCl solution (1:9); (v) the last, washing with ultra-pure water. In sequence, the leaves were placed into paper bags and dried in a forced-air oven at 60°C. The analyses were processed at the Forest Ecology Laboratory (LABEFLO) of the Federal University of Santa Maria (UFSM). The plant material collected was dried again in a forced-air oven at 70°C up to constant weight, and then ground in a Wiley mill [0.841 mm sieve (20 mesh)] and placed into sealed containers for subsequent chemical analysis. Nutritional analyses were based on 2 g of the dry mass of leaves. The N content was determined by sulfuric acid digestion (H_2SO_4 + H_2O_2) using Kjeldahl method (Büchi, Autokjeldahl K-370); The P, K, Ca, Mg, S, Mn, Zn, Fe and Cu content were determined by nitric-perchloric digestion [HNO_3 + $HClO_4$ (3:1)], using atomic absorption spectrometry method (Perkin Elmer, Analyst 200) for Ca, Mg, Mn, Zn, Fe and Cu at 422.67, 285.21, 279.48, 213.86, 248.33 and 324.75 wave-length, respectively; flame photometry method (Digimed DM – 62) for K content; spectrophotometry (Unico 2100) at 660 wave-length for P content; and turbidimetry (Unico 2100) at 420 wave-length for S determination. Results were expressed as g kg^{-1} of dry weight (DW) for macronutrients (N, P, K, Ca, S and Mg) and μg kg^{-1} DW for micronutrients (Fe, Cu, Mn and Zn). At 4 DAS, the fifth and sixth leaves of four replicates were individually digitalized at a resolution of 300 dpi, with the aid of a scanner. For each leaf, the proportion of phytotoxicity symptoms area was determined using the QUANT software (24). In the four nondestructive replicates, at 7 and 14 DAS was measured the chlorophyll SPAD index using mobile chlorophyll measurer (SPAD-502, Minolta®). Ten readings were taken from the trifoliate leaf of the fifth node, at the central leaflet of all plants.

Statistical analysis. Normal distribution of data was evaluated by the Kolmogorov-Smirnov test at 5%. Statistical analyses were performed using ASSISTAT software 7.7 beta

(22) and the data of all variables were subjected to an analysis of variance (ANOVA). Within each sampling time, the means of the control and TRIF+PROT treatments (for the –mancozeb or +mancozeb treated plants) or the means from the –mancozeb and +mancozeb (for the control or TRIF+PROT treatments) were compared based on the t-test ($P \leq 0.05$).

RESULTS AND DISCUSSION

All the factors and their interactions were significant for the phytotoxicity (4 DAS) and chlorophyll SPAD-502 index (7 and 14 DAS) (Table 1). There was no significant interaction for macronutrients concentration within leaves. The factor multi-site fungicide (+mancozeb or –mancozeb) was significant for S, Mn and Zn nutrients concentration at 4 and 8 DAS. The factor systemic fungicide (control or TRIF+PROT) was significant for S content at 8 DAS.

The presence of mancozeb significantly affected the nutrient content in soybean leaves (Fig. 1). The increases were observed mainly for S, Mn and Zn. The rest of the nutrients assessed did not show any significant change. Such conclusions suggest that, ions present in fungicide molecules might be released, absorbed by the leaves and then used by the plants to its assimilation pathways. The sulfur concentration increased 29% due to mancozeb application at 4 DAS (Fig. 1a) compared to –mancozeb. At 8 DAS both fungicides increased the S concentration, remarkably the combination mancozeb + trifloxystrobin + prothioconazole showed the highest increment (25%) (Fig. 1b). Good levels of chlorophyll and protein are typically dependent on a good supply of sulfur (4). The "green effect" observed may be a reflex of the gain on S content. Furthermore, sulfur plays a crucial role as component of amino acids working as functional groups (R-SH) on metabolic reaction (11) and great part (≥90%) as constituent of the tripeptide glutathione (2). Glutathione has several functions in plants, and its roles in the metabolism have been extensively reviewed by

Rouhier et al. (19), being the antioxidant power, one of its major role. Thus, the gains of S levels in the leaves may be further studied towards glutathione role, in plants exposed to mancozeb and submitted a some abiotic stress.

Manganese content had increases of 42% at 4 DAS and up to 49% at 8 DAS at the presence of mancozeb (Fig. 1c; 1d). The manganese plays an important role, due to its participation in the photosynthesis (O_2 evolution and water photolysis), and in the nitrogen metabolism, especially on the nitrate sequential reduction. Yet, Mn makes part of cyclic compounds as precursors of amino acids, hormones (auxin), phenols and lignin (6). The concentration of manganese in plants has shown high correlation to soybean yield, being foliar applications more responsive than soil application (10).

When sprayed mancozeb the Zn concentration increased 26 and 31% at 4 and 8 DAS, respectively (Fig. 1e; 1f). Zinc is essential to enzymes structure and activation, proteins synthesis, tryptophan, indole-3acid (IAA) metabolism, membrane integrity, RNA and DNA metabolism as well (11), taking over different roles in crops, such formation, partitioning and usage of photosynthesis assimilates. Additionally, zinc performs important role to attenuate the abiotic stresses, like saline stress in soybean (25). The zinc supply has been positively related to yield increase of soybean (8). At the 4 DAS there was a reduction on iron content in plants that received mancozeb application (Fig. 1g). Nevertheless, eight after the application, such response was not evident (Fig. 1h). This may be a reflex of an antagonistic interaction between Zn and Fe that has been reported in crops, which high concentration of zinc affect the absorption and translocation of iron and vice-versa (26). Even though, it was not statistically significant, there was an increment of nitrogen concentration of 5.8 and 4.4 % at 4 and 8 DAS, respectively, as a result of mancozeb application. Such increase may be attributed to the absorption of secondary compounds from mancozeb metabolism such as ethylenediamine

(EDA), ethylenethioureia (ETU), ethyleneurea (EU) and 2-imidazoline, which take attached amine groups (3).

Based on these findings, the green effect observed after mancozeb application is, in part, explained by gains on S, Mn and Zn concentration in leaves. The fungicide TRIF + PROT caused phytotoxicity on soybean plants (6.5%) (Fig. 2), reducing the SPAD index in both dates of evaluation (Fig. 3). Plants sprayed with mancozeb showed increases on SPAD index at 14 DAS. The SPAD index data make evident and aid to prove the "green effect" verified. The SPAD index was greater when plants were treated with mancozeb. It has been noticed that the association trifloxystrobin + prothioconazole demands some attention to spray technology, because of detrimental situations, like high temperature, low air humidity and plant water deficit may increase the intensity of phytotoxicity. However, it can be noticed that there was a reduction on the phytotoxic effect when adding mancozeb to the mixture TRIF+PROT.

Clearly the gains on S, Mn and Zn concentration may be benefit to the soybean crop. These benefits may be better viewed whenever there are limiting factors to a suitable nutritional status, like on soybean crop grown in soils with low fertility (10), in soils presenting high pH, where the micronutrient availability is commonly reduced (1,16), areas with high concentration of NH_4^+ (14), soils under intensive use of phosphate (12), saline soils (25), and even in situations of low water availability, where these nutrients uptake can be compromised (1). In addition, the mechanisms of plant resistance to abiotic and biotic stresses are highly correlated to micronutrients status of the plants (11,17).

In conclusion, mancozeb promoted increases in the concentrations of sulfur, manganese and zinc in the leaves. Such increments explain, in parts, the visual "green effect" in areas treated with mancozeb. These nutritional increments can promote benefits to plants as a better response to abiotic stresses such as fungicide phytotoxicity. Future studies should

investigate possible influences of nutritional gains on resistance to abiotic stresses and on crop yield.

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Table 3. Analysis of variance of the effects of mancozeb spraying (M), trifloxistrobina + prothioconazole spraying (TP) and their interaction for phytotoxicity (Phyto), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu) and chlorophyll SPAD-502 index (SPAD).

Variables	4 DAS			8 DAS		
	M	TP	M × TP	M	TP	M × TP
Phyto	<0.0001*	<0.0001*	<0.0001*	–	–	–
N	0.0511ns	0.9448ns	0.7089ns	0.0553ns	0.1626ns	0.5948ns
P	0.9348ns	0.6926ns	0.3657ns	0.1775ns	0.2249ns	0.6917ns
K	0.8964ns	0.3674ns	0.8364ns	0.2247ns	0.1771ns	0.1771ns
Ca	0.7954ns	0.9542ns	0.3208ns	0.6549ns	0.6198ns	0.1522ns
Mg	0.4807ns	0.1223ns	0.1797ns	0.5022ns	0.4203ns	0.9593ns
S	0.0262*	0.0617ns	0.5628ns	0.0226*	0.0260*	0.4324ns
Mn	0.0001*	0.7266ns	0.2336ns	0.0011*	0.8097ns	0.3026ns
Fe	0.0404*	0.7552ns	0.8960ns	0.4222ns	0.3281ns	0.8478ns
Zn	0.0001*	0.9575ns	0.4944ns	0.0007*	0.9640ns	0.3016ns
Cu	0.8213ns	0.4728ns	0.0876ns	0.0631ns	0.4463ns	0.6561ns
		7 DAS			14 DAS	
SPAD	0.0016*	0.0001*	0.0030*	<0.0001*	<0.0001*	0.0115*

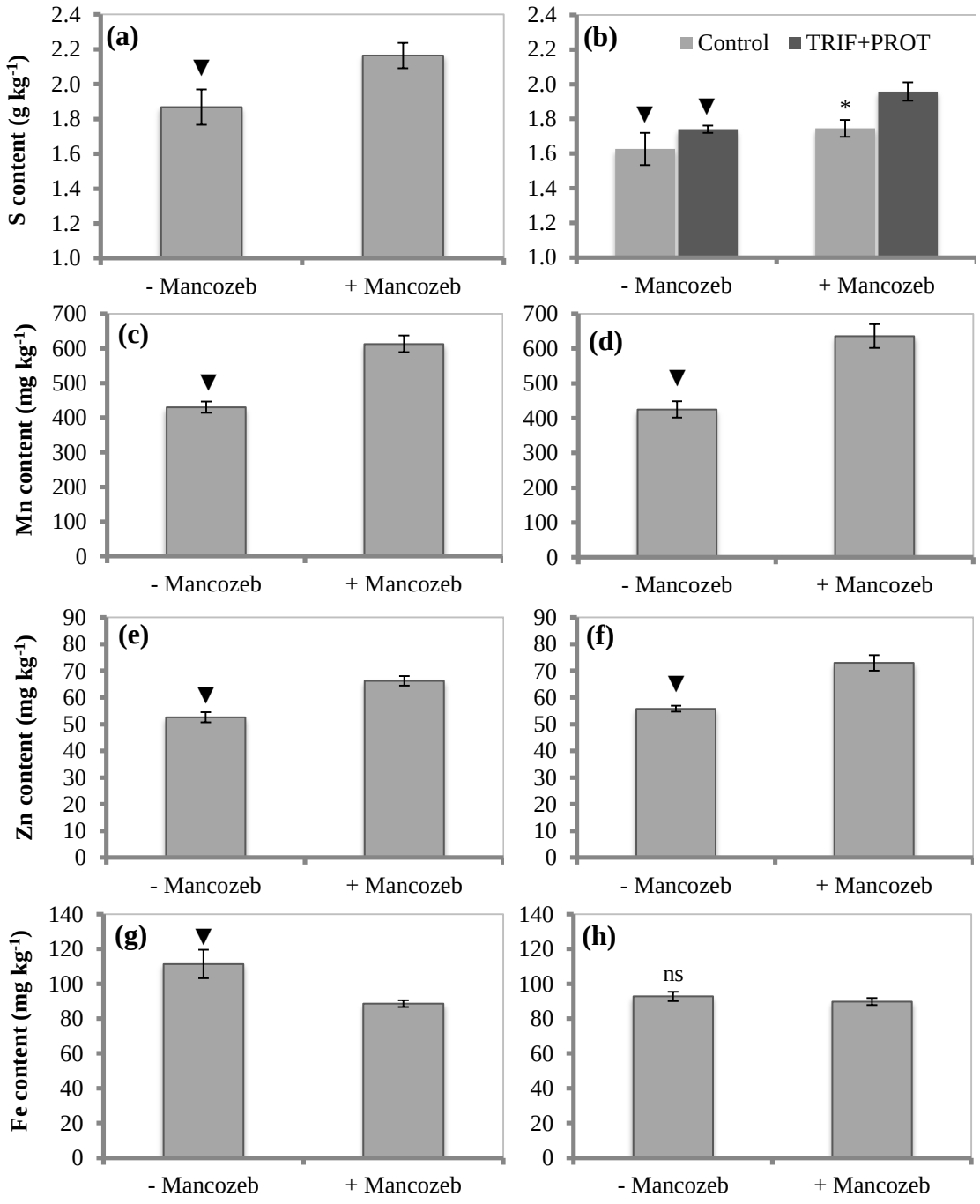

Figure 7. Nutrient concentration on soybean leaves, S (a;b), Mn (c;d); Zn (e;f) and Fe (g;h). Assessment at 4 DAS (a; c; e and g) and at 8 DAS (b; d; f and h). Means of the –Mancozeb and +Mancozeb treatments that are followed by the symbol (▼) are significantly different ($P \leq 0.05$) based on the t-test. Means of the control and TRIF+PROT treatments that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the t-test. Bars represent the standard error (SE) of the means (n = 4).

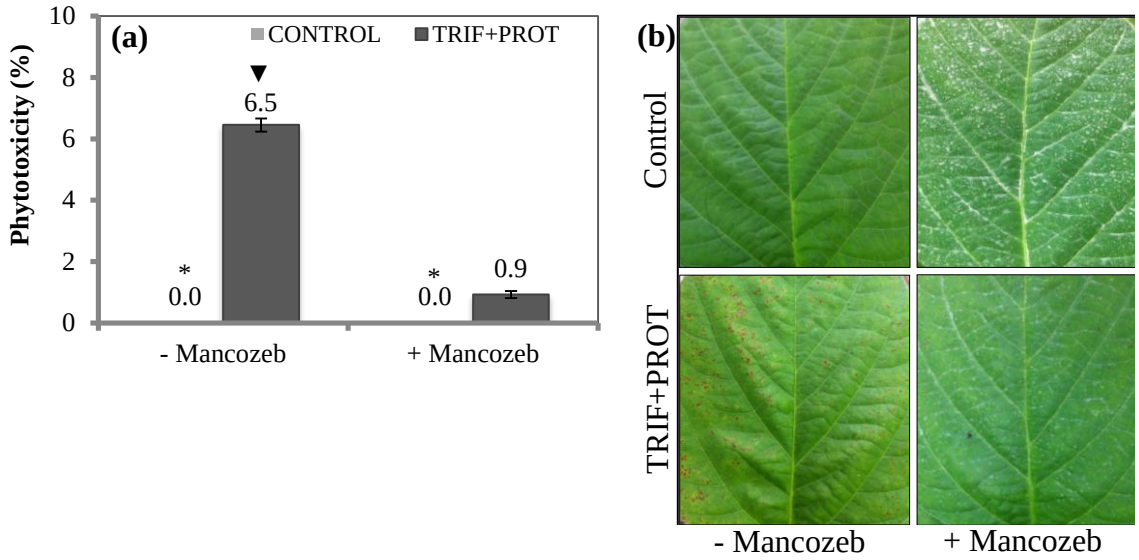

Figure 8. Area with phytotoxicity symptoms (%) (a) and detail of symptoms (b) on soybean leaves at 4 DAS of fungicides treatments on shoot. Means of the –Mancozeb and +Mancozeb treatments that are followed by the symbol (▼) are significantly different ($P \leq 0.05$) based on the t-test. Means of the control and TRIF+PROT treatments that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the t-test. Bars represent the standard error (SE) of the means (n = 4).

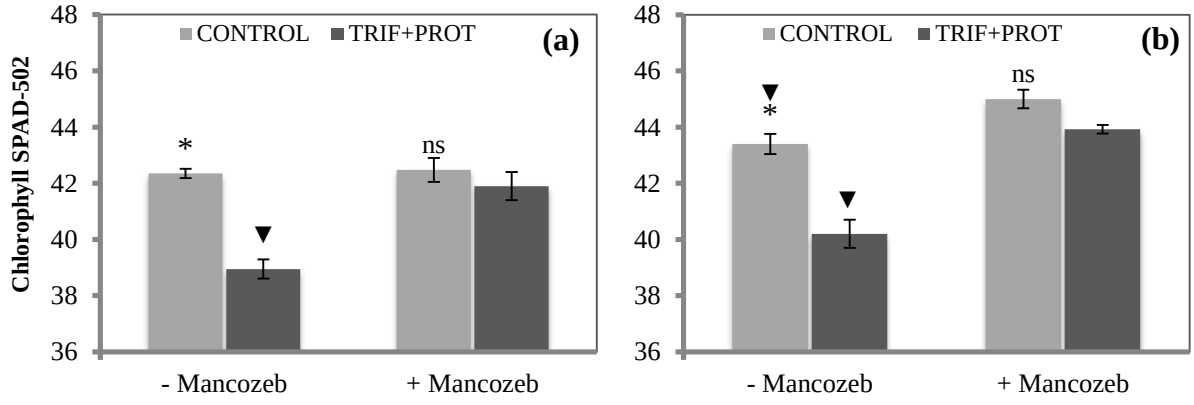

Figure 9. Chlorophyll SPAD-502 index in soybean leaves at 7 DAS (a) and 14 DAS (b) with different fungicides treatments on shoot. Means of the –Mancozeb and +Mancozeb treatments that are followed by the symbol (▼) are significantly different ($P \leq 0.05$) based on the t-test. Means of the control and trifloxystrobin + prothioconazole (TRIF+PROT) treatments that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the t-test. Bars represent the standard error (SE) of the means (n = 4).

CONSIDERAÇÕES FINAIS

Mancozebe apresenta-se como ferramenta importante no controle de ferrugem da soja. Dentre os propósitos de uso dessa molécula, o incremento de controle junto aos fungicidas acompanhantes tem sido marcante. Os incrementos na eficácia de controle foram variáveis em relação à eficácia do fungicida acompanhante. Em associação com produtos de eficácia reduzida, os ganhos são maiores sugerindo como resultado uma interação sinérgica. Em associação com produtos de maior eficácia o incremento é menor, porém espera-se no mínimo uma interação aditiva.

Ficou evidente, que nas condições experimentais utilizadas nesse trabalho, o fungicida sistêmico trifloxistrobina + protioconazol induziu alterações fisiológicas e bioquímicas nas plantas, incluindo aumento da atividade de enzimas antioxidantes, aumento no conteúdo de malondealdeído (peroxidação lipídica), redução de pigmentos fotossintetizantes, redução da atividade fotossintética e assimilação líquida de CO2. Tais alterações foram correlacionadas à ocorrência de sintomas visuais de fitotoxidade nas folhas. No entanto, vale ressaltar que as metodologias utilizadas neste trabalho objetivaram submeter as plantas à situações que favorecessem a ocorrência de fitotoxidade, objeto de estudo desse trabalho. No entanto, a ocorrência de fitotoxidade não é uma regra e a forma como o produto é utilizado pode mitigar tais efeitos deletérios. O produto em si possui um potencial fitotóxico o qual pode ser revertido se condições adequadas de aplicação forem adotadas.

A exposição das plantas a mancozebe refletiu em benefícios fisiológicos nas plantas. Mancozebe teve um efeito atenuador dos efeitos fitotóxicos do fungicida acompanhante trifloxistrobina + protioconazol. Houve melhorias significativas nos parâmetros avaliados quando mancozebe esteve associado com o fungicida sistêmico. Os resultados não permitem definir com clareza qual o mecanismo envolvido da ação de mancozebe como atenuador do estresse abiótico. Investigações futuras poderão fornecer tais esclarecimentos utilizando ferramentas "ômicas". Mancozebe promoveu também melhorias no estado nutricional de folhas de soja aumentando os níveis de enxofre, manganês e zinco.

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