University of São Paulo
"Luiz de Queiroz" College of Agriculture

Yield losses of soybean due to target spot (*Corynespora cassiicola*), its genetic and chemical management

Juan Pablo Edwards Molina

Thesis presented to obtain the degree of Doctor of Science.
Area: Plant Pathology

Piracicaba
2018

Juan Pablo Edwards Molina
Agronomist

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Dr. **LILIAN AMORIM**

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To Evangelina and Amanda, my everyday motivation…

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O rio de Piracicaba vai jogar água para fora

Quando chegar a água dos olhos de alguém que chora

(Tião Carreiro e Pardinho)

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RESUMO

Perdas de rendimento da soja causadas por mancha alvo (*Corynespora cassiicola*) e manejo genético e químico da doença

A mancha alvo é uma doença foliar que pode ocasionar perdas de rendimento na cultura da soja. A doença tornou-se recentemente uma preocupação nos principais países produtores de soja (EUA, Brasil e Argentina), devido à crescente intensidade de suas epidemias. Os objetivos deste estudo foram: i) estimar a eficiência de controle da mancha alvo por fungicidas comerciais registrados no Brasil e a resposta em rendimento da soja, identificando fatores que afetam o desempenho dos fungicidas; ii) caracterizar a relação entre a severidade da mancha alvo e o rendimento da soja, utilizando técnicas meta-analíticas e identificar padrões que permitam compreender a heterogeneidade existente nessa relação; iii) explorar a variabilidade da resistência genética de cultivares de soja e verificar a metodologia mais eficiente para discriminar cultivares suscetíveis de resistentes; iv) explorar os efeitos da interação entre a origem do isolado de *C. cassiicola* e a cultivar de soja no desenvolvimento da epidemia; v) comparar a sensibilidade da posição do estrato do dossel afetado por *C. cassiicola* na redução do rendimento de grãos. Fungicidas compostos pelos ingredientes ativos fluxapyroxad + piraclostrobina foram os mais eficientes para controlar a mancha alvo, com níveis de controle de até 75%, e sua resposta ao rendimento dependeu da pressão da doença (PD, PD_{baixa} < 35% de severidade da mancha alvo na testemunha não tratada ≤ PD_{alta}). Na PD_{baixa}, a aplicação de fungicidas não foi lucrativa, e em PD_{alta} os fungicidas compostos por fluxapyroxad + piraclostrobina apresentaram os melhores desempenhos, superando o rendimento em relação à testemunha em 469 kg ha^{-1} (+ 19,1%). O rendimento potencial geral da soja, na ausência da mancha alvo, foi estimado em 3507 kg ha^{-1} para 41 ensaios distribuídos no Brasil. A redução no rendimento para cada ponto percentual de incremento na severidade da mancha alvo foi calculada em 0,48%. Com isso, níveis (hipotéticos) de severidade de mancha alvo de 50% ocasionariam uma redução de rendimento de 24% (variando entre 8% a 42%). A cultivar de soja teve um efeito significativo para explicar esta grande amplitude de respostas: reduções potenciais (com 50% de severidade) de 11%, 18,5% e 42% foram calculadas, respectivamente, para as cultivares BMX Potência RR, TMG803 e M9144RR. Foi constatada alta variabilidade na resistência genética no germoplasma testado, desde cultivares com intensidade de mancha alvo muito baixa (BRS360) até cultivares altamente suscetíveis. Avaliações da severidade da doença em uma única data não foi tão confiável quanto a avaliação integrada de duas ou três datas. Em experimentos onde 3 isolados de diferentes regiões do Brasil foram inoculados isoladamente em 3 cultivares contrastantes nos níveis de resistência, observou-se que o período de incubação, a severidade da doença, a densidade de lesões e o tamanho das lesões aos 14 dias após a inoculação foram influenciados pela cultivar. Porém, não foi observado efeito da origem geográfica do isolado de *C. cassiicola* nos componentes monocíclicos avaliados. A severidade da doença em diferentes posições da planta, juntamente com as avaliações de desfolha ao longo dos estádios de crescimento reprodutivo da soja, foi correlacionada ao rendimento de grãos. A melhor correlação entre o rendimento de grãos e a intensidade da doença (severidade + desfolha) foi observada nas avaliações em R5.5 na seção média do dossel.

Palavras-chave: *Glycine max*, manejo, eficiência dos fungicidas, meta-análise, resistência genética.

ABSTRACT

Yield losses of soybean due to target spot (*Corynespora cassiicola*), its genetic and chemical management

Target spot is a foliar disease of soybean that can produce yield losses. The disease has recently become a concern due to increasing intensity of its epidemics in the main soybean growing countries (USA, Brazil and Argentina). The goals of this study were to i) estimate the target spot control efficiency and yield response of labeled fungicides for the main soybean growing region of Brazil and identify factors affecting their performance; ii) characterize the relationship between target spot severity and soybean yield using meta-analytic techniques, and to identify patterns which allow understanding the heterogeneity in the relationship; iii) to explore the variability of genetic resistance of cultivars and verify the less time-consuming methodology for doing it; iv) explore the pathogen-host interaction effects on the epidemic development; v) compare the sensitiveness of the canopy strata position injured by *C. cassiicola* to reduce grain yield. Fungicides containing fluxapyroxad + pyraclostrobin were the most efficient ones to control target spot, with control levels of 75% and their yield response depended on the disease pressure (DP, DP_{Low} < 35% target spot severity at untreated checks ≤ DP_{High}). At DP_{Low} was unprofitable the use of fungicides and at DP_{High} the latter fungicides had the best performances increasing yield relative to the untreated check in > 469 kg ha^{-1} (+19.1%). Potential yield of soybean in absence of target spot was estimated in 3507 kg ha^{-1} for 41 trials in Brazil and the percentual reduction for each target spot severity point was calculated in 0.48%, what would represent a reduction of 24% in a hypothetical target spot severity of 50% (ranging from 8% to 42%). The soybean cultivar had a significant effect to explain this wide range of responses: potential losses of 11%, 18.5% and 42% was calculated for cultivar BMX Potência RR, TMG803 and M9144RR respectively at target spot severity = 50%. We observed high variability on the genetic resistance in the tested germplasm: cultivars with very low target spot intensity (BRS360) to highly susceptible cultivars. A single-point disease severity assessment was not as reliably as an integrative three-point assessment, which had no difference with a less time-consuming two-point disease assessment. In trials where 3 *C.cassicola* isolates from different regions of Brazil were inoculated individually on 3 soybean cultivars contrasting in their resistance level we observed that the incubation period, disease severity, lesion density and lesion size at 14 days after inoculation, were influenced by the cultivar and not by isolate geographical origin. Using disease severity coupled with defoliation assessments throughout the reproductive growth stages, we performed correlations between grain yield and the leaf area injury at different plant positions – growth stages. The best correlation was observed with the assessments at R5.5 for injuries at middle canopy section.

Keywords: *Glycine max*, disease management, fungicide efficacy, meta-analysis, genetic resistance

1 THE RE-EMERGENCE OF SOYBEAN TARGET SPOT IN THE AMERICAN CONTINENT

Abstract

Soybean target spot has been considered a minor disease since its first report in USA, Brazil and Argentina. However, changes in the agro-systems have established favorable conditions for the inoculum continuous multiplication and survival. A re-emergence of target spot is being experienced in the main soybean growing areas of the American continent. The high frequency and intensity of recent target spot epidemics have influenced growers to consider the use of foliar fungicides for minimizing economic losses. The limited studies assessing soybean yield losses due to target spot or absence of weather-based forecasting systems difficult farmers' decision-making to plan a fungicide application. The high genetic diversity provides the pathogen a great ability to adapt to different environments and infect a considerable range of crops. This is the first review of this multifaceted pathogen that can act as necrotrophic or even endophytic depending on the circumstances, extracting nutrients from leaves, roots, stems, pods or seeds.

Keywords: *Glycine max*; *Corynespora cassiicola;* disease management; yield loss

1.1 Introduction

Soybean (*Glycine max* [L.] Merril) is an annual legume of the Fabaceae family, considered the most important oilseed crop worldwide. The first historical evidence places the emergence of soybean as a food crop in Northeastern China around 1700–1100 B.C (Hartman *et al.*, 2011). Such evidence is based on observations that semi-natural wild soybeans are extensively distributed in this area but not in other regions (Fukuda, 1933). Soybean seed is composed by 40% of protein and 20% of oil, approximately. Such characteristics represent the highest protein content and the highest gross output of vegetable oil among the cultivated crops in the world (Singh, 2010). The USA Department of Agriculture (USDA) estimates that the global soybean production for 2016/2017 was 338 M Ton (61% of the world's oilseed production). USA (35%), Brazil (30%), and Argentina (17%) are responsible for around 82% of this total production (Faostat, 2016).

Several important abiotic and biotic stresses threaten soybean production by reducing seed yields and/or seed quality. Abiotic stresses, which include extreme levels (high or low) of nutrients, temperature and moisture, reduce soybean production directly or also indirectly through increases in pathogens and pest attacks. Differently from the latter, biotic stresses tend to be geographically and environmentally restricted. Around 11% of the soybean attainable production is endangered by pathogens (Oerke, 2006). More than 40 fungal species are reported to cause significant yield losses to soybean crops (Hartman *et al.*, 2011), and are closely associated to environmental conditions (Yang & Feng, 2001). No-till along with retention of crop residue, has been an agronomic revolution in the farming systems of the soybean region that improves water conservation. However several necrotrophic fungi found a great substrate to grow and survive (Baird *et al.*, 1997). One of them is *Corynespora cassiicola* (Berk & M.A. Curtis) C.T. Wei, the causal agent of soybean target spot.

1.2 Disease background

In the early 1900s, cowpea (*Vigna unguiculata* [L.] Walp) and soybean plants in China were infected by a fungus that produced large and slender, pale olivaceous brown conidia, (Wei, 1950). In the 1930´s, the fungus was identified as *Cercospora vignicola* Kawamura (Tai, 1936; Teng, 1939). A decade later, Liu identified a fungus as *Helminthosporium vignae* Olive on cowpeas in Japan (Liu, 1948).

First reports of target spot at western hemisphere refer to 1944. Olive *et al.* (1945) attributed the defoliation of cowpea crops at La Place, Louisiana and Florida, to a hitherto undescribed species of *Helminthosporium* (*H. vignae* Olive), and was subsequently found to be associated with specimens of the same host from North and South Carolina, and also with soybeans similarly affected in Florida in 1943.

Nineteen years after the detection in the USA, target spot was first reported in Canada in 1963 occurring on roots of mature soybeans grown at three locations in Ontario (Seaman *et al.*, 1965). By that time, in the Southeastern USA, the fungus had already caused premature defoliation of susceptible varieties of soybean (Hartwig, 1959), cowpea (*Vigna sinensis* [Torner] Savi), and sesame (*Sesame indicum* L.), and a leaf spot of cotton (*Gossypi hirsutum*) (Jones, 1961). Despite target spot disease of soybean foliage had not been reported in the northern USA, root and stem rot of soybean in Nebraska has been attributed to infections by *C. cassiicola* (Seaman *et al.*, 1965).

In South America, soybean target spot was first reported in Brazil, in São Paulo state, in 1976 (Almeida *et al.*, 1976). Yorinori *et al.* (1977) stated that the disease had been first observed in Mato Grosso state in 1974. In Rio Grande do Sul state it was identified by Veiga in 1978 in experimental plots at Federal University of Santa Maria. In 1986 root rot symptoms and leaf spots were found at Castro county, Paraná state, and in the following year the disease was detected in Mato Grosso, Mato Grosso do Sul and Rio Grande do Sul states (Yorinori, 1988).

In Argentina target spot was first detected in the Northern region in the late 1980s (Ploper & Ramallo, 1988), although it was not until the late 1990s that it started increasing its prevalence and severity, mainly by the second half of the crop reproductive phase, which led to include target spot in the late season diseases group (Ploper, 2010). This latter group is an artificial classification of the diseases that infect soybean leaves, stems, pods and seeds, and can potentially cause premature senescence, reducing grain yield or seed quality. However, several reports consider target spot different from this disease complex since first symptoms can be detected from flowering stage, simultaneously with the canopy closure, when periods of high humidity are observed (Teramoto *et al.*, 2013)

1.3 Life cycle and disease symptoms

Corynespora cassiicola overwinter on infected soybean debris and seeds and can survive in fallow soil for at least two years (Almeida *et al.*, 2001), acting as primary inoculum source for new epidemics. Alternatively, the pathogen can colonize a wide range of plant residues on soil surface as well as the cysts of the soybean cyst nematode (Carris & Glawe, 1986). It can also survive in stems and roots in the form of chlamydospores (Snow & Berggren, 1989, Oliveira *et al.*, 2012). Wind is the agent responsible for conidia removal and transport, so these processes are favored by dry weather. Maude (1996) called these propagules as “dry spores”. Rainfall is also responsible for the spread of this pathogen at short distances, mainly for secondary infections within a same crop.

Foliar infections are favored when free moisture is present on leaves and the relative humidity is 80% or above (Sinclair, 1999). Under this conducive environment, a germinative tube is formed (Fig. 1), allowing the fungus to penetrate the host tissue (dry weather inhibits infection and colonization in both leaves and roots). At high humidity environment and temperatures from 20 to 30°C, symptoms appear 5-7 days after infection.

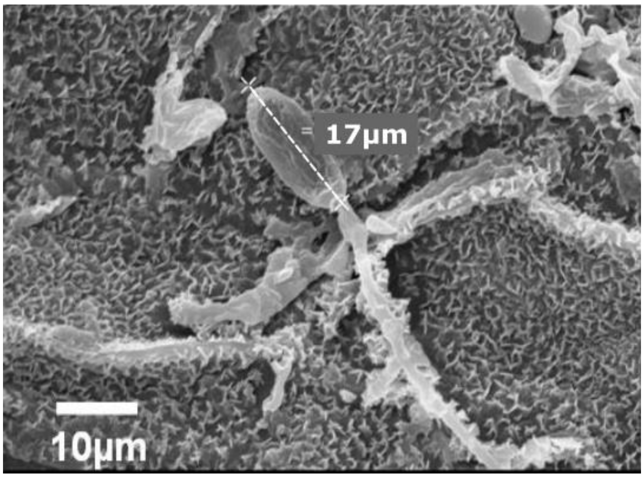

Figure 1 Conidia germination after 48 h of leaf wetness on soybean leaf (electron microscopy photograph). Picture: Mesquini R, *unpublished*.

In vitro experiments showed that *C. cassiicola* conidia germinate within a temperature range of 7°C to 39°C with an optimum at 23°C (Sinclair, 1999; Melo & Reis, 2010) and mycelium grew normally from 7.2°C to 32°C (Mesquini *et al.*, 2011). These latter authors also observed rates of conidia germination of 16%, 33% and 90% after 12, 24 or 48 h of leaf wetness respectively (Fig. 1). Stems and roots infections can occur since cotyledonary stage (Raffel *et al.*, 1999). Soil temperature from 15°C to 18°C are optimal for root infection and disease development. Root rots are common in no-till areas, evidenced by a dry rot that starts as a red to purple spot on the cortical tissue, evolving into a black color. The occurrence of leaf and root symptoms seems to be independent because they are not frequently observed simultaneously at the same field (Yorinori, 1992). Snow & Berggren (1989) reported the existence of at least two different races of *C. cassiicola* affecting soybeans: the race that infects the hypocotyl, roots and stem of soybean, responsible for root rot, and the race that infects leaves, pods and seeds, causing the target spot symptoms. They added that both races are morphologically different from each other. The morphological differences between isolates that cause roots and stems rots and those that cause target spot could indicate that two pathogen species are involved with this disease (Sinclair & Shurtleff, 1975). However, Yorinori (1992) stated that isolates from root were capable of causing typical target spots under artificial inoculations.

Once inside the host plant, the pathogen can release “cassiicolin”, a toxin that kills tissues adjacent to the infection site. The pathogen colonizes and reproduces over the necrotic tissues. Several plant species, including soybean, show similar symptoms disregarded if they come from conidial inoculations or by injecting the purified toxin. This demonstrated that cassiicolin behaves as a host-specific toxin sharing the same host range as the pathogen it was originates from (Barthe *et al.*, 2007).

Symptoms caused by *C. cassiicola* on soybean leaves include roughly circular to irregular necrotic lesions, which may have alternating light and dark rings surrounded by a dull green or yellowish-green halo (Snow & Berggren, 1989). In reference to these symptoms, the disease was commonly referred as ‘target spot’ (Fig. 2A).

Target spot is a typical representative of the light stealer disease group (Boote *et al.*, 1983) since it reduces the soybean photosynthetic leaf area by its symptoms itself and by accelerating the natural senescence process (Fig. 2B). Symptoms are commonly founded in the lower canopy strata (Fig. 2C), moving up in the canopy in case of favorable environmental conditions. Some differences can be observed from this typical field symptom when artificial inoculations are performed at greenhouse. Three types of leaf spots are observed in greenhouse screening assays with conidial inoculations: dark infection point surrounded by chlorotic halo (Fig. 2D); necrotic spot without chlorotic halo (Fig. 2E); and brown-reddish specks restricted to the infection point (Fig. 2F).

Target spot lesions may also form on pods, petioles, and stems like frogeye leaf spot, however laboratory diagnosis are needed to distinguish between both diseases symptoms. When the host leaf tissues are running out, the fungus finally can reach the pods and the seeds or stay in plant residues on the soil until new soybean cultivation is re-established (Almeida *et al.*, 2005).

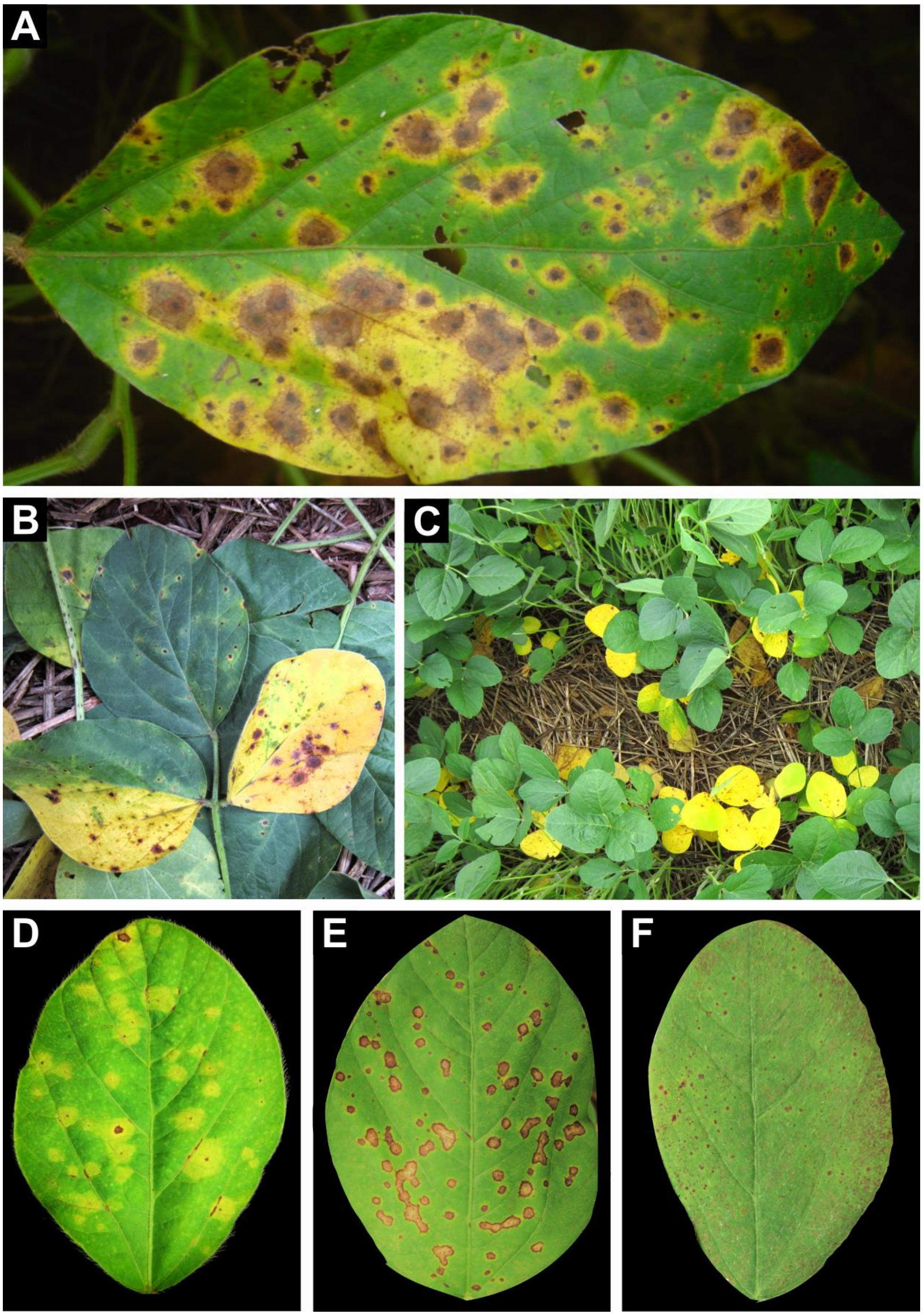

Figure 2 Target spot foliar symptoms. At field environment: A) Typical leaf spot with black center, concentric rings of necrosed tissue and chlorotic halo; B) Induced defoliation at diseased leaf; C) Defoliation at lower canopy. At greenhouse environment, 14 days after conidia inoculation: D) infection point surrounded by chlorotic halo, and venial necrose; E) Necrotic spot without halo (cultivar M9144RR); F) Brown-reddish specks without lesion expansion

1.4 Host range

There is abundant evidence reporting the wide host range of *C. cassiicola* worldwide what led plant pathologists to consider it as a cosmopolitan and unspecific species (Ellis & Holliday, 1971). In addition to soybean, some crops of great economic value are included in this list of susceptible species as: cotton, cowpea, cucumber (*Cucummis sativus*), eggplant (*Solanum melongena*), papaya (*Carica papaya*), rubber tree (*Hevea braziliensis*), sesame, tomato (*Lycopersicum esculentum*), and tobacco (*Nicoteana tabacum L.*), among others (Snow & Berggren, 1989).

Smith (2008) reported that 530 plant species from 380 genera, including monocots, and dicots, can be infected by *C. cassiicola*. On some hosts *C. cassiicola* is also reported to grow as an endophyte or non-specific saprophyte (Gond *et al.*, 2007; Promputtha *et al.*, 2007).

Cross-inoculation assays with *C. cassiicola* and different host species have been performed by several authors examining the host specificity on species of economic importance including soybean. Most of soybean disease compendium or soybean target spot research articles contain extensive lists including host species for which *C. cassiicola* has not even been reported to be crossed-pathogenic with soybean. Olive *et al.* (1945) reported the positive cross infections between *C. cassiicola* (named *Helminthosporium vignae* Olive, by that moment) strains obtained from soybean and cowpea collected from different areas of southern USA. Isolates from soybean, sesame, cowpea and cotton in Mississippi presented no differences in pathogenicity on different hosts (Jones, 1961). Spencer & Walters (1969) confirmed the crossed susceptibility of cotton and soybean. Onesirosan *et al.* (1974) found soybean isolates from southern USA and Mexico to be highly virulent on soybean, sesame, eggplant and cotton. However, different isolates from one host can present differences on their virulence on other hosts. Only one out of two isolates of *C. cassiicola* isolates obtained from tomato were able to infect on soybean (Cutrim & Silva, 2013). Oliveira *et al.* (2006) evaluated the pathogenicity of 15 isolates of *C. cassiicola* obtained from several hosts, to four Japanese cucumber hybrids (*Cucumis sativus*) and they reported that only three isolates from cucumber and one isolate from pumpkin were able to infect the four cucumber hybrids; only two hybrids were infected by the two isolates from soybean.

Cross inoculations performed with three cotton isolates and two soybean isolates on six cotton cultivars and six soybean cultivars under greenhouse conditions showed that both group of isolates were virulent to all hosts. Similar banding patterns were observed for cotton and soybean isolates using ERIC/REP-PCR (Galbieri *et al.*, 2014). They observed that while there was some genotypic variation within the cotton and within the soybean isolates, no clear indication of variation was detected between host species isolates. Similarly, results of both molecular techniques indicated that the *C. cassiicola* isolates attacking cotton and soybean belong to the same strain of the pathogen in Brazil.

A quantitative summary of cross *C. cassiicola* inoculations studies (hosts included in at least five studies) showed that the highest soybean compatibility was observed with cotton, eggplant and sesame (100% of crossed infections) and in lower level (from 40 to 60%) with cowpea, cucumber, tomato and papaya (Table 1). It may be important to consider that planting soybean, near or in a sequence with cotton, may represent a high-risk situation for target spot epidemics at both crops. The latter is a common context in Mato Grosso state in Brazil or the Mid-South states in USA, where the biggest target spot epidemics including important yield losses were observed in the last years (Galbieri *et al.*, 2014).

Table 1 Cross-inoculation studies for *Corynespora cassiicola* isolates obtained from soybean, cotton, cowpea, cucumber, eggplant, papaya, sesame or tomato.

	Inoculation Direction[a]	Cotton	Cowpea	Cucumber	Eggplant	Papaya	Sesame	Tomato
Soybean	→	13/13 (2-5,10,11)	5/5 (1,3,5,9)	9/21 (5,7,8,9)	7/7 (5)	8/9 (5,8)	8/8 (3,5)	10/11 (5,7,9)
	←	6/6 *(2,5,10,11)*	2/8 (1,2,9)	7/14 (5,8,9,10,12)	1/1 (5)	0/10 (5,9)	1/1 (5)	4/20 (5,6,8,9,10,12)
Hosts compatibility		100%	54%	46%	100%	42%	100%	45%

[a] Right arrow indicates studies where *Corynespora cassiicola* strains isolated from soybean, were inoculated on the alternative hosts, and the opposite direction is indicated by the left arrow. References: [1]Olive *et al.*, 1945; [2]Jones *et al.*, 1961; [3]Seaman *et al.*,1965; [4]Spencer & Walters,1969; [5]Onesirosan *et al.*, 1974; [6]Cutrim & Silva, 2003; [7]Oliveira *et al.*, 2006; [8]Oliveira *et al.*, 2007; [9]Dixon *et al.*, 2009; [10]Teramoto *et al.* 2013; [11]Galbieri *et al.*, 2014; [12]Aguiar, 2015.

Not only are several plants species alternative hosts for soybean pathogenic *C. cassiicola* isolates. In Illinois, Carris & Glawe (1986) isolated *C. cassiicola* from *Heterodera glycines* cysts extracted from soybean field soil and re-isolated the fungus after spraying soybean leaves with a conidial suspension at the greenhouse: foliar symptoms, colony, conidia and conidiophores characteristics resembled those typically reported.

Dixon *et al.* (2009) performed phylogenetic analyses using nucleotide sequences of four genes on 143 isolates of *Corynespora spp*., and observed a lack of recombination within the species (asexual reproduction) and six phylogenetic lineages among the pathogen isolates that correlated with host of origin, pathogenicity and growth rate, but not with geographic location of collection.

1.5 Current status of target spot of soybean in the American continent

As we described previously, the first occurrence of soybean target spot in the American continent date back to 1945 in the USA and 1963 in Canada. Hartwig (1959), observed in a five years period study at Mississippi´s Deltas soybean yield losses due to target spot ranging from 18 a 32%. After this period, Hartwig stated that the disease did not developed enough to cause important damage probably due to lower accumulation of rainfalls during the crop. In 2004 in Florida, yield losses from 20 a 40% were estimated for several commercial crops (Koenning *et al.*, 2006). The increasing appearance of first reports of target spots in soybean crossed-hosts as cotton (Fulmer, 2012) or sesame is probably an indication of the pathogen expansion across the Southern USA regions. At the beginning of 2017, target spot turned one of the hot topics in Mississippi due to the severe occurrence of the disease during 2016. Environment was extremely conducive for target spot in 2016, which allowed the detection of extremely susceptible varieties, even one of the local best yielding varieties, including severe defoliation. The problem ranged from the south Delta of the Mississippi all the way up into northeast Arkansas and northeast Mississippi (Allen 2017).

Soybean target spot has been considered a disease of limited importance since it first report in 1976 in Brazil. However, due to the massive adoption of no-till cultivation practices, susceptible cultivars and loss of sensitivity of the fungus to some of the sprayed fungicides, the disease has spread throughout the entire Brazilian soybean growing area in last decade. Currently the disease can cause yield losses all abroad the country. In 2006, an important target spot epidemic was reported with reduction of yield between 10 and 20% (Carregal, 2008). A meta-analysis of fungicide field experiments across Brazil estimated potential overall losses of 24%, however this response was significantly moderated by the cultivar, since some of them (BMX Potência RR) resulted to be highly tolerant to the disease, with potential maximum losses of 8%, meanwhile, for other cultivars (as M9144RR) potential yield losses were estimated in 42% (Chapter 3). Since 2010, target spot is an endemic disease with important yield losses not only for soybean but also for cotton in the Center - Northern agricultural region of Brazil, and over the years, the disease has been found earlier in soybean or cotton crops.

In Argentina, soybean target spot was first detected in 1990 in the Northwest region under subtropical climate In this region after the year 2000 the disease started increasing its prevalence and severity, probably favored by successive years of continuous soybean mono-cropping and no-tillage agro systems (Ploper *et al.*, 2011). In the last seasons, it was considered the most prevalent soybean disease in that region. More than 80% of Argentina’s total soybean production is concentrated in the Pampas region, a temperate area, with cold winter located at the center-east of the country. Since 2015, the disease expanded outside of the subtropical limits and showed the first symptoms in the main soybean-growing region of Argentina.

It can be considered that *C. cassiicola* took its time to settle endemically in the soybean crop at the main producers countries, since its first reports: around 60 years at USA, 28-30 years in Brazil and around 20 years in Argentina (Fig. 3)

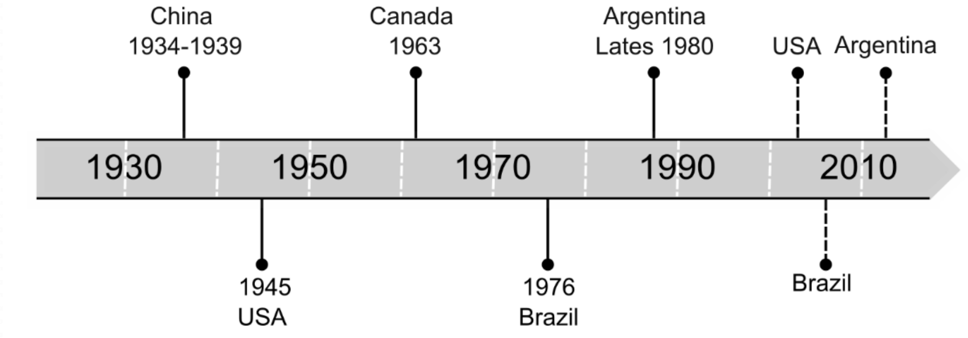

Figure 3 First reports of soybean target spot (solid black lines) and re-emergence reports (dashed black lines).

In Bolivia, about 95% of the soybean crop is produced in Santa Cruz de la Sierra state. Based on climate and soil types, the country could be divided into two different regions, Northern and Southern Santa Cruz. Since 2001 target spot is known as an endemic disease mainly in the Northern region, where under conditions of considerable rainfall may appear early (from flowering: R1 to R2) and reach high levels of severity, causing significant reductions in yield due to premature defoliation. In Southeastern region, the presence of this disease becomes evident later (grain filling stages: R5), due to environmental conditions predisposing and possibly because only soybean is grown during the summer. The increasing intensity of *C. cassiicola* since summer 2005/06, has made it an important disease economic, including important yield losses and difficulty of obtaining satisfactory levels of disease control with traditional fungicides.

1.6 Disease management

The effect of target spot on soybean yield can vary greatly from region to region or from year to year inside a same site. The convergence of at least three factors seems to be needed to cause soybean yield losses: i) varieties with high susceptibility or low tolerance to the disease; ii) intense rainfalls during soybean reproductive stages (Sinclair. 1982, Teramoto *et al.*, 2013); iii) viable inoculum in the field, with presence of lesions at the middle portion of canopy (Chapter 5) (Fig. 4).

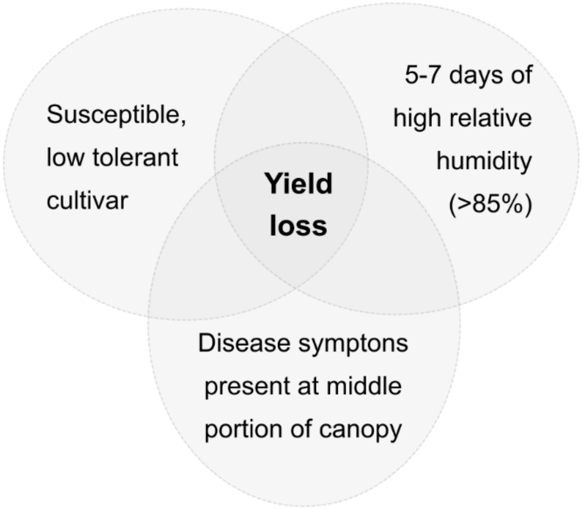

Figure 4 Risk factors associated to soybean yield losses due to target spot.

An efficient target spot management includes reducing the primary inoculum by the use of healthy seeds, however studies evaluating the fungicide efficacy to control target spot on seeds are scarce. A recommended treatment for soybean seeds consists of a mixture of thiabendazol + thiram or carbendazim + thiram (Reis *et al.*, 2010). Low efficiency control of carbendazim has been reported (Xavier *et al.*, 2013) therefore the use of this active ingredient could be compromised for the treatment of seeds in the control of *C. cassiicola*.

The efficiency of crop rotations is a controversial point: while Almeida *et al.* (2001) suggested the adoption of monocot crops to reduce *C. cassiicola* primary inoculum, the reported non-specific saprophytic activity of the pathogen may reduce the interest of this management technique for some technicians and plant pathologists. Avoid planting cotton and soybean in a sequence since, as was demonstrated before, both are 100% compatible hosts for *C. cassiicola* infections.

Cultivar selection based on the resistance to *C. cassiicola* should be a primary action for an integrated disease management. Silva *et al.* (2008) reported that the increase in the target spot intensity in Brazil could be due to factors related to the genetic improvement in the development of new cultivars, which usually seek resistance to nematodes, resulting in greater susceptibility to *C. cassiicola*. Recent cultivar screenings to target spot resistance showed variability in the current commercial Brazilian germplasm, with genotypes highly resistant as BRS360RR (Chapter 4) or with high disease tolerant, showing stable yield even with increasing target spot severity levels.

In case of convergence of the three risk factors, soybean yield losses can be minimized by means of chemical control. Three groups of efficiency were determined in a meta-analysis of labeled fungicides tested from 2012 to 2016 (Chapter 2): higher efficiency group with fluxapyroxad + pyraclostrobin and fluxapyroxad + pyraclostrobin + epoxiconazole (~76% of control efficiency); prothioconazole + trifloxystrobin with intermediate control efficiency (66%) and lowest efficiencies were observed in mancozeb (49.6%), azoxystrobin + bixafen (46.7%) and carbendazim (32.4%). Additionally, this study determined that yield responses depended on the disease pressure (assessed at the untreated plots at R5-R6 growth stage): when target spot severity (whole plant mean) was higher than 35% yield responses of fungicides based on fluxapyroxad + pyraclostrobin had the best performances increasing yield relative to the untreated check: fluxapyroxad + pyraclostrobin + epoxiconazole 503 kg ha^{-1} (+20.2%) and fluxapyroxad + pyraclostrobin 469 kg ha^{-1} (+19.1%). *In vitro* evaluation of six fungicides at two concentrations (50 and 100 ppm) revealed that four fungicides i.e. fluxapyroxad, propiconazole, tebuconazole and hexaconazole completely inhibited the growth of the pathogen (Kurre *et al.*, 2017)

Research effort should be done determining the conducive weather conditions, mainly focused on accumulated rainfalls, what has been informally reported as a risk factor. Cultivar resistance and tolerance should also be furthered studied. Improvement programs may incorporate parental with good resistance level to target spot for the region where the disease is endemic. Fungicide spraying timing and active ingredients mixtures efficacy against the foliar disease complex of soybean, since commonly target spot occurs early than the late season foliar diseases.

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2 META-ANALYSIS OF DISEASE CONTROL EFFICIENCY AND YIELD RESPONSE OF FUNGICIDES FOR SOYBEAN TARGET SPOT IN BRAZIL

Abstract

Target spot (*Corynespora cassiicola*) is an endemic disease on soybean in Brazil. Public and private Brazilian research institutes initiated in 2011 a collaborative network of field Uniform Fungicide Trials (UFTs) to study the efficiency of fungicides to control target spot. The included fungicides were: azoxystrobin + benzovindiflupyr (AZ_BF); carbendazim (CZM); fluxapyroxad + pyraclostrobin (FLUX_PYRA); epoxiconazole + fluxapyroxad + pyraclostrobin (EPO_FLUX_PYRA); mancozeb (MZB); prothioconazole + trifloxystrobin (PROT_TRIF). Sprays (3 for all the fungicides and 4 for MZB) initiated at 45-50 days after sowing and the following applications in up to 21 days intervals. We used network meta-analysis to synthetize and test moderator variables (disease pressure - DP, year of experiment) in the UFTs database from 2012-2016 across the main Brazilian soybean growing region. Modeling fungicide disease control efficiency was not improved by adding any moderator variable, therefore we estimated the overall values and performed contrasts between fungicides. Three groups of efficiency were determined: the fungicides with higher efficiency to control target spot were FLUX_PYRA (76.2%) and EPO_FLUX_PYRA (75.7%); PROT_TRIF had intermediate control efficiency (66.5%) and the lowest efficiencies were observed in MZB (49.6%), AZ_BF (46.7%) and CZM (32.4%). The inclusion of the moderator variable DP ($DP_{Low} < 35\% \leq DP_{High}$) was significant to model the yield response: at DP_{Low} the highest yield response was observed with PROT_TRIF (+342 kg ha^{-1}, +12.8%) and EPO_FLUX_PYRA (+295.5 kg ha^{-1}, +11.2%), however only CZM (+211 kg ha^{-1}, +7.3%) paid the application costs. At DP_{High} fungicides based on fluxapyroxad + pyraclostrobin had the best performances increasing yield relative to the untreated check: EPO_FLUX_PYRA 503 kg ha^{-1} (+20.2%) and FLUX_PYRA 469 kg ha^{-1} (+19.1%). The probability of recovery the investment of spraying the fungicides ($0 \leq p \leq 1$) for a wide simulated ratios of application cost / soybean grain price ranged from 0.26 to 0.56 at DP_{Low} or from 0.34 to 0.66 at $DP_{High.}$

Keywords: *Glycine max*; *Corynespora cassiicola;* chemical control; network meta-analysis

2.1 Introduction

Target spot caused by *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei is a common disease in the tropics and subtropics and can infect more than 380 plant species (Dixon *et al.* 2009). The disease was first reported on soybean in the United States in 1945 (Olive *et al.*, 1945), and now it can be found in most of soybean growing countries. Since its first report in 1976 in Brazil (Almeida *et al.* 1976) target spot has been considered a disease of limited importance. However, due to the massive adoption of no-till cultivation practices, sowing of susceptible cultivars and a decreased sensitivity of the pathogen to single-site fungicides (Xavier *et al.* 2013), this disease has increase its prevalence in Brazilian soybean growing regions, Paraguay, Bolivia and northern Argentina in the recent decade (Ploper *et al.* 2013).

The disease affects leaves, stems, pods, seeds, hypocotyls and roots. The reddish brown leaf lesions, initially observed in the lower to middle part of canopy, are round to irregular varying from specks to mature spots of a centimeter or more in diameter (Snow & Berggren 1989). A yellow halo commonly surrounds the lesions, which often become concentrically ringed at maturity (hence the name target spot). Symptoms can be observed during all the soybean cycle and susceptible cultivars can present intense defoliation. The latter (defoliation up to 50%) happened in 2009 and 2016 in soybeans in mid-South states of USA (Arkansas, Mississippi), coinciding with really wet years (McGee 2017). Some of the key environmental factors that favor disease progression include prolonged conditions (typically 5-7 days) of high relative humidity, or free moisture provided by rain or heavy dew plus warm temperatures (Snow & Berggren 1989).

In the USA, the recommended management practices are the use of cultivars with genetic resistance levels and fungicide seed treatment. In regular years, both management practices can be effective enough to prevent soybean losses due to target spot. However, years with higher than normal rainfalls or long duration of rainfall events at specific growth stages can cause significant yield losses, then fungicide sprays may be adopted to minimize yield losses. A considerable amount of resources has been addressed since 2011 by public and private Brazilian research institutes to study the efficiency of fungicides to control target spot. A collaborative network of field Uniform Fungicide Trials (UFTs) have been evaluated annually the control efficiency of current label fungicides, and estimate the impact of the target spot on yield in the main soybean region of Brazil including a wide range of environments combining locations and growing seasons.

Fungicide effectiveness and yield response studies are mostly limited to report diseases control efficiency and yield data, however, a significant yield response is not information enough to guide farmers' decision-making for crops pest management. In order to maximize growers' profit, and therefore minimize unnecessary, wasteful and environmentally damaging fungicide sprays, technical reports may include at least the cost of the tested technology and calculations of the profit of using it. One further step would be to estimate the probability of recovering the investment what could aid growers to spray or not, or even to select the most suitable fungicide for each particular situation.

Every year, technical reports are published summarizing the UFTs results, however these type of summaries do not model the effects of factors that influence the estimates and variability of fungicide control efficiency or yield response. Meta-analysis, provides a suitable alternative for integrating and interpreting results from multiple individual studies, and particularly in agriculture topics as crop losses due to diseases or fungicide efficiency (Lipsey & Wilson 2000; Madden, Piepho & Paul 2016).

The objectives of this quantitative review synthesis were to: i) determine the target spot control efficiency and yield response of fungicides evaluated in the main soybean growing region of Brazil across the period 2012-2016; ii) identify factors affecting the efficacy of the products included; iii) estimate the probability of economic benefit of applying a fungicide under a range of scenarios of grain market prices and application costs.

2.2 Material and methods

A total of 56 UFTs carried out during five growing seasons (2012-2016, years of harvesting) across five Brazilian states (Paraná - PR, Mato Grosso do Sul – MS, Mato Grosso - MT, Goiás - GO and Tocantins - TO), was available for the present analysis (Fig. 1) (Godoy *et al.*, 2012, 2013, 2014, 2015, 2016). All cultivars used were classified as susceptible. UFTs followed the regional farming practices, and that most trials were sowed at the beginning of the recommended planting date to minimize the probability of soybean rust infections.

Treatments consisted of 3 or 4 applications of fungicides currently labelled for soybean diseases at the Ministry of Agriculture, Livestock and Food Supply in Brazil (Agrofit 2017) (Table 1). A CO^2-pressurized backpack sprayer equipped with a spray wand was calibrated to deliver 200 L ha^{-1} of the fungicide solution on plots. First sprays were performed at 45-50 days after sowing, and the following applications in up to 21 days intervals.

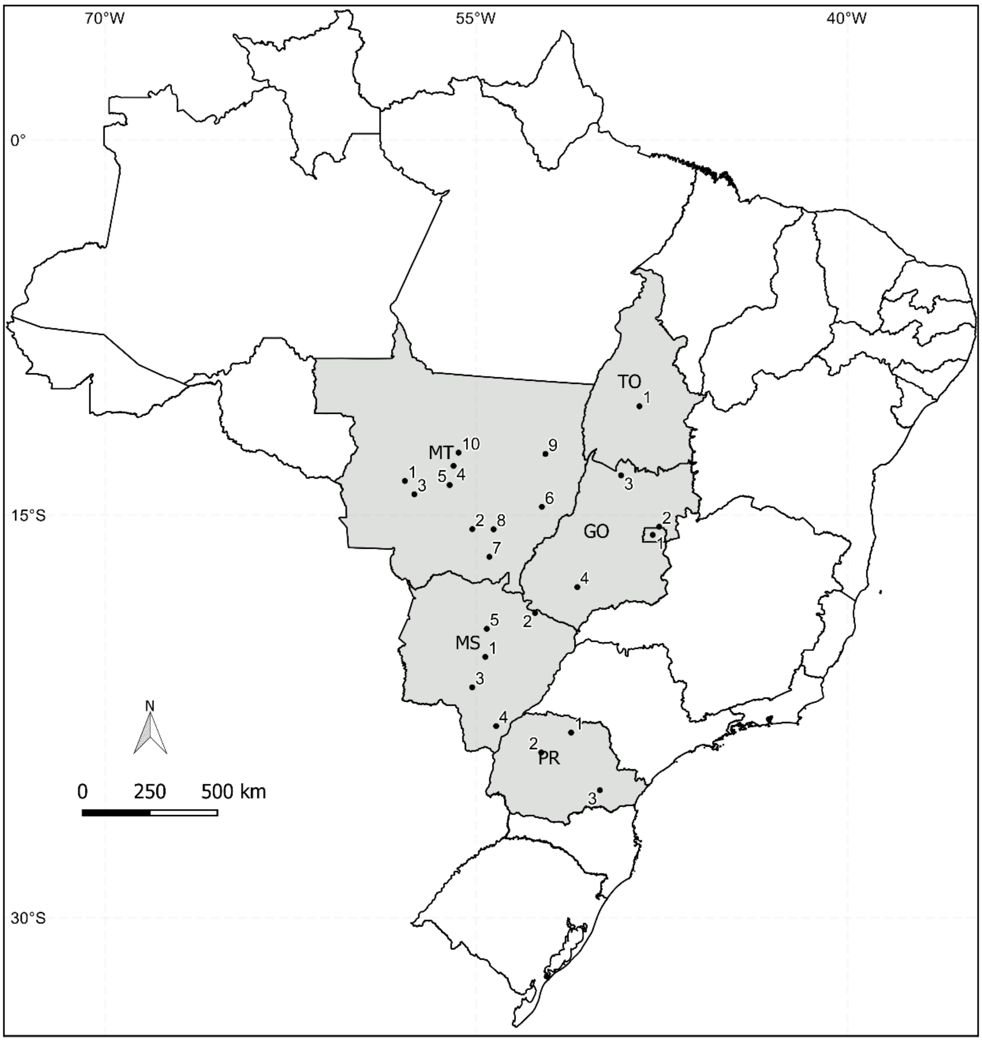

Figure 1 Brazilian states and locations of the Uniform Fungicide Trials sites of conduction. Numbered points inside each state (dark grey) correspond to the experimental locations. Goiás (GO): 1-Brasilia, 2-Planaltina, 3-Porangatu, 4-Rio Verde; Mato Grosso do Sul (MS): 1-Campo Grande, 2-Chapadão do Sul, 3-Maracaju, 4-Navirai, 5-São Gabriel do Oeste; Mato Grosso (MT): 1-Campo Novo do Parecis, 2-Campo Verde, 3-Deciolândia, 4-Lucas do Rio Verde, 5-Nova Mutum, 6-Nova Xavantina, 7-Pedra Preta, 8-Primavera do Leste, 9-Querência, 10-Sorriso; Paraná (PR): 1-Londrina, 2-Campo Mourão, 3-Palmeira; Tocantins (TO): 1- Porto Nacional. Refer to Table S1 for other field-specific information

Table 1 Fungicide treatments included in the meta-analysis: product and active ingredients doses, FRAC classification, owner company, trade name and dose market price.

Fungicide Code (sprays)	Product Dose (l/ha)	Active ingredient (%)	g i.a./ ha	Chem.Group (FRAC code)	Trade name (Company)	US$/ Dose[a]
AZ_BF (3)	0.2	Azoxystrobin (30) Benzovindiflupyr (15)	60 30	QoI (11) SDHI (7)	Elatus (Syngenta)	32.8
CZM(3)	1	Carbendazim (50)	500	MBC (1)	Carbendazim (Nortox)	5.6
FLUX_PYRA (3)	0.35	Fluxapyroxad (16.7) Pyraclostrobin (33.3)	58.5 116.5	SDHI (7) QoI (11)	Orkestra (Basf)	31.5
EPO_FLUX_PYRA (3)	0.8	Epoxiconazole (5) Fluxapyroxad (5) Pyraclostrobin (8.1)	40 40 64.8	DMI (3) SDHI (7) QoI (11)	Ativum (Basf)	31.2
MZB (4)	1.5	Mancozeb (75)	1125	Multi-site contact	Unizeb Gold, (UPL)	12.1
PROT_TRIF (3)	0.4	Prothioconazole (17.5) Trifloxystrobin (15)	70 60	DMI (3) QoI (11)	Fox (Bayer)	31.3

[a] Price consulted in July, 2017, for the state of São Paulo- Brazil.

The experimental design was a randomized complete block with four or five replications. Each replicate plots was at least six rows wide and 5 meters long. Target spot severity was assessed between growth stages R5-R6 with aid of a diagrammatic scale (Soares, Godoy & de Oliveira 2009). Disease severity represented the mean of assessments from the three canopy layers (lower, middle and upper) taken at four locations within each plot. The two center rows were harvest on full maturity and the yield was converted for 13% seed moisture content.

All fungicides included in the present study are currently labelled for soybean diseases at the Ministry of Agriculture, Livestock and Food Supply in Brazil (Agrofit 2017) (Table 1). Evaluated fungicides belong to methyl benzimidazole carbamate (MBC), demethylation inhibitors (DMI), quinone outside inhibitors (QoI), succinate dehydrogenase inhibitors (SDHI) single-site group and one multi-site dithiocarbamate (Table 1). The specific fungicide treatments were selected because they were included at least in 20 studies along the 2012-2016 period. However, throughout this period, the field experiments included four different combinations of these fungicides, i.e. they were not present simultaneously at all the studies, but in four different sets. An untreated check was used as the control.

In meta-analysis, an effect size is any statistic (ratio of means, difference between treatments and its control, etc.) that can be used to evaluate the overall effect of some treatment or the strength of a relationship between variables (Lipsey & Wilson 2000; Borenstein *et al.* 2007). To estimate an overall effect size, random-effects meta-analysis techniques handle the two types of variability that acts in a multi-environment study, giving a weight to each experiment that is an inverse function of the within-study variance (the higher the variance, the lower the precision) and a the between-study variance (inherent differences among trials).

2.2.1 Fungicide control efficiency

The (natural log-transformed) ratio of target spot severity (L^{sev}) was the effect size used to model and test the control efficiency for a specific fungicide, which, within each trial, is given by:

$$L^{sev}{}_{j} = \ln \frac{\overline{Sev}_{Trt}}{\overline{Sev}_{Check}} = \ln(\overline{Sev}_{Trt}) - \ln(\overline{Sev}_{Check}) \qquad (1)$$

Where $\overline{Sev}_{Trt}$ is the mean disease severity at each j-th fungicide treatment and $\overline{Sev}_{Check}$ is the mean disease severity at their respective experiment control plots. Right side of equation is the equivalent form of the ln(ratio) as the difference between logarithmic means (Hedges *et al.*, 1999). For an easier interpretation, $\bar{L}^{sev}{}_{j}$ (overall k-studies mean estimated by a meta-analytic model) can be transformed to fungicide control efficiency (%), calculated as:

$$\bar{C}_j = \left(1 - \exp\left(\bar{L}^{sev}{}_{j}\right)\right) \cdot 100 \qquad (2)$$

By definition, the higher efficiency as the larger negative $\bar{L}^{sev}$, corresponding to a larger positive $\bar{C}$. Only those trials in which mean target spot severity at untreated plots was higher than 15% were kept for assessing the fungicides control efficiency.

2.2.2 Yield response

Two effect sizes were used to estimate the fungicide yield response: the absolute difference in yield (D, estimated directly by the difference between the estimates for the fungicide treatment and the respective untreated check); and the yield response (L^{yld}), following the same form of equation 1, then back transforming and calculating yield response ($\bar{R}$, %) as:

$$L^{yld}{}_{j} = \ln \frac{\overline{Yld}_{Trt}}{\overline{Yld}_{Check}} = \ln\left(\overline{Yld}_{Trt}\right) - \ln\left(\overline{Yld}_{Check}\right) \qquad (3)$$

where $\overline{Yld}_{Trt}$ is the mean soybean yield at each j-th fungicide treatment and $\overline{Yld}_{Check}$ is the mean yield at their respective experiment control plots, then:

$$\bar{R}_j = \left(\exp\left(\bar{L}^{yld}{}_{j}\right) - 1\right) \cdot 100 \qquad (4)$$

where $\bar{L}^{yld}$ is the overall mean estimated (by meta-analysis) yield response for the j-th fungicide. The variances of L (within-study or sampling variance) for each individual study were calculated as $\mathrm{s}_{\mathrm{j}}^{2} = \mathrm{V}/(\mathrm{n} \cdot \bar{Y}_j)$ where, the j subscript refers to the specific treatment, n is the number of replicates (4 or 5) and $\bar{Y}_j$ is the within trial variable mean (disease severity or yield). V is the residual variance or mean square error (MSE) from the ANOVA for the individual study in which Y was analyzed (Paul *et al.* 2008). The variance of D_j was calculated as $\mathrm{s}_{\mathrm{j}}^{2} = \mathrm{V}/\mathrm{n}$, with the same meaning for the equation components as below. Trials with incidence of soybean rust were removed for the analysis of yield response.

2.2.3 Quantitative synthesis of effect sizes

Multi-treatment (or network) meta-analysis was used to summarize L and D since the six fungicide treatments were simultaneously analyzed in different combination of treatments. In this kind of situations researchers often perform a separate meta-analysis for each effect size of interest (e.g., for each pair of treatment means), however they are ignoring the correlation of effect size estimates within studies, which could lead to biased overall results (Higgins *et al.* 2012). Two different ways can be opted for modeling the effect sizes in network meta-analysis: the most common one is based on the contrasts of the treatment of interest with a common reference (e.g. control treatment) also known as the conditional modeling approach, or contrast-based meta-analysis. A simpler approach (adopted in this study), commonly used in plant pathology, is to fit a two-way linear mixed model directly to the treatment means from each study in a two-stage analysis (Greco *et al.* 2015), also known as the unconditional modeling approach or arm-based meta-analysis.

We fitted the model directly to L (for both target spot severity and soybean yield) or directly to the mean yield of treatments to further estimate the yield difference (D) by setting the untreated check as the reference level, following the model:

$$Y_i \sim N\ (\mu, \Sigma + S_i\ )$$

where Y_i is the vector of responses across the k studies (i =1,…,k) for which a normal distribution with a mean μ and a variance-covariance matrix $\Sigma + S_i$ was assumed, since the effect sizes values within a study are functionally related because they were all referenced to a same treatment (the untreated check). Thus the variance-covariance matrix $\Sigma + S_i$ where Σ is a 7 × 7 between-study variance-covariance matrix should be accounted for in the meta-analysis (Higgins *et al.* 2012).

We used an unstructured Σ matrix and the models were fitted to the data with a maximum-likelihood parameter. R "Metafor" package (Viechtbauer 2010) was used to fit all the meta-analytical models. Statistical analysis was performed using the computing environment R (R Core Team 2013) and plots were generated using "ggplot2" package (Wickham 2011).

2.2.4 Moderator variables

The among-study variance (σ^2) reflects the heterogeneity of treatment effects on the estimated effect size across studies. There can be multiple causes of this heterogeneity, such as the diversity in the ways the studies were conducted and other characteristics of the studies (Borenstein *et al.* 2010). Account for study-specific factor effects can be possible by incorporating "moderator variables" in the meta-analytical model (Houwelingen *et al.*, 2002). The effect of the moderator variable for the i-th study on the response vector is given by the vector δi, with seven rows (for the six treatments, plus the check). The model can now be re-written as:

$$Y_i \sim N\ (\mu + \delta_i, \Sigma + S_i\ )$$

where δi is the moderator variable for the i-th study and all other terms are as defined previously. The expected log means for each treatment was no longer a constant vector (μ) but depended on the moderator variables in the particular study (μ + δi). For our purposes, we tested the inclusion of two categorical moderator variables: disease pressure (DP), where target spot severity (TSs) at the untreated check classified trials into two groups, considering 10%, 15%, 20%, 25%, 30%, 35% or 40% as thresholds to classified the trials as low DP (DP_{Low} < threshold) or high DP (threshold ≥ DP_{High}). Year was tested as a factor or continuous variable to verify whether there was a trend of decreasing control efficacy over the years, what could represent, for example, loss of sensitivity to the active ingredients from the *C. cassiicola* populations.

Network meta-analyses involve the simultaneous analysis of both direct and indirect comparisons among multiple treatments across multiple studies, usually randomized trials. A simple indirect comparison may be confounded if the studies involving one of the treatments of interest are fundamentally different from the studies involving the other treatment of interest. Statistical conflicts were called inconsistency (Lu & Ades 2004) and can be tested adding the effect of the "design" (where design indicates here the set of treatments in the study) interacting with the treatments (Piepho 2014). In case of significance, based on the Wald test statistic, is an indicator of inconsistency (Piepho *et al.*, 2015; Machado *et al.* 2017).

In an attempt to explore the effect of cultivar effect over the fungicide yield response, the original dataset was reduced by keeping only the cultivar used at least in five experiments and the fungicides tested at least in 30 trials: cultivars BMX Potência RR, M9144RR, NA_5909_RG, TMG803, and fungicides CZM, EPO_FLUX_PYRA and PROT_TRIF. In this reduced network meta-analysis "cultivar" was tested as moderator variable.

2.2.5 Economic analysis

Treatments yield difference and between-study variance ($\overline{D}$, σ^2) from the meta-analysis reported here were used to calculate the probability (p) of the expected yield response being sufficient to offset the cost in a given simulated fungicide application cost (C, product + operational costs (USD ha^{-1}) and soybean grain market price (Sp, USD kg^{-1}): the C/Sp ratio. The lower the C/Sp ratio, the more favorable context for growers to obtain a profit from spraying a fungicide, under a same level of yield response, since, it turns lower the quantity of soybean grain needed to pay for the fungicide application. This probability is estimated as: $p = \phi[(\overline{D} - \frac{C}{Sp})/\sigma]$, where $\phi(\bullet)$ is the cumulative standard-normal function and σ is the estimated between-study standard deviation (Paul *et al.* 2011; Salgado *et al.* 2014; Machado *et al.* 2017). For the current C/Sp ratio, we fixed the operational costs at $8 USD ha^{-1}; fungicide prices were calculated as the average of three market prices consulted in May 2017 (Table 1); and we used as exchange rate \$3.3 BRL = \$1 USD. Soybean price was the average of the period 2012-2016 as \$330 USD MT^{-1}. Probability of breaking-even the application costs (p) was calculated for a simulated C/Sp combination grid considering both reference cost/prices ± 10%.

2.3 Results

From the 56 trials contained in the dataset 39% were conducted in the state of MT, 23% in GO, 20% in MS and 9% in PR or TO, during seasons 2012 (14%), 2013 (16%), 2014 (28%), 2015 (23%) and 2016 (18%). Only five out of a total of 23 cultivars were used at least five times: BMX Potência RR, M9144RR, TMG803, NA 5909 RG, TMG1179RR.

Target spot severity, based on the untreated plots means, ranged from 6.8% to 75%, with a median value of 29% (Fig. 2A). As expected, the median level of target spot severity was lower in fungicide-treated plots than in the untreated check: 10% to 20% for AZ_BF, CZM or MZB and lower than 10% for EPO_FLUX_PYRA, FLUX_PYRA or PROT_TRIF. Soybean yield at untreated plots ranged from 1160 to 4252 kg ha^{-1}, with a median value of 3174 kg ha^{-1} (Fig. 2B). The six fungicide treatments had higher yield median values than the untreated check but lower than 3500 kg ha^{-1}.

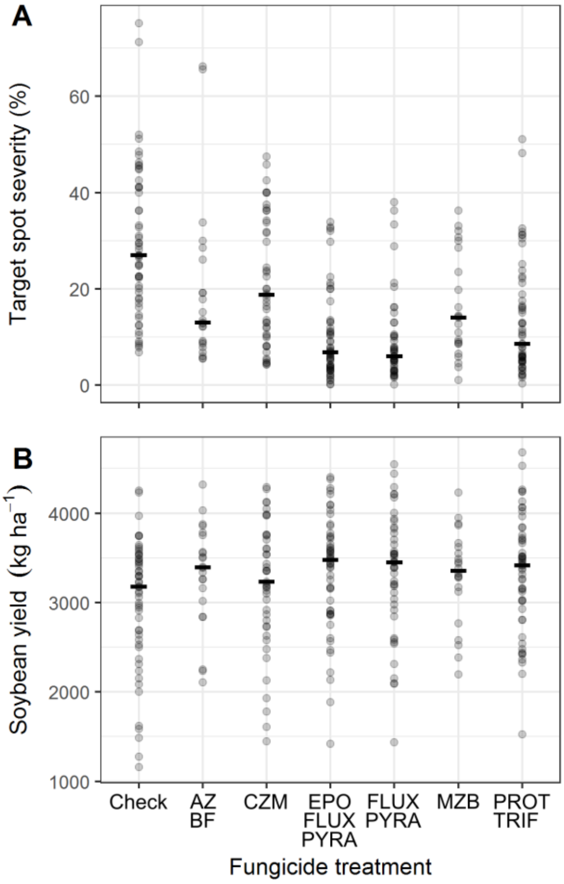

Figure 2 Trial mean values (points) and overall treatment median values (horizontal bold line) of target spot severity (A) and soybean yield (B). AZ_BF = azoxystrobin + benzovindiflupyr; CZM = carbendazim; EPO_FLUX_PYRA = epoxiconazole + fluxapyroxad + pyraclostrobin; FLUX_PYRA = fluxapyroxad + pyraclostrobin; MZB = mancozeb; and PROT_TRIF = prothioconazole + trifloxystrobin.

2.3.1 Fungicides efficiency to control target spot

For all fungicides, $\bar{L}^{sev}$ differed significantly from zero, based on the standard normal test in the meta-analysis (P = 0.009 for CZM and P < 0.001 for the rest of fungicides) (Table 2). Estimated $\bar{L}^{sev}$values ranged from -1.433 to -0.392, corresponding to $\bar{C}$ of 76.2% (FLUX_PYRA) and 32.4% (CZM) respectively. Linear contrasts between $\bar{L}^{sev}$ resulted in significant differences between treatments and three groups of efficiency were determined: the best fungicides to control target spot ($\bar{C}$) were FLUX_PYRA (76.2%) and EPO_FLUX_PYRA (75.7%); PROT_TRIF had an intermediate control efficiency (66.5%) and the lowest efficiencies were observed in MZB (49.6%), AZ_BF (46.7%) and CZM (32.4%).

Table 2 Log of the response ratio (effect size), percent control and corresponding statistics for the effect of six fungicides treatments on soybean target spot. Estimates values based on network meta-analysis of Uniform Fungicide Trials conducted in the main soybean-growing region of Brazil from 2012 to 2016.

		Effects sizes[c]					Control efficacy (%)[d]	
Fungicides[a]	k[b]	$\bar{L}^{sev}$	SE	95%CI	Z	P	$\bar{C}$	95%CI
AZ_BF	20	-0.628	0.179	(-0.28; -0.98)	-3.50	<0.001	46.7	(24.2; 62.4)
CZM	35	-0.392	0.151	(-0.09; -0.68)	-2.59	0.009	32.4	(9.1; 49.7)
EPO_FLUX_PYRA	44	-1.416	0.156	(-1.11; -1.72)	-9.09	<0.001	75.7	(67.1; 82.1)
FLUX_PYRA	37	-1.433	0.164	(-1.11; -1.75)	-8.76	<0.001	76.2	(67.1; 82.7)
MZB	20	-0.684	0.181	(-0.33; -1.04)	-3.78	<0.001	49.6	(28.1; 64.6)
PROT_TRIF	44	-1.092	0.149	(-0.79; -1.38)	-7.31	<0.001	66.5	(55.0; 75.0)

[a] Active ingredients: AZ_BF: azoxystrobin + benzovindiflupyr; CZM: carbendazim; EPO_FLUX_PYRA: epoxiconazole + fluxapyroxad + pyraclostrobin; FLUX_PYRA: fluxapyroxad + pyraclostrobin; MZB: mancozeb; PROT_TRIF: prothioconazole + trifloxystrobin.
[b] Total number of studies used for each specific fungicide treatment and their respective control.
[c] Mean log response ratio ($\bar{L}^{sev}$) for the mean effect of each fungicide treatment on target spot severity relative to check mean (obtained with equation 1); standard error (SE) of $\bar{L}^{sev}$ and 95% confidence interval containing $\bar{L}^{sev}$; Z (standard normal) statistic from the meta-analysis model. P = probability value (significance level).
[d] Mean percent control ($\bar{C}$) (obtained with equation 2) and 95% confidence interval containing $\bar{C}$.

The inclusion of moderator variables was not significant for modeling the fungicides control efficiency: disease pressure or year (factor or continuous variable) did not reduce significantly the between study variability. Null hypothesis of consistency test was not rejected by testing the “design” as moderator variable ($P > 0.05$).

2.3.2 Yield response

Yield coefficient of variation was 23.9% considering only the untreated check means and 20.7% when including all the treatments (n = 238 entries). Therefore, the use of D as effect size was correctly supported (Madden and Paul 2011). D values ranged from -294 to 1024 kg ha^{-1} with a mean value of 296.2 kg ha^{-1}. All the treatments had at least three values D < 0 (from 11 to 17% of all the entries, at each treatment) (Fig. 3). The overall estimated $\bar{D}$ was significantly different from zero for all the treatments, based on the standard normal test (Z) in the meta-analysis ($P < 0.001$). In other words, spraying the fungicides significantly increase the yield relative to untreated plots. For the six fungicides, the estimated $\bar{D}$ values were content in the range 200-400 kg ha^{-1}: EPO_FLUX_PYRA 365 kg ha^{-1}; PROT_TRIF 348 kg ha^{-1}; FLUX_PYRA 330 kg ha^{-1}; MZB 267 kg ha^{-1}; AZ_BF 238 kg ha^{-1}; CZM 209 kg ha^{-1}. Based on the Wald test statistic, lack of inconsistency was observed in the present network (no significant design-by-treatment interaction was found, $P > 0.05$).

2.3.3 Influence of moderator variables on fungicide yield response

The inclusion of moderator variable “disease pressure” with target spot severity = 35% ($DP_{Low} < 35\%\ TSs \leq DP_{High}$) as threshold was significant to model the effect sizes D (P = 0.0252) and L^{yld} (P = 0.037). At DP_{Low} $\overline{D}$ was significantly higher than 0 for all fungicides. The highest yield response in this category was observed with PROT_TRIF (342 kg ha^{-1}, 12.8% higher than the check) and EPO_FLUX_PYRA (295.5 kg ha^{-1}, 11.2% of yield increment) (Fig. 4 for $\overline{R}$ values and Fig. 5 for $\overline{D}$ estimates). AZ_BF presented the lowest estimated $\overline{D}$ value: 182.6 kg ha^{-1} (7% of yield increment). On the other hand, at DP_{High}, the higher values of $\overline{D}$ were observed for EPO_FLUX_PYRA (503.5 kg ha^{-1}) and FLUX_PYRA (469.5 kg ha^{-1}), corresponding to 20.2 % and 19.1% respectively. These two latter fungicides had significantly higher $\overline{D}$ values in comparisons to the same fungicides at DP_{Low} (P = 0.025 and P = 0.011). AZ_BF yield response was marginally significant higher at DP_{High} relative to itself performance at DP_{High} (P = 0.052). Therefore, yield response of CZM, MZB or PROT_TRIF was not affected by DP.

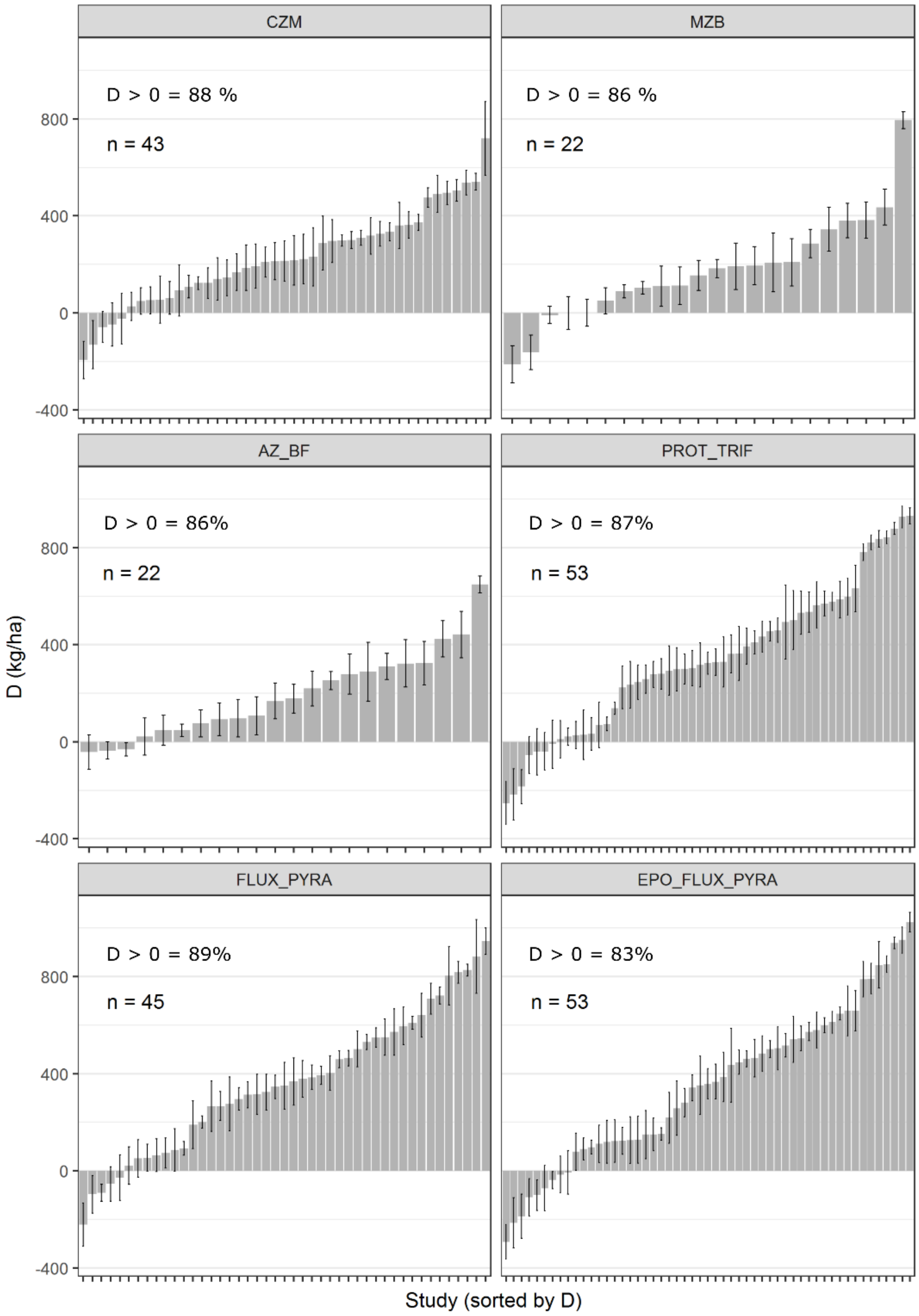

Figure 3. Mean yield differences (*D*, grey bars) between fungicide treated and untreated soybean plots (standard error in black lines) sorted from lowest to highest in x-axis. For each fungicide it is included the percentage of trials with D>0 and their respective number of entries (n). Fungicide codes: A) CZM = carbendazim; B) MZB = mancozeb; C) AZ_BF = azoxystrobin + benzovindiflupyr; D) PROT_TRIF = prothioconazole + trifloxystrobin; E) FLUX_PYRA = fluxapyroxad + pyraclostrobin; F) EPO_FLUX_PYRA = epoxiconazole + fluxapyroxad + pyraclostrobin. All the fungicides sprayed three times, for exception of MZB sprayed four times.

Table 3 Estimated mean soybean yield difference ($\bar{D}$) between fungicide-treated and non-treated plots (with the related statistics) and calculated percent yield return relative to non-treated plot ($\bar{R}$), for the effect of six fungicides on soybean target spot included in the Uniform Fungicide Trials conducted in the main Brazilian soybean growing region from 2012 to 2016.

			Effect sizes					Yield response (%)[e]	
	Fungicide[b]	k[c]	$\bar{D}$[d]	SE	95%CI	Z	P	$\bar{R}$	95%CI
Low disease press (< 35%)[a]	AZ_BF	16	182.6	38.3	(108; 257)	4.8	<0.001	7.0	(4.0; 10.2)
	CZM	29	211.7	34.1	(144; 279)	6.2	<0.001	7.3	(4.8; 10.0)
	EPO_FLUX_PYRA	36	295.5	52.1	(193; 398)	5.7	<0.001	11.2	(6.9; 15.6)
	FLUX_PYRA	33	276.8	42.0	(194; 359)	6.6	<0.001	10.3	(6.7; 14.1)
	MZB	16	209.9	48.1	(115; 304)	4.4	<0.001	8.4	(4.3; 12.7)
	PROT_TRIF	36	342.0	51.6	(240; 443)	6.6	<0.001	12.8	(8.2; 17.6)
High disease press (≥35%)[b]	AZ_BF	6	320.0	70.7	(181; 458)	1.9	0.052	13.4	(4.1; 23.4)
	CZM	14	231.0	60.1	(113; 349)	0.3	0.737	9.6	(2.3; 17.3)
	EPO_FLUX_PYRA	17	503.5	92.5	(322; 684)	2.2	0.025	20.2	(7.7; 34.2)
	FLUX_PYRA	12	469.5	76.1	(320; 618)	2.5	0.011	19.1	(8.4; 30.9)
	MZB	6	300.0	89.0	(126; 474)	1.0	0.311	13.1	(1.1; 26.5)
	PROT_TRIF	17	387.8	91.4	(208; 567)	0.5	0.616	16.3	(3.6; 30.7)

[a] Mean target spot severity for the untreated plots baseline classes

[b] Active ingredients: AZ_BF: azoxystrobin + benzovindiflupyr; CZM: carbendazim; EPO_FLUX_PYRA: epoxiconazole + fluxapyroxad + pyraclostrobin; FLUX_PYRA: fluxapyroxad + pyraclostrobin; MZB: mancozeb; PROT_TRIF: prothioconazole + trifloxystrobin.

[c] Total number of studies used for each specific fungicide treatment and their respective control.

[d] Mean yield difference ($\bar{D}$, kg/ha) for each fungicide treatment relative to check; standard error of $\bar{D}$ (SE) and 95% confidence interval around $\bar{D}$.

[e] Mean yield return ($\bar{R}$, %), calculated by back-transformation of the estimated $\bar{L}^{yld}{}_j$ (following equation 4), lower and upper limits of the 95% confidence interval for $\bar{R}$ (95%CI).

A summary of the overall fungicide efficiency control ($\bar{C}$) and yield response ($\bar{R}$) (this latter modeled with the inclusion of DP) can be observed at Fig. 4. Correlation analysis and simple linear regression were tested, using the disease control and yield response estimates as the independent and dependent variables, respectively. At both DP classes it was observed a significant positive correlation between $\bar{C}$ and $\bar{R}$: DP$_{Low}$ [r = 0.81 (P = 0.049); R^2: 0.66] and DP$_{High}$ [r = 0.98 (P = 0.001); R^2: 0.97].

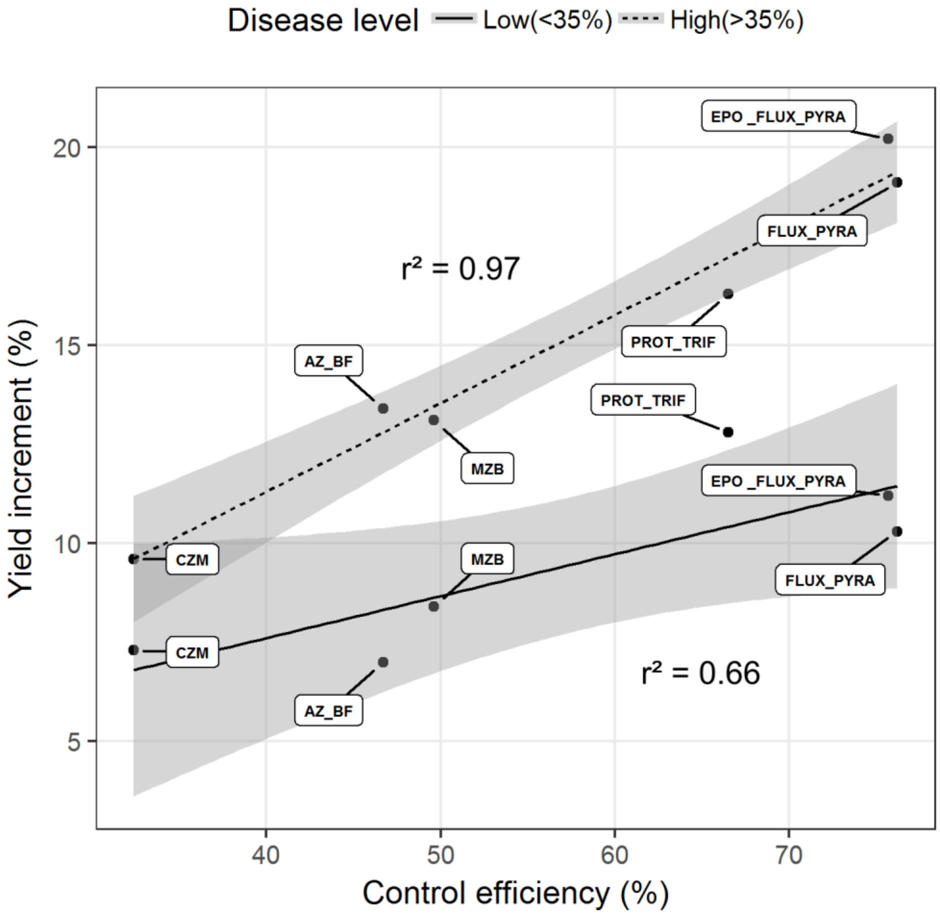

Figure 4 Overall mean control efficiency (%) and yield response (%) for each tested fungicide. AZ_BF = azoxystrobin + benzovindiflupyr; CZM = carbendazim; EPO_FLUX_PYRA = epoxiconazole + fluxapyroxad + pyraclostrobin; FLUX_PYRA = fluxapyroxad + pyraclostrobin; MZB = mancozeb; and PROT_TRIF = prothioconazole + trifloxystrobin.

2.3.4 Economic analysis

The cost of spraying the fungicides (expressed in kg of soybean grains) were calculated in: CZM = 123 kg; MZB = 240 kg; FLUX_PYRA = 350 kg; PROT_TRIF = 350 kg; EPO_FLUX_PYRA = 352 kg; AZ_BF = 373 kg. CZM was the unique fungicide that overcome the application cost at both DP levels: +89 kg ha^{-1} at DP$_{Low}$ or +108 kg ha^{-1} at DP$_{High}$ (Fig. 5). The opposite occurred with AZ_BF, which at both DPs it did not pay the application cost: - 190 kg ha^{-1} (DP$_{Low}$) or -53 kg ha^{-1} (DP$_{High}$). The other estimated fungicides profits at DP$_{High}$ were: EPO_FLUX_PYRA (151 kg ha^{-1}), FLUX_PYRA (120 kg ha^{-1}), MZB (60 kg ha^{-1}) and PROT_TRIF (38 kg ha^{-1}).

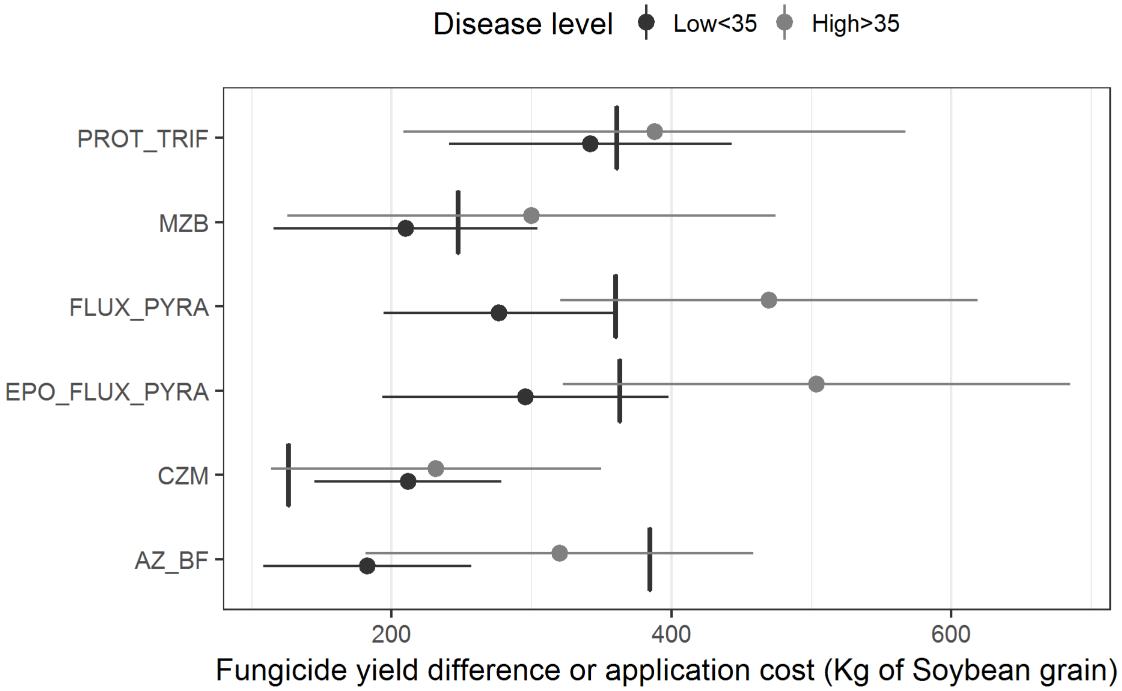

Figure 5 Yield difference ($\bar{D}$, dots) and 95% confidence interval (horizontal lines) for each tested fungicide used to control target spot at both disease baseline classes (Low < 35% TSs ≤ High). Vertical bold lines are the each fungicide application cost (product + sprayings) represented in soybean grain volume (kg) calculated with product prices of July 2017 and mean soybean grain price of period 2012-2016. Fungicide code and respective application costs: AZ_BF = azoxystrobin + benzovindiflupyr (373 kg ha^{-1}); CZM = carbendazim (123 kg ha^{-1}); EPO_FLUX_PYRA = epoxiconazole + fluxapyroxad + pyraclostrobin (352 kg ha^{-1}); FLUX_PYRA = fluxapyroxad + pyraclostrobin (350 kg ha^{-1}); MZB = mancozeb (240 kg ha^{-1}); PROT_TRIF = prothioconazole + trifloxystrobin (350 kg ha^{-1}). All the fungicides were sprayed three times, for exception of MZB that was sprayed four times. $\bar{D}$ values and 95% confidence interval were estimated by network meta-analysis of a database obtained from trials conducted during 2012-2016 in the main Brazilian soybean-growing region.

The probabilities of breaking-even the application costs (p) for the range of simulated C/Sp ranged from 0.26 to 0.56 at DP_{Low} and from 0.34 to 0.66 at DP_{High} across all the fungicides (Fig. 6A and 6B).

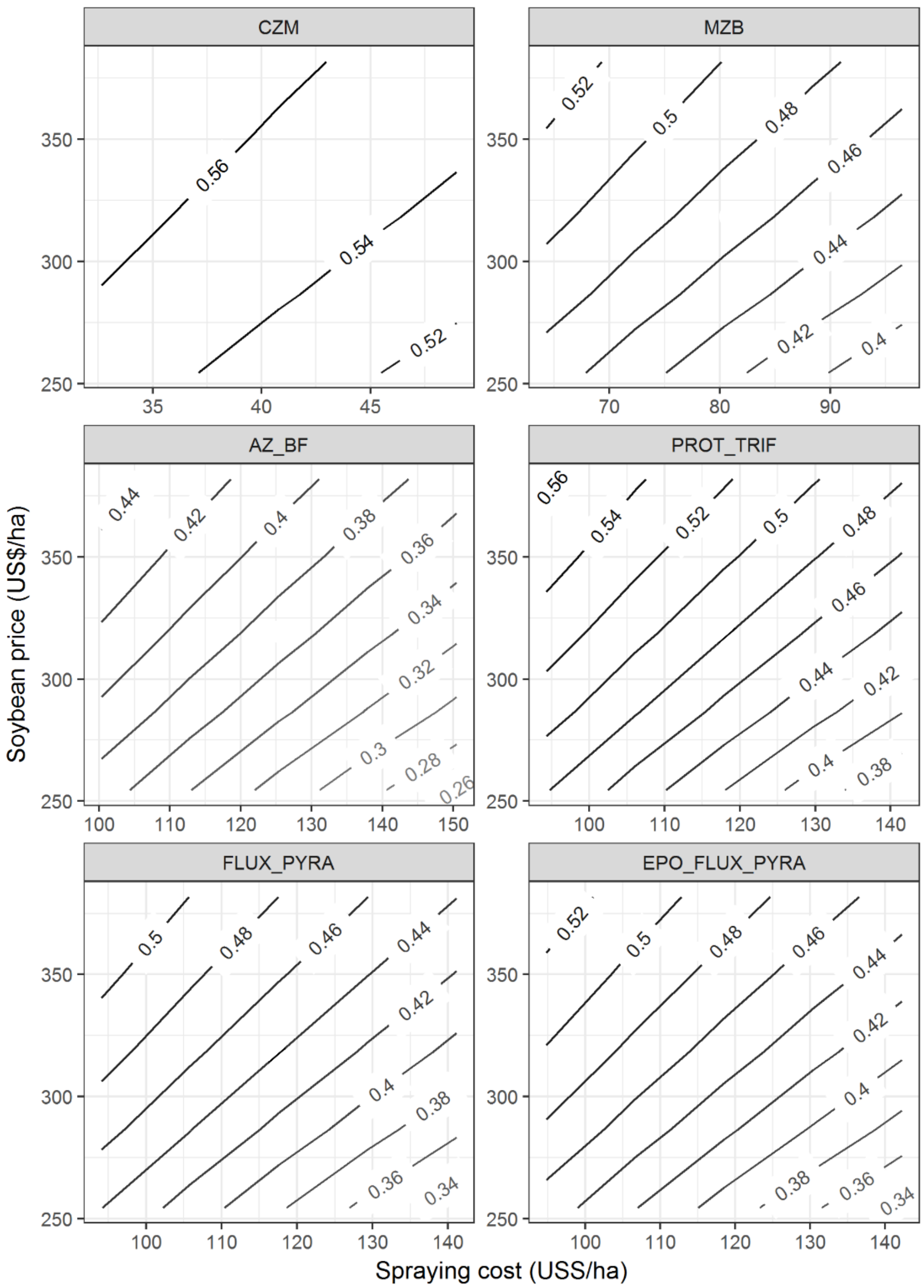

Figure 6A Probability (diagonal lines) of breaking even on the fungicide investment for a simulated range of costs (product + application, x-axis) and soybean trading prices (y-axis), in a low disease pressure scenery (target spot severity < 35% at trial untreated checks) for the fungicide treatments: A) CZM = carbendazim; B) MZB = mancozeb; C) AZ_BF = azoxystrobin + benzovindiflupyr; D) PROT_TRIF = prothioconazole + trifloxystrobin; E) FLUX_PYRA = fluxapyroxad + pyraclostrobin; F) EPO_FLUX_PYRA = fluxapyroxad + pyraclostrobin + epoxiconazole. All the fungicides were sprayed three times, for exception of MZB that was sprayed four times. D values were estimated by network meta-analysis of a database obtained from trials conducted during 2012-2016 in the main Brazilian soybean-growing region.

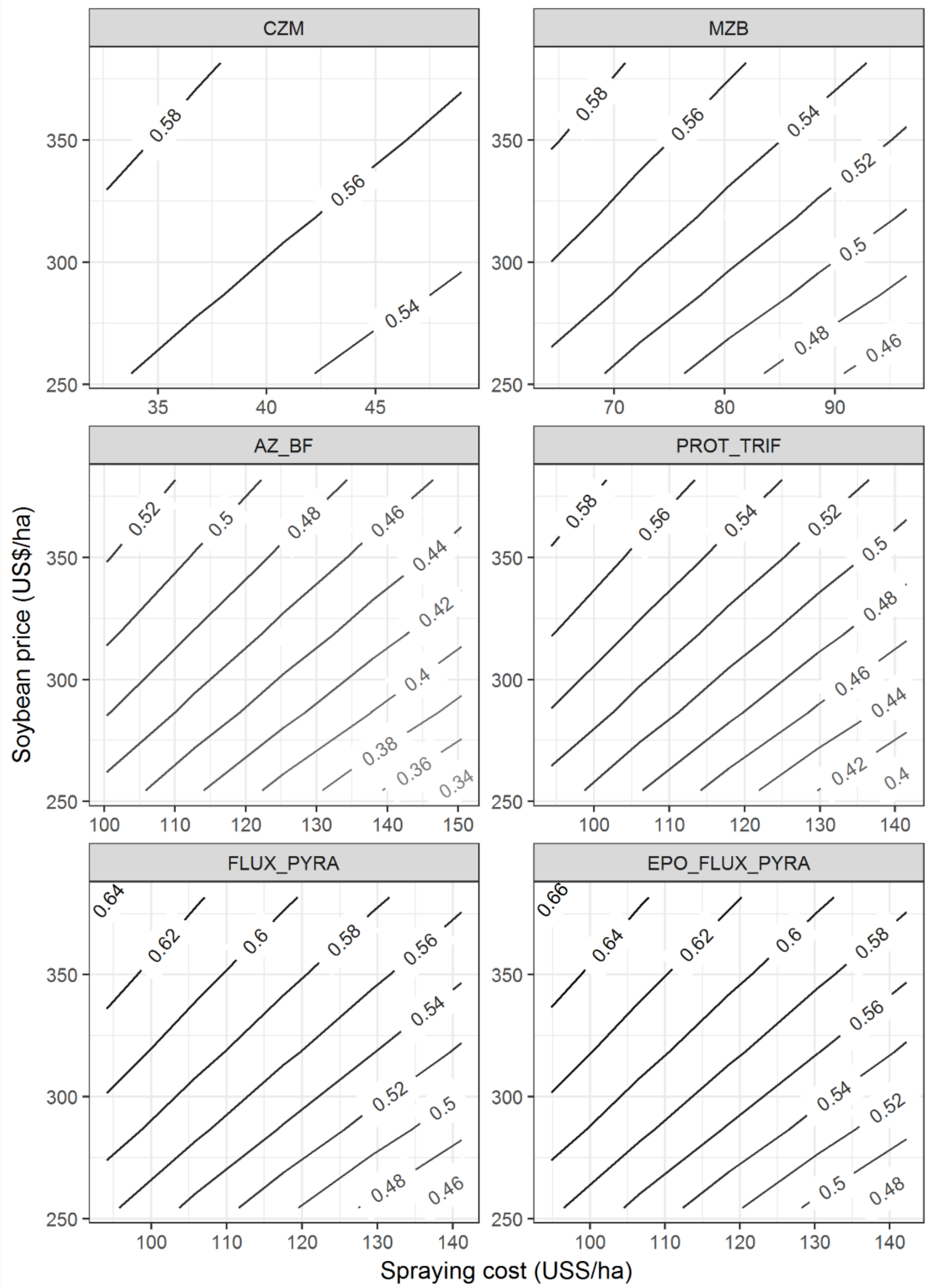

Figure 6B Probability (diagonal lines) of breaking even on the fungicide investment for a simulated range of costs (product + application, x-axis) and soybean trading prices (y-axis), in a high disease pressure scenery (target spot severity > 35% at trial untreated checks) for the fungicide treatments: A) CZM = carbendazim; B) MZB = mancozeb; C) AZ_BF = azoxystrobin + benzovindiflupyr; D) PROT_TRIF = prothioconazole + trifloxystrobin; E) FLUX_PYRA = fluxapyroxad + pyraclostrobin; F) EPO_FLUX_PYRA = fluxapyroxad + pyraclostrobin + epoxiconazole. All the fungicides were sprayed three times, for exception of MZB that was sprayed four times. D values were estimated by network meta-analysis of a database obtained from trials conducted during 2012-2016 in the main Brazilian soybean-growing region.

At DP_{Low} - highest C/Sp levels (most pessimistic economic simulated situation, bottom right plots area) CZM had a $p = 0.52$, and for the rest of fungicides p were lower or equal than 0.4, with a minimum value of 0.26 corresponding to AZ_BF. At the most optimistic simulated situation (lowest C/Sp, top-left plots area) p was higher than 0.5 for all the fungicides with exception of AZ_BF (0.44) (Fig. 6A). At DP_{High} - highest C/Sp (bottom-right) p ranged from 0.34 (AZ_BF) to 0.54 (CZM) and at lower C/Sp (top-left), EPO_FLUX_PYRA and FLUX_PYRA had the highest values of p: 0.66 and 0.64 respectively (Fig. 6B).

2.3.5 Cultivar effect in fungicide yield responses

The interaction cultivar - fungicide had significant effect (P <0.001) on the yield response ($\overline{D}$). For cultivars BMX Potência RR, NA 5909 RG, or TMG803 the effect of fungicides did not have significant differences in $\overline{D}$ (Fig. 7). On the other hand, for cultivar M9144RR the fungicides influenced significantly the yield response: PROT_TRIF (696 kg ha^{-1}) and EPO_FLUX_PYRA (647 kg ha^{-1}) had higher performance than CZM (195 kg ha^{-1}).

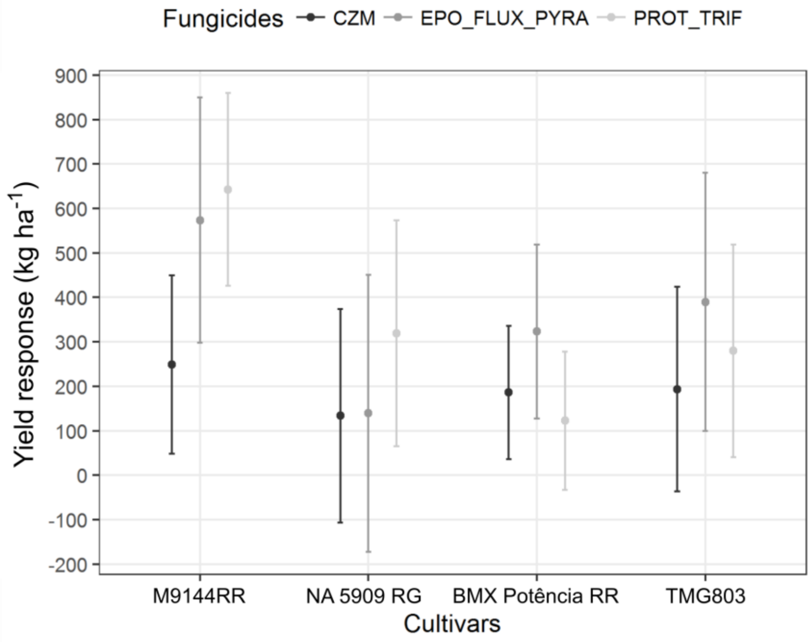

Figure 7 Yield response ($\overline{D}$) estimated by network meta-analysis of fungicides CZM = carbendazim; PROT_TRIF = prothioconazole + trifloxystrobin; EPO_FLUX_PYRA = epoxiconazole + fluxapyroxad + pyraclostrobin (all fungicide sprayed three times), on cultivars M9144RR, NA 5909 RG, BMX Potência RR or TMG803. Trials were conducted during 2012-2016 in the main Brazilian soybean-growing region.

2.4 Discussion

Since target spot turned as an endemic disease in Brazil a general controversy remains on the use of fungicide in soybean crop to improve yield among growers and technicians. This present study does not only synthetize the results for the database across the multi-environments fungicide trials (2012 to 2016 in five states) carried out by public and private Brazilian research institutes, but also it brings new basic insights about the chemical management of soybean target spot.

We detected a wide variability in the fungicide efficacy to control target spot: from very low performance (as carbendazim ≈ 30 %) to products containing fluxapyroxad (SDHI) and pyracostrobin (QoI) fungicides in mixture that presented a consistent efficacy, with control values higher than 75%. These results confirm the stated by several authors from Brazil or even from other soybean growing regions. Field plot fungicide experiments performed in Mato Grosso also reported the best control efficiency with the actives FLUX_PYRA and PROT_TRIF in a same set of four sequential sprayings, reducing the area under the curve of target spot progress in 75% with applications since vegetative crop stages (Belufi, Kempim & Pasqualli 2015). Also in Brazil, two sprays of FLUX_PYRA (at R1 and R1 + 15 d) reduced 47% target spot severity and 22% defoliation relative to the untreated control plots, what led to a yield increment of 13% (285 kg ha^{-1}) in Tocantins state (Ribeiro *et al.* 2017). The latter fungicide treatment, in Mato Grosso state, reduced disease AUDPC in 50% without significant yield increment, but also good control of anthracnose (*Colletotrichum truncatum*) (Basso, Bonaldo & Ruffato 2015). In field experiments conducted in northern of Argentina (subtropical climate) in 2016, the fungicide EPO_FLUX_PYRA had the best performance to increase soybean yield among several tested fungicides, however brown spot (Septoria glycines) was also present together with target spot (De Lisi 2016). The untreated check plots had a mean of ≈ 40 % of brown spot severity and ≈ 25 % target spot, which were reduced by a rate of 77% and 50% respectively by spraying EPO_FLUX_PYRA twice (R3 + R5). In fungicide trials (2014-2015 x 3 locations) in cotton (also host for *C. cassiicola*), spraying FLUX_PYRA significantly slowed target spot development more efficiently than the other tested products (Price 2017).

The low efficiency of carbendazim reported here can be explained by the low fungi-toxicity of the active ingredient itself or to the increase of the proportion of reported resistant isolates in the local *C. cassiicola* populations (Xavier *et al.* 2013; Avozani 2011; Teramoto *et al.* 2012).

To address this particular issue we performed an individual analysis (results not presented) to test the effect of year (as factor or numeric variable) on the carbendazim control efficiency, including at least 8 trials per year from 2012 to 2015: any of the included forms of year resulted significant (as factor P = 0.817 or as continuous P = 0.599). The same results was observed for yield response: year as factor (P = 0.545) or as continuous variable (P = 0.233), confirming a uniform (low) efficiency control throughout the period 2012-2015.

In comparison with other pathosystems, fungicidal control of soybean target spot in Brazil is less effective than soybean rust (Phakopsora pachyrhizi) for which can be found disease reduction of 90-100% relative to their respective non-sprayed plots (Scherm *et al.* 2009). Meta-analysis of fusarium head blight of wheat presented a range of control from 30 to 70 % (Paul *et al.* 2007) indicating similar fungicide efficacy than target spot.

In field experiments conducted in 2016 in Mississippi (USA), fungicide products did not reduce the severity of target spot within the soybean canopy: products applied (at R4) either contained a stand-alone strobilurin (QoI), triazole (DMI), or were applied as a commercially available pre-mix (QoI + DMI) and in one case a three-way pre-mix fungicide (QoI + DMI + MBC - thiophanate-methyl) (Allen 2017). This negative reported effect can be explained by two factors: the use of non-effective fungicides to control target spot (QoI – DMIs) and the delayed timing of application, probably after canopy had closed. Because target spot starts low in the canopy, generally observed from the time the canopy closes, efforts may be focused to identify the early presence of the pathogen. This early spray timing differs from the general soybean fungicide application advice for the mid-southern states of USA, where based on a data set over the last 12 years (without presence of the disease), they observed the greater yield benefit from applying a fungicide between R3 and R4 (T. Allen, personal communication). Fungicide efficacy to control soybean rust was meta-analyzed in Brazil for the period 2003-2007 (Scherm *et al.* 2009) and authors concluded that two well-timed applications are optimal for maintaining yield. In this same line Miles *et al.* (2007), for a broader soybean region, argued that the addition of a third application to control soybean rust was inconsistent in further improving yield.

The UFT trials for target spot is carried out on susceptible cultivars based on field observation since no breeding program release this information in the folders. The main target disease for fungicide application in Brazil is soybean rust and in the case that the cultivar is susceptible to *C. cassiicola,* a fungicide with spectrum for this disease is used.

At low disease pressure context, the highest yield response was observed with the application of PROT_TRIF, however the only fungicide that paid the application costs for the current economical context was carbendazim. Similar trend was observed in a set of fungicide experiments at growers' fields in Texas (USA) under absence of disease pressure: the mixture prothioconazole + trifloxystrobin (sprayed at R3 + R5) was the only fungicide product tested that reported significant yield increments of 23% and 14% in 2010 or 2011 relative to the same cultivar without the fungicide, however net increase in dollars per hectare over the unsprayed check was only observed in 2 out of 8 experiments (Grichar 2013). On the other hand, no changes in leaf area index, dry matter, respiration, transpiration, stomatal conductance, leaf temperature, number of pods or weight of 1000 seeds were observed for the treatment fluxapyroxad, neither alone nor in combination with pyraclostrobin in a non-diseased experiment (Carrijo 2014). The low probability (<0.56 for all simulated sceneries and fungicides) of breaking even the application cost reported here for target spot in low disease pressure context agree with reported by the founds of Mesquini (2012) in field plot experiments during two seasons which not significant yield losses were observed up to 37% of target spot severity in the lower canopy, then considering the whole canopy, and with very low disease severity in the mid-upper sections, it would represent an overall disease severity < 15%. Other soybean canopy necrotrophic disease as frogeye leaf spot (Cercospora sojina), presented a same pattern of yield response to foliar fungicides in Illinois trials with ≈ 200 kg ha^{-1} yield difference in low disease pressure versus ≈ 600 kg ha^{-1} in moderate to high disease pressure on susceptible varieties (Bradley 2009). The same was reported for other crops like corn for which was stated that unless the crop is at risk of developing fungal disease, farmers would be smart to skip fungicide treatments that promise increased yields, adding that fungicides used in fields where conditions were optimal for fungal diseases may improve yields and paid for themselves (Paul *et al.* 2011).

As expected, the most efficient fungicides, had the highest yield difference relative to the untreated check at high disease pressure (≥ 35%): EPO_FLUX_PYRA or FLUX_PYRA, with yield increments of 503.5 and 469.5 kg ha^{-1} respectively (∼19-20%). This threshold of disease severity corresponded with the supported defoliation levels without yield reductions of 33% reported by Begum & Eden (1965). In southern USA, the current defoliation threshold is based on research by (Nettles *et al.*, 1968), who suggested a threshold of 35% defoliation from emergence to flowering and 20% defoliation from flowering until maturity. Despite the high disease efficacy levels of these fungicides, the probability of breaking even at the high disease pressure, and the highest ratio soybean price / fungicide spraying cost context, was 0.65 ± 0.1.

Yield response due to chemical control of soybean rust in Brazil can rise up to higher values than target spot: for example Gasparetto *et al.* (2011) reported 59% of yield increase due to fungicidal sprays; or yield difference of 63% was observed for the best treatments with a same pattern than target spot (highest yield differences at high disease pressure) (Scherm *et al.* 2009). pyraclostrobin and azoxystrobin applied at the R3 growth stage significantly reduced final levels of brown spot; however, significant increases in yield occurred in only three of the six location-years (Cruz *et al.* 2010) what can be comparable with the results obtained in our meta-analysis. In Brazil, similar economic risk analysis was performed for fusarium head blight and the probability of breaking even was >50% what can be considered higher than target spot fungicide control. Despite the lower disease control efficacy, spraying carbendazim resulted in both disease pressure context economical profit, mainly due to the lowest product price, representing 123 kg of soybean grain the cost of 3 applications.

There is a big need for applied research to better understand how to manage soybean target spot, however, based on what was reported here some basic principles of disease management can be establish. One of the main factor than can influence the yield response is the cultivar selection. We observed variability in the cultivar tolerance, i.e. some varieties can maintain yield even in presence of high target spot severity and with other ones yield decrease dramatically with the increase of target spot severity. Brazilian soybean breeding programs have usually seek resistance to nematodes, such as the cyst nematode (Heterodera glycines Ichinohe) widely spread in the Midwest region of the country what could lead to maintain cultivars highly susceptible to *C. cassiicola* (Silva *et al.,* 2008). Therefore, soybean varieties that were observed to be susceptible to target spot should be avoided in fields with a known presence of *C. cassiicola*, or close to specific alternative crop host as cotton (what is a common situation in the southern USA states).

The results obtained in the present work should be taken with caution since sequential applications of the same fungicide are not recommended to prevent the raising of fungicide resistance isolates in the *C. cassiicola* local populations. However, these present results can serve as a guide for estimate and compare the range of disease control of the tested fungicides, their yield response and thus the likelihood of a return on the investment of chemical control as a technique to manage soybean target spot. Therefore, it would be interesting to continue studying the efficiency of different sets of fungicides - number of sprays - timing. Reducing one fungicide spray, from 3 (used in the UTFs) to 2, without differences in control efficiency would reduce application costs and thus, increase the probability of offset the application costs. In a same level of priority, interaction cultivar – fungicide and meteorological conditions that conduce to intense epidemics of target spot need further studies for the specific environments of the soybean growing areas. Interestingly, in both field experiments conducted in 2016 in

Mississippi as in northern Argentina target spot occur simultaneously with brown spot, what support the idea that in general soybean necrotrophic foliar diseases occur as a complex and rarely is only one disease the target of a fungicide application. This important aspects of soybean foliar disease epidemics may be taken into account for assessing all the present symptoms and evaluate the fungicide efficacy for the whole complex of diseases. Planting and production practices that promote a quick canopy closer may represent high risk factors for target spot development, which needs also to be further investigated.

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2.6 Supporting information

Table S1. Dataset description summary of the 56 UTFs conducted during 2012 to 2016 in the main Brazilian soybean-growing region.

Study	Year[a]	State[b]	Cultivar	Blocks	TS Sev_{Check}	$Yield_{Check}$
1	2012	MT	TMG803	4	42.5	2640
2	2012	MT	TMG803	4	36.2	2996
3	2012	MT	TMG803	4	41.2	2366
4	2012	MS	5G830 RR	4	30.6	3347
5	2012	MS	BMX Potência RR	5	27.0	3971
6	2012	MT	TMG803	4	44.8	3230
7	2012	GO	M8336 RR	4	47.8	3178
8	2012	GO	M8336 RR	4	8.2	3447
9	2013	MT	TMG1179 RR	4	40.0	3534
10	2013	MT	5G830 RR	4	48.5	2687
11	2013	MS	NA 5909 RG	4	9.1	3124
12	2013	GO	BRSGO 9160 RR	4	41.1	2827
13	2013	PR	BMX Potência RR	4	8.0	2314
14	2013	PR	BMX Potência RR	4	45.7	2690
15	2013	GO	BRSGO 9160 RR	4	29.5	1160
16	2013	GO	Syn1180 RR	4	26.5	2501
17	2013	MT	TMG7188 RR	4	22.2	1274
18	2014	MS	NA 5909 RG	4	28.1	3641
19	2014	MT	TMG1179 RR	4	51.2	3298
20	2014	MT	TMG1179 RR	4	6.8	3404
21	2014	MT	TMG1179 RR	4	22.5	3546
22	2014	GO	BRSGO 8151 RR	4	19.3	3106
23	2014	MT	TMG803	4	33.2	3618
24	2014	MS	BMX Potência RR	4	7.8	4252
25	2014	GO	BRSGO 8151 RR	4	12.5	3750
26	2014	GO	BRSGO 9160 RR	3	20.1	3750
27	2014	TO	M9144RR	4	41.0	1618
28	2014	MT	BRSGO 8661 RR	4	11.0	2152
29	2014	MT	M9144RR	4	18.0	1486
30	2014	PR	NA 5909 RG	4	14.5	2946
31	2014	GO	ST 810	4	24.8	2511
32	2014	GO	P98Y30	4	12.4	3195
33	2014	PR	BMX Ativa RR	4	29.4	2234

Study	Year[a]	State[b]	Cultivar	Blocks	TS Sev_{Check}	$Yield_{Check}$
34	2015	MS	NA 5909 RG	4	17.0	3219
35	2015	MT	M9144RR	4	36.2	2530
36	2015	MS	BMX Potência RR	4	17.8	4228
37	2015	MS	BMX Potência RR	4	31.0	3488
38	2015	GO	AS3730 IPRO	4	14.0	3577
39	2015	GO	TMG1175 RR	4	20.6	2912
40	2015	PR	NS5445_IPRO	4	10.4	2584
41	2015	MT	TMG803	4	25.1	3742
42	2015	TO	M9144RR	4	45.0	3031
43	2015	MT	TMG1180RR	4	22.5	3488
44	2015	MT	TMG1180RR	4	46.2	2968
45	2015	MT	TMG1180RR	4	28.8	3062
46	2015	TO	M9144RR	4	52.0	1588
47	2016	MT	M8210 IPRO	4	22.5	3170
48	2016	MT	TMG1179RR	4	24.8	3598
49	2016	TO	M9144RR	4	71.2	2084
50	2016	GO	TMG2281 IPRO	4	27.0	3431
51	2016	MT	TMG803	4	45.5	3534
52	2016	MT	NA 5909 RG	4	16.2	3458
53	2016	MS	6968RSF	5	26.8	3293
54	2016	MS	BMX Potência RR	5	32.7	3348
55	2016	MS	8473RSF	5	8.7	3295
56	2016	TO	M9144RR	4	75.2	2001

[a] Harvest year.

[b] States: GO = Goiás, MS = Mato Grosso do Sul, MT = Mato Grosso, PR = Paraná, TO = Tocantins.

[c] Target spot severity at R5-R6 at the non-sprayed treatment.

[d] Soybean yield at crop maturity at the non-sprayed treatment

3 EFFECT OF TARGET SPOT ON SOYBEAN YIELD: META-ANALYSIS OF UNIFORM NETWORK FIELD STUDIES IN BRAZIL

Abstract

Target spot has been reported to inconsistently affect soybean yield, therefore growers may not always benefit from spraying fungicides to control the disease. The lack of robust estimates of soybean losses due to target spot led us to perform this study. Our objective was to verify if soybean yield at R8 (W, kg ha^{-1}) is related to target spot severity at soybean stage R5-R6 (S, %) and to identify patterns that could moderate this relationship. Results from 41 selected Uniform Fungicide Trials carried out in the main Brazilian soybean growing region during 2012-2016 were used to estimate both linear regression coefficients (intercept: β_0, free-diseased yield; slope: β_1, W decrease per unit of S) with mixed models; and Pearson´s *r* correlation coefficient using meta-analysis. Overall coefficients were estimated in $\widehat{\beta_0}$ = 3507 kg ha^{-1}, $\widehat{\beta_1}$ = -17.1 kg ha^{-1} %$^{-1}$; $\hat{r}$ = -0.46. The significant inclusion of two moderator variables split the overall estimates in four context of yield losses resulting from a model containing baseline yield (Low < 3300 kg ha^{-1} ≤ High) effect on β_0 and yield response (to a reference effective fungicide, Low < 10% ≤ High) effect on β_1: potential losses (at S = 50%) ranged from 8% to 42%. Cultivar effect was also a significant moderator: potential losses of 11%, 18.5% and 42% for cultivar BMX Potência RR, TMG803 and M9144RR respectively. We confirmed the reported range of yield losses due to target spot, highlighting the cultivar selection as a key management factor to minimize soybean losses by the disease.

Keywords: *Glycine max*; *Corynespora cassiicola*; yield loss; fungicide; cultivar

3.1 Introduction

Soybean target spot caused by *Corynespora cassiicola* is a common disease in the tropics and subtropics (Dixon *et al.*, 2009). Since its first report in 1976 in Brazil, target spot has been considered a disease of limited importance (Almeida *et al.*, 1976). However, due to the massive adoption of no-till cultivation practices, sowing of susceptible cultivars and a decreased sensitivity of the pathogen to single-site fungicides (Xavier *et al.*, 2013), this disease has spread throughout the entire Brazilian soybean growing area recently (Godoy, 2015).

The pathogen, a cosmopolitan and necrotrophic organism can overwinter on crop debris, alternative hosts or seeds. Infection occurs with the combination temperatures from 20 to 30°C and relative humidity around 80% (Sinclair, 1999). Foliar symptoms are reddish-brown rounded to irregularly shaped lesions that are often surrounded by yellow halos ranging in diameter from 10 to 15 mm. The lesions may develop concentric rings and diseased tissue may become dry hence the common name of the "target spot" disease.

First symptoms are commonly observed in lower strata of canopy, evolving vertically in the plant (Almeida *et al.*, 2005). On very susceptible cultivars defoliation may occur prematurely (Sinclair, 1999). Favorable environment for target spot epidemics commonly occurs in Brazil from mid to late season, at the beginning of reproductive stages simultaneously with crop canopy closure (Teramoto *et al.*, 2013) distinguishing the disease from the soybean late season disease complex: frogeye leaf spot (*Cercospora sojina*), brown spot (*Septoria glycines*) and Cercospora leaf blight (*C. kikuchii*).

Target spot foliar symptoms present a great visual impact in the crop leaf area, however its effect on soybean yield still remains controversial. It was observed that high target spot severity, up to 37 %, at the lower plant canopy do not cause yield reduction in a susceptible cultivar (Mesquini, 2012). This was probably due to the low contribution of this portion of the canopy to the seed formation and filling, in contrast to the medium canopy with higher light interception (Sakamoto & Shaw, 1967).

Target spot is found in most of the US soybean-growing regions, but it also has been considered to be a minor soybean disease. A re-emergence of the disease was detected in 2004-2005 in the Southeastern US probably as a consequence of the changes in weather patterns and in pathogen virulence, and/or the introduction of more susceptible host genotypes (Wrather & Koenning, 2006). A survey carried out in 2006 in that region estimated yield losses due to target spot of 20 % in average to maximum levels of 40 % (Koenning *et al.,* 2006). Even with an increasing incidence of target spot, spraying fungicides to control the disease was considered non-profitable by extensions professionals in Arkansas in 2011 (Faske & Kirkpatrick, 2011). In a field experiment carried out at the subtropical region of Argentina fungicide treatments significantly reduced disease severity but had no effect on yield compared to non-sprayed treatment (Ploper *et al.*, 2013). On the other hand, target spot became the most prevalent disease in the 2014/2015 season in the same subtropical region, spreading into the main Argentine soybean growing area (De Lisi & Ploper, 2015).

Most published crop loss studies are based on results from a small number of locations or years. This type of studies could lead to weak conclusions because of the narrow range of scenarios where the trials were performed (Savary *et al.,* 2006). Ideally, identical experiments should be conducted in all geographical areas where the crop is important, over a period of at least 3 years, using the major cultivars under the range of conditions observed at normal farming practice scenarios (James, 1974).

The increasing occurrence of target spot in Brazil, United States and Argentina stimulated the installation of fungicide field experiments in the last years. However results from multiple environments and years are still rare. The increasing interest in target spot of Brazilian growers, companies and agricultural technicians led public and private research institutes to create a collaborative network of field experiments. This network assesses the control efficiency of current label and pre-label fungicides, and the impact of the target spot on yield in several states. This network is known as Uniform Fungicide Trials (UFTs) and provides a valuable database to perform different type of studies for the most planted cultivars.

Meta-analysis has emerged to stay among crop protection researchers and can be suitable statistical technique to synthesize large amount of information and quantify specific topics as crop losses due to diseases (Madden & Paul, 2011). Our main objectives in this work were: i) to characterize the relationship between target spot severity and soybean yield using meta-analytic techniques, and ii) to identify patterns that allow understanding of the heterogeneity in the relationship disease severity-soybean yield.

3.2 Material and methods

A total of 56 target spot UFTs carried out across five Brazilian states (Goiás, Mato Grosso, Mato Grosso do Sul, Paraná, and Tocantins) during five growing seasons (2012-2016, years of harvesting) was available to study the relationship between soybean yield (kg ha^{-1}) and target spot severity (%). All cultivars used were classified as susceptible and, except by the fungicide treatments, all UFTs followed the agronomic standard management as described by Godoy *et al.* (2012, 2013, 2014, 2015, 2016). Treatments consisted of 2-4 applications of label or pre-label fungicides using CO^2 pressurized sprayers with a volume of 200 L ha^{-1}. First sprays were performed at 45-50 days after sowing (before canopy closure) and the following applications at 15 to 21 days intervals. Treatments were applied upon a randomized complete block with four or five replications, in plots of at least 6 rows, measuring 5 meters lengthwise. Target spot severity was assessed between growth stages R5-R6 (Fehr *et al.* 1971). Disease assessment was performed with aid of a diagrammatic scale (Soares *et al.*, 2009). This particular soybean growth stage is considered the most sensitive to reductions in leaf area with high impact on yield (Fehr *et al.*, 1981). The two center rows were harvested on full maturity and the yield was converted for 13% seed moisture content.

3.2.1 Criteria of study selection

Only those trials in which the range of target spot severity (difference between the minimum and maximum plot severity) was higher than 10% and mean disease severity at untreated check was also higher than 10% were included in the analysis. No trials with significant presence of soybean rust were kept. This selection criteria was accounted by 41 trials that constituted independent studies for this analysis (Table S1). With exception of two trials (located in Paraná state) all of them were located in the tropical savanna ecoregion of Brazil known as “Brazilian Cerrado” with semi-humid tropical climate, with annual temperatures between 22 and 27 °C and average rainfall between 800–2000 mm (Ratter *et al.*, 1997).

In meta-analysis, each primary study is considered independent and has a single result, which is called "effect size". Examples of effect sizes that summarize the relationship between two continuous variables are the Pearson's *r* correlation coefficient (r) and both linear regression coefficients, the intercept (β_0) and the slope (β_1). Linear regressions have been widely used to characterize critical-point models, in which the independent variable is disease intensity at and percentage loss in yield is the dependent variable (Paul *et al.*, 2006; Dalla Lana *et al.*, 2015; Lehner *et al.*, 2017).

3.2.2 Regression coefficients

For the regression coefficients estimates we fitted a mixed multi-level model, allowing the intercepts and slopes to (randomly) vary across subjects (also called a random coefficients model) (Madden & Paul, 2009; Lehner *et al.*, 2017). The study-specific expectation of yield, indicating the mean yield for a given disease index for each individual study is given by:

$$W_{ij} = (\beta_{0i} + u_{0i}) - (\beta_{1i} + u_{1i})\, TS_{ij} + e_{ij} \qquad (1)$$
$$u_i \sim N(0, \tau^2) \; and \; e_{ij} \sim N(0, v_i)$$

where j subscript represents the j-observation (plot) and i subscript represents the i-study, both for the yield (W, at R8), or target spot severity (TS, assessed at R5-R6). β_0 and β_1 are the population average intercept (kg ha^{-1}) and slope (kg ha^{-1} %$^{-1}$); u_{0i} and u_{1i} are the effect of the i-study on the intercept and the slope, respectively, considered as random variables (with mean 0 and variances τ_{u0} and τ_{u1}), also known as the study-specific deviations; and v_i are the (approximately) known sampling variances of the observed outcomes. The sum of β_0 and u_{0i} or β_1 and u_{1i} yield the “Best linear unbiased prediction” (BLUP) for both parameters respectively.

The lmer function in the lme4 R package (Bates *et al.*, 2015) was used to fit the data using maximum likelihood method.

The inclusion of the moderator variables expanded the random model to a mixed model:

$$W_{ijk} = (\beta_{0i} + \delta_k + u_{0i}) - (\beta_{1i} + \theta_k + u_{1i})\,TS_{ijk} + e_{ijk} \qquad (2)$$

A third subscript was added for the estimated yield and observed target spot severity (W, TS) for the j-observation of the i-study of the k-level of the moderator variable; δ_k and θ_k represent the fixed effect of k-level of moderator variable in the intercept and in the slope respectively.

Five moderators variables that can potentially account for the variability between studies were tested: year of experiment (from 2012 to 2016); “Disease pressure” based on the study mean untreated check disease tested as a continuous variable or factor considering the severity level that significantly moderated the fungicide yield response in Chapter 2 (DP: Low $< 35\% \leq$ High); “Baseline yield” based on the mean study yield of the most efficient fungicide treatment (healthy plots) available: epoxiconazole + fluxapyroxad + pyraclostrobin (RF: Reference fungicide, Low < studies median yield = 3445 kg ha^{-1} ≤ High) (Chapter 2) included in all the studies; “Yield response” based on the % difference between non sprayed check and the latter fungicide treatment (YR, [yield$_{(RF)}$ / yield$_{(Check)}$] *100: Low < 10 % ≤ High) (Scherm *et al.*, 2009); and cultivar growth habit (determinate: interruption of vegetative growth at flowering stage; indeterminate: persistence of vegetative growth after flowering).

3.2.3 Correlation effect sizes

Pearson’s r correlation coefficients were estimated and then transformed to Fisher’s z (with best statistical properties) for each i-study at the plot level with their corresponding variance estimated with the following equations:

$$z_i = 0.5 \ln\left(\frac{1+r_i}{1-r_i}\right) \qquad (3)$$

$$Vz_i = \frac{1}{n_i - 3} \qquad (4)$$

where z_i is the Fisher's *z* transformation of Pearson's *r* coefficient for the i-study; Vz_i is the variance of z_i and n_i is the sample size, or the number of pairs plots used to estimate *r* in the i-study.

To estimate the population average effect size of the Fisher's z coefficients a standard univariate random effect meta-analytic model was fit via maximum likelihood method following:

$$\gamma_i = \mu + b_i + e_i \qquad (5)$$

where γ is the i-vector of effect sizes (z); μ is the population average of *z;* and b_i is the estimated random effect of the i-study, and e_i is the residual from each i-study. Overall means and 95 % confidence interval (95 % CI) were calculated. All steps of the correlation meta-analysis were performed using the "metafor" R package: the calculation of z_i and Vz_i via *escalc* function and the fitting of the random or mixed models via *rma* function (Viechtbauer, 2010).

The inclusion of the moderator variables (detailed at regression coefficient section) expanded the standard random-model to a univariate mixed-model:

$$\gamma_i = \mu + b_i + \delta_k + e_i \quad (6)$$

where γ is the i-vector of effect sizes (z); μ is the population average value of z*;* and b_i is the estimated random effect of the i-study; δ_k represent the effect of the k-level of the moderator variable (for instance, the two levels of the baseline yield: low or high), and e_i is the residual from each i-study.

3.2.4 Subset database for testing cultivar effect

The full database with selected studies, was reduced to 23 studies containing the most used cultivars in the UFTs: BMX Potência RR, n = 7; M9144RR, n = 8; and TMG8003, n = 7. The objective was to test cultivar effect and estimate both linear regression coefficients of the relationship soybean yield – target spot severity for each selected cultivar and summarize Pearson´s *r* correlation coefficient.

3.2.5 Appropriateness of the models

The Akaike Information Criterion (AIC, the lower the better) and the likelihood ratio test was used to determine if the additional parameters for moderator variables (on the intercept, the slope or both) increased the likelihood function significantly, allowing us to define the model structure with best goodness of fit.

3.2.6 Prediction and relative yield loss

To allow the comparison of this study results with other published reports, a damage coefficient (DC) was calculated by dividing the estimated slope (kg ha^{-1} %$^{-1}$) by the respective intercept (kg ha^{-1}) and multiplying by 100. Besides this, the damage coefficient (%$^{-1}$) can be used to predict the relative crop loss at any level of target spot severity (Madden & Paul, 2011):

$$L_i = \left(\frac{\beta_1}{\beta_0}\,100\right) TS_i \qquad (7)$$

where L_i is the percent yield reduction (%) for the *i*-severity level of target spot severity (TS_i), β_0 and β_1 are the parameters (intercept and slope) estimated by the meta-analytic models. For example, one can predict the potential yield loss at the maximum level of target spot severity commonly observed at the field (50%) adding a subscript to L to indicate the level of TS for what L was calculated: L_{50} (obtaining a potential yield loss).

3.3 Results

3.3.1 Variables description for primary studies

A considerable **v**ariability was observed in the 41 selected studies: target spot severity in the untreated plots ranged from 11% to 55% with a median of 29.4% (Fig. 1A), and soybean yield at the reference fungicide treatment ranged from 2134 to 4401 kg ha^{-1} with a median value of 3537 kg ha^{-1} (Fig.1B).

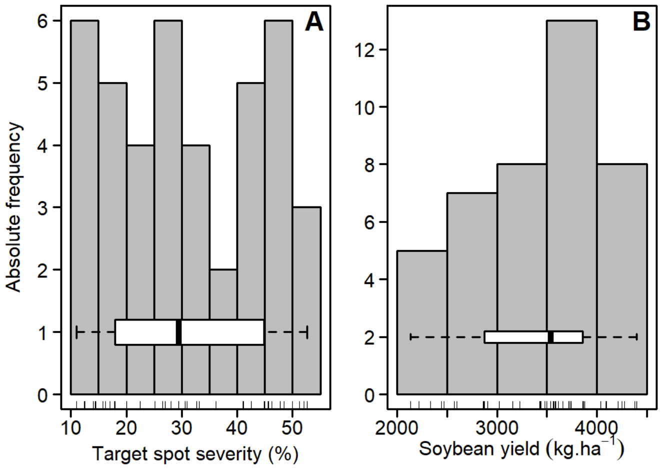

Figure 1 Histograms of observed mean target spot severity at untreated check (A) and soybean yield at the reference fungicide treatment (epoxiconazole + fluxapyroxad + pyraclostrobin) (B), for the 41 selected uniform trials performed in Brazil in seasons 2012 to 2016. Horizontal white boxplots indicate interquartile range (IQR) and black thick marks are the median values.

With the exception of three studies, a general trend showed a negative linear relationship between soybean yield and target spot severity: the higher the levels of the disease severity, the lower was the yield. The study-specific linear regression coefficients varied from 2203 to 4850 kg ha^{-1} (intercepts) and from -60.8 to 9.1 kg ha^{-1} %$^{-1}$ (slopes). Considering all the plots (sprayed with fungicides varying in their efficacy), soybean target spot severity had a median value of 13.7% and soybean yield of 3366 kg ha^{-1} (Fig. 2).

The Pearson's r correlation coefficients had a median of -0.49 and a slightly right skewed – bimodal distribution. However, Fisher's z transformation reduced partially the skewness showing a more symmetrical distribution around the mean with a median value of - 0.54 (Fig. S1).

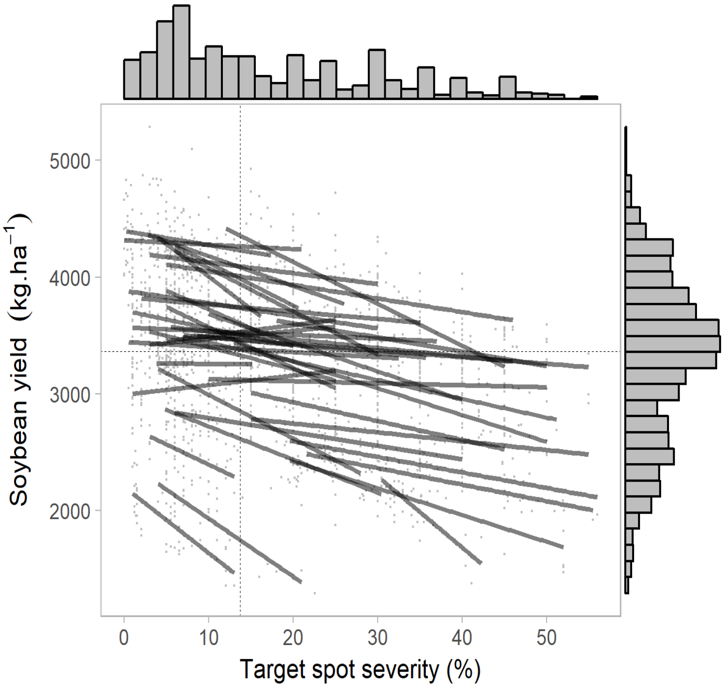

Figure 2 Linear regressions of the relationship soybean yield ~ target spot severity for the 41 selected uniform fungicide trials performed in Brazil in seasons 2012 to 2016. Marginal histograms of soybean yield (vertical) and target spot severity (horizontal). Dashed lines inside plot represent median values of each variable.

3.3.2 Linear regression coefficients

A significant (P < 0.001) likelihood ratio test suggested that the model (equation 1) considering the intercept and slopes as random effects was the best model to summarize the overall relationship between soybean yield and target spot severity. The population-average estimated regression coefficients were 3564 kg ha^{-1} for the intercept and -17 kg ha^{-1} %$^{-1}$ for the slope) (Table 1, Fig. 3).

With the estimated regression coefficients the damage coefficient was calculated as 0.48 %$^{-1}$ what would represent a yield loss L_{50} = 24%. BLUPs histograms showed a slight left skewed distribution for the intercepts with a highest accumulation from 3000 to 4000 kg ha^{-1}, and a bimodal distribution at the slopes.

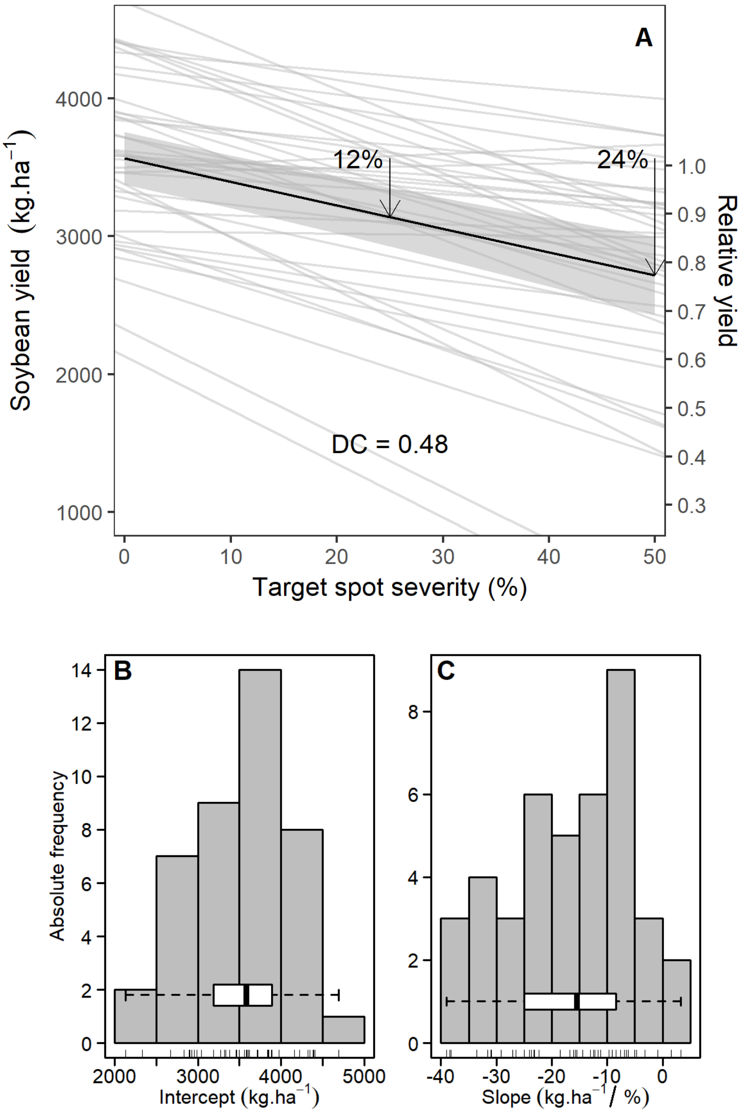

Figure 3 Overall fitted linear regression and respective 95% confidence interval (black solid and grey shaded area) and study-specific prediction lines (BLUPs, grey lines) (A) of the relationship soybean yield ~ target spot severity for the 41 selected fungicide studies performed in Brazil in seasons 2012 to 2016 and the histograms of their linear regression coefficients: intercepts (B) and slopes (C).

Table 1 Fitted linear regression coefficients for the overall model and the full model including the significant moderator variables.

	Coefficient	Estimated	SE	CI_{Low}	CI_{High}
Overall model	Intercept	3564	99.36	3376	3753
	Slope	-17.1	2.27	-21.4	-12.5
Full model					
BY_{Low}[1]	Intercept	2932	99.1	2737	3125
BY_{High}	Intercept	3916	143.3	3770	4063
YR_{Low}[2]	Slope	-6.34	2.73	-11.1	-1.6
YR_{High}	Slope	-23.7	2.55	-27.9	-19.3

[1] BY: Baseline Yield, based on the yield at reference fungicide treatment (Low < 3445 kg ha^{-1} ≤ High)
[2] YR: Yield response, based on the % increment of the reference fungicide relative to the untreated check (Low < 10% ≤ High)

3.3.3 Moderator variables inclusion

Two out of the five moderator variables were significantly incorporated to the overall model: effect of baseline yield on the intercept (P < 0.001) and effect of yield response on the slope (P < 0.001), thus, the overall model was split into four regression equations combining both fixed effects (Table 1, Fig. 4).

$$Yield\ (BY_{Low}\ YR_{Low}) = 2955\ \mathrm{kg\ ha^{-1}} - 5.5\ \mathrm{kg\ ha^{-1}}/\ \%\ \cdot TS$$

$$Yield\ (BY_{Low}\ YR_{High}) = 2955\ \mathrm{kg\ ha^{-1}} - 25.1\ \mathrm{kg\ ha^{-1}}/\ \%\ \cdot TS$$

$$Yield\ (BY_{High}\ YR_{Low}) = 3964\ \mathrm{kg\ ha^{-1}} - 5.5\ \mathrm{kg\ ha^{-1}}/\ \%\ \cdot TS$$

$$Yield\ (BY_{High}\ YR_{High}) = 3964\ \mathrm{kg\ ha^{-1}} - 25.1\ \mathrm{kg\ ha^{-1}}/\ \%\ \cdot TS$$

The resulted damage coefficients for each combination are presented at Fig. 4. Then the highest damage coefficient corresponded to the combination $BY_{Low}\ YR_{High}$ DC = 0.81 $\%^{-1}$ with potential losses of L_{50} = 42%; followed by $BY_{High}\ YR_{High}$: DC = 0.6 $\%^{-1}$ (L_{50} = 31.5%) then $BY_{Low}\ YR_{Low}$: DC = 0.22 $\%^{-1}$ (L_{50} = 9.5%); and finally $BY_{Low}\ YR_{Low}$: DC = 0.16 $\%^{-1}$ (L_{50} = 7%).

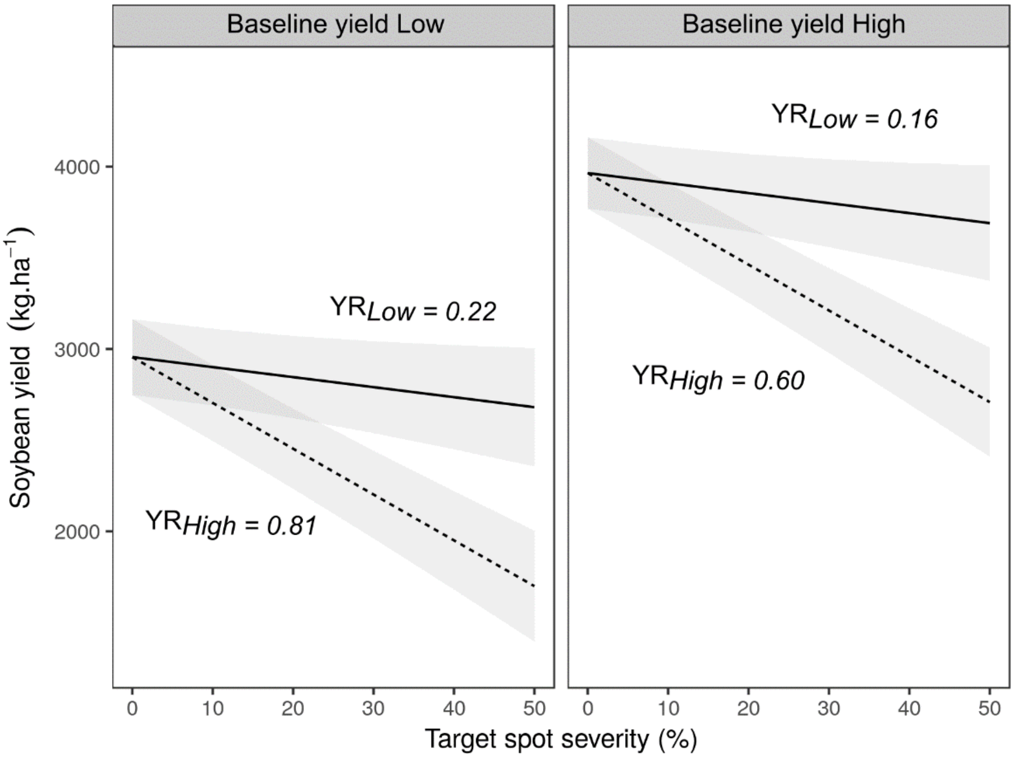

Figure 4 Prediction lines and 95%CI (grey-dashed area) for the fitted linear regressions of the model including the moderator variables: Baseline yield (Low < 3300 kg ha^{-1} ≤ High) and Yield response (YR, Low < 10% yield increase of reference fungicide ≤ High, full or dashed lines respectively). In italics are displayed the damage coefficients (slope/intercept * 100) for each factors combination.

At the reduced dataset, we inspected whether cultivars BMX Potência RR, M9144RR or TMG803 were homogeneously distributed across the moderator combination or if they had some aggregation at some factor classes. We observed that cultivar BMX Potência RR was predominant in $BY_{Low}\,YR_{Low}$ (5/8 studies); cultivar M9144RR was predominant in $BY_{Low}\,YR_{High}$ (7/8 studies); and TMG803 was more homogeneously distributed in the four moderators variables combinations (Fig. 5; Fig.S2). The model including cultivar effect for both intercept and slope was the one with best goodness of fit (effect of cultivar on the intercept P = 0.003 and on slope P = 0.03). The estimated coefficients are presented at Table 2 and the damage coefficients in Table 2 and Fig. 5. We observed a great variability among the cultivars: BMX Potência RR resulted to be the most tolerant cultivar (L_{50} = 10%); M9144RR the least tolerant one (L_{50} = 41%), and TMG803 in an intermediate level (L_{50} = 18.5%).

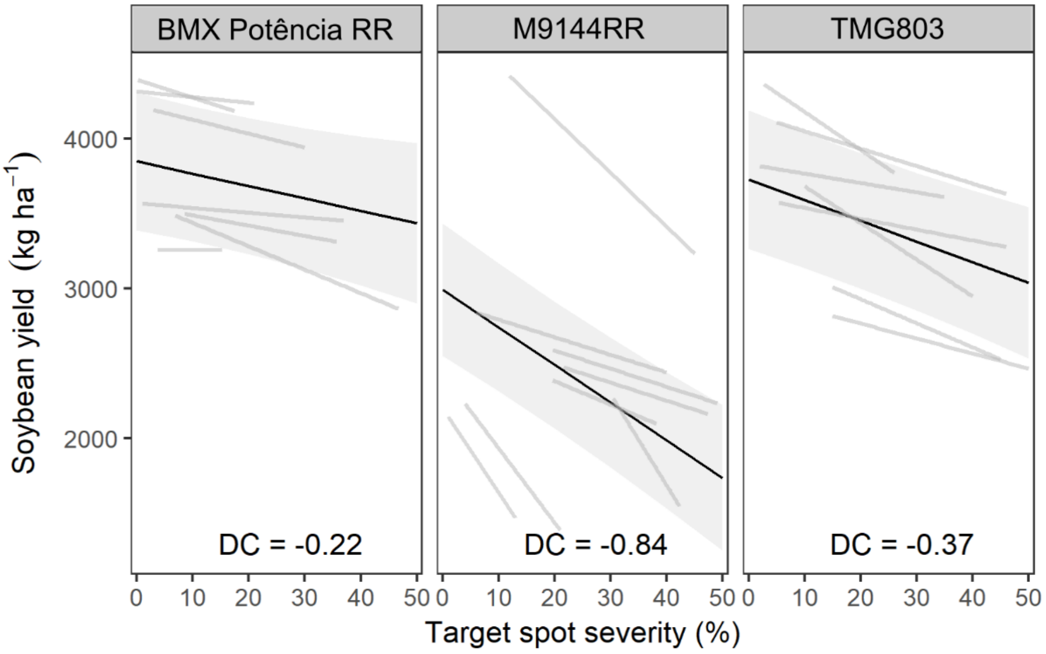

Figure 5 Fitted regression lines of cultivars BMX Potência RR, M9144RR and TMG803 (black lines, and 95% CI in grey shaded area) and observed study-specific models (grey lines). Damage coefficient (DC = slope/intercept * 100) for each cultivar represent the loss in kg ha^{-1} for each target spot severity % unit increment.

3.3.4 Correlation coefficients

A standard univariate random model was fit to the 41 selected studies to estimate the overall value of Fisher' z. The null hypothesis that all studies evaluated the same effect was rejected in the Q test ($P < 0.0001$). The overall mean estimated value for *z* was -0.48 (95% CI = -0.6, -0.36) corresponding to a back transformed Pearson's *r* value of -0.45 (95% CI =-0.54, -0.35) (Fig. 6). The statistic I^2 = 78.6% indicated that of the total variation in study estimates, that proportion was due to heterogeneity in the true effect size (*z*).

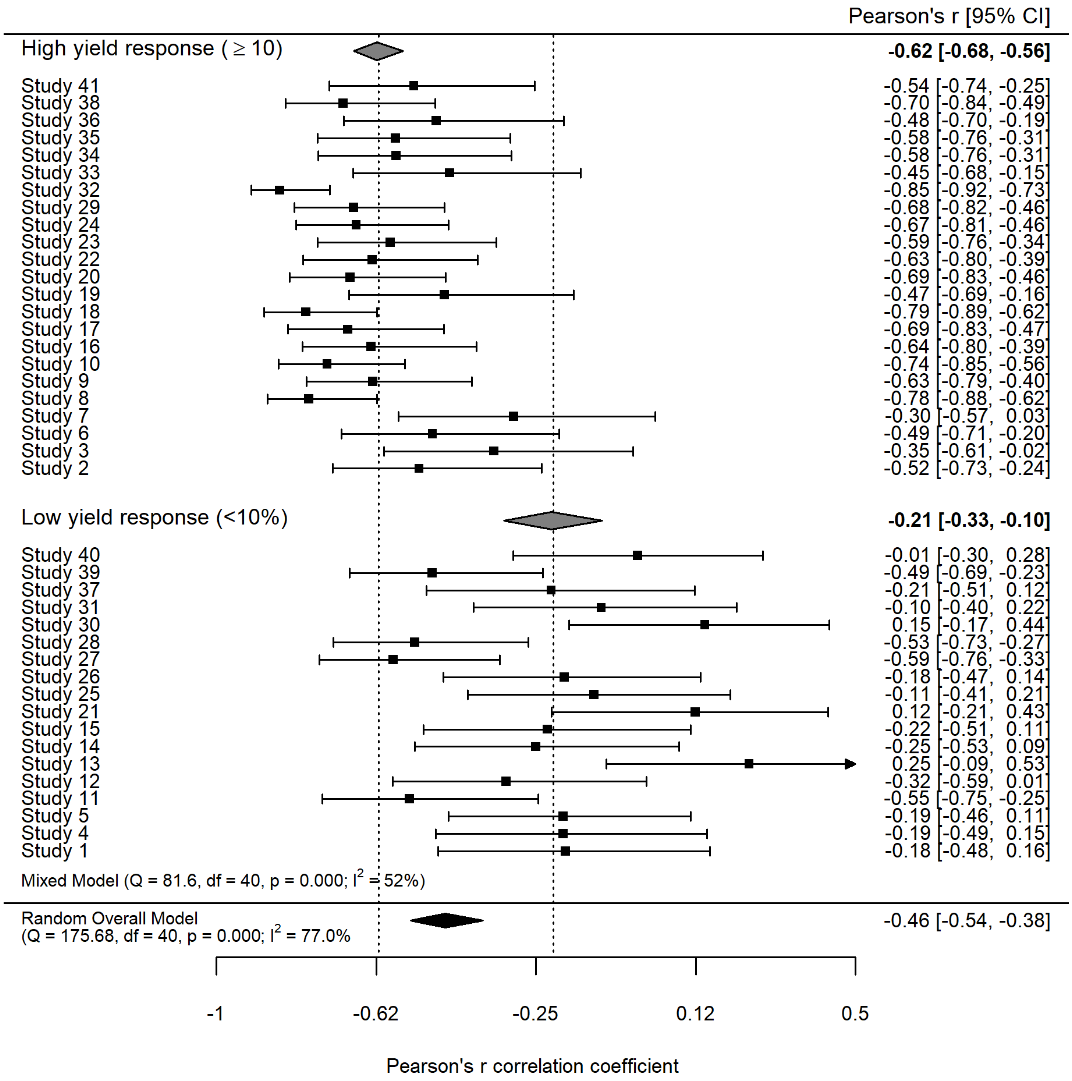

Figure 6 Forest plot of Pearson´s r coefficient between target spot severity and soybean yield for the 41 selected uniform trials performed in Brazil in seasons 2012 to 2016. Grey diamonds are the k-levels of moderator yield response mean estimates and black diamond is the overall mean.

Only YR resulted significant to improve the simple random effect model ($P < 0.001$) reducing the between-study variablity (τ^2) from 0.081 (reduced model) to 0.0247 (full model). The mean estimated Fisher' *z* values for both classes of yield response were: YR_{High} = -0.71 (95% CI = -0.83, -0.603) and YR_{Low} = -0.19 (95% CI = -0.32, -0.07). The back transformation to Pearson's *r* scale for the estimated values of z were -0.62 for YR_{High} and -0.19 for YR_{Low} (Fig. S3). The estimated Fisher´s z and Pearson´s r for cultivars BMX Potência RR, M9144RR and TMG803 and the respective statistics are presented in Table 2.

Table 2: Predicted intercepts ($\widehat{\beta_0}$) and slopes ($\widehat{\beta_1}$) for the selected cultivars and their statistics.

	Regression coefficients					
Cultivar	$\widehat{\beta_0}$	SE[1]	$\widehat{\beta_1}$	SE	DC[2]	L_{50}[3]
BMX Potência RR	3850	233.9	-8.31	3.96	0.22	11%
M9144RR	2992	324.0	-25.1	5.43	0.84	42%
TMG803	3726	332.4	-13.7	5.27	0.37	18.5%

	Correlation coefficients							
Cultivar	Fisher´s Z	SE	p-val	*CI_{lower}	*CI_{upper}	Pearson´s r	*CI_{lower}	*CI_{upper}
BMX Potência RR	-0.29	0.09	0.0009	-0.47	-0.121	-0.29	-0.44	-0.12
M9144RR	-0.87	0.08	<.0001	-1.02	-0.713	-0.70	-0.77	-0.61
TMG803	-0.44	0.08	<.0001	-0.60	-0.267	-0.41	-0.54	-0.26

[1]Standard error; [2] Damage coefficient; [3]Yield losses at target spot severity = 50%; * 95% Confidence intervals

3.4 Discussion

Target spot can potentially cause important yield losses to soybean crops. Soybean yield losses due to target spot has been reported to be inconsistent, from null (Faske & Kirkpatrick, 2011; Ploper *et al.*, 2013) up to 40% (Koenning *et al.*, 2006). We agreed with these reports and additionally we could partially explain the specific context in which low or high yield losses can occur.

As suggested by James (1974) in order to span a wide range of growing conditions, we used a five season's database of Uniform Fungicide Trials (41 studies) spread across the main Brazilian soybean production region. To the extent of our knowledge, this is the first study to estimate and model the damage of target spot on soybean yield at multiple locations and years. We observed an overall yield reduction of 0.48 %$^{-1}$ (kg ha^{-1} of soybean per unit increment of target spot severity, based on a free-disease yield of 3564 kg) resulting in a potential yield loss of 24%. The most efficient fungicides to control target spot in the main Brazilian soybean-growing region were the mixture of fluxapyroxad + pyraclostrobin (from SDHI and QoI chemical groups respectively) and this same mixture with the addition of the DMI fungicide epoxiconazole. The adoption of these mixtures provided disease control levels of ~75% (Chapter 2). Such levels of disease control on the treated plots provided, on average, 19-20% higher yields than the untreated plots with high disease severity (>35%).

This overall value should be used cautiously, since it has been reported that target spot damage depends on specific environments and agronomic conditions (Sinclair, 1999). The best model we could fit to split this overall response into more specific conditions included the baseline yield (with effect on the intercept) and the yield response (with effect on the slope). A wide range of potential losses: from 8-11% to 30-40% was also observed (Fig. 4). The inclusion of the baseline yield was a factor also considered by Faske & Kirkpatrick (2011) suggesting the use of high-yielding soybean cultivars, as a practice for target spot management.

A dichotomous scenery could then be considered: null to low damage or highly important losses due to target spot. For the first group, fungicide sprayings may not be profitable however it would be strongly recommended to protect with fungicides for the second one. Therefore yield losses reported by Koenning *et al.*(2006) are included in the range of losses estimated in our study.

The heterogeneous response of yield reduction due to target spot can be summarized by the yield response moderator variable (yield response to a reference efficient fungicide treatment). Different agronomic aspects could affect the crop yield response to fungicides sprayings. Probably the main factor is the water availability at critical periods for yield definition (R3 to R5). Above-normal seasonal rain, is considered to be beneficial to both crop growth and disease development, thus leading to a high difference in the yield response between protected and unprotected plots (Dalla Lana *et al.*, 2015). Some information is available regarding the relationship between rainfall and Asian rust severity (Del Ponte *et al.*, 2006) or foliar late season diseases severity (Carmona *et al.*, 2015). However all the reported weather - based assessment of soybean target spot have been reported from empirical observation.

Some agronomical practices that can affect the yield response to a fungicide can be the crop arrangement or plant population. For instance, yield increases from benomyl treatment for Septoria brown spot control in soybean tended to be greater in 17 cm row width than in 75 cm row width (Copper, 1989). Spraying pyraclostrobin led to yield gains in tilled (ranging from 1 to 17% increments) and no yield gain was observed in no-till field because frog eye leaf spot severity was not reduced as significantly in no-till as in treated tilled plots. The heterogeneity of yield response to fungicide exposed above are linked to differential differences in the management. However in our work we observed different yield reductions at same levels of target spot severity and management practices, varying only in the cultivar.

Zadoks & Schein (1979) called “tolerance” to one of the plant internal factors that allow some cultivars to suffer less damage than others at the same level of injury. If crop loss results are being compared among cultivars, $\beta 1$ from the linear regression model represent, in a sense, the tolerance of a cultivar to a given disease (Madden *et al.*, 2007). We observed that cultivar BMX Potência RR presented a very weak strength in the linear relationship between both variables, evidenced by a low correlation coefficient (Fig. S3) and also a small damage coefficient (Fig. 5), which applying to a maximum potential disease severity of 50% could reduce yield in 11%. At the other extreme, cultivar M9144RR resulted dramatically affected by the disease severity increase: a moderate to high linear relationship was observed at the Pearson´s r coefficient (Fig. S3) and a high damage coefficient (Fig. 5), highlighting the low tolerance of the cultivar to the disease. In a potential context of target spot severity of 50% cultivar M9144RR would reduce its yield in 42%, which corroborates with the maximum reported yield losses (Koenning *et al.*, 2006).

Soybean tolerant genotypes were also reported for Asian rust in Brazil: Cultivars BRS 239 and BRSGO 7560 did not reduce their yield with or without disease control (Melo *et al.*, 2015). Further studies should be done exploring which compensation mechanisms allow cultivar BMX Potência RR to stabilize its yield even with increasing target spot severities rates.

Fortunato *et al.* (2015) observed variability in the activity of defense-related enzymes in soybean cultivars inoculated with *C. cassiicola*. The low tolerance showed by cultivar M9144RR can be associated to the specific host-pathogen reaction, mainly to the toxin cassiicolin.

We did not observe significant effect of other moderator variables for modelling target spot effect on yield: the cultivar growth habit (determinate or indeterminate) did not affect the regression coefficients nor the correlation between target spot severity and soybean yield. Similar results were obtained by Copper (1989) stating that yield reductions due to septoria brown spot vary by genotype, but were not associated specifically with plant type (determinate vs. indeterminate). Disease pressure was not significantly included in the models (as categorical or as continuous variable) despite having significant effect on yield response to fungicides. In higher severities, above 35%, some fungicides result in higher yield difference relative to the untreated plots (Chapter 2)

In developing countries where funding for research organizations is limited, priority setting is necessary to optimize research initiatives in plant protection (Strange & Scott, 2005). Previous works meta-analyzed the relationship between soybean yield - rust severity (Dalla Lana *et al.*, 2015) or soybean yield - white mold (*Sclerotinia sclerotiorum*) incidence (Lehner *et al.*, 2017) for the main soybean Brazilian growing region. A two years study with similar approach using chemical control as a source of disease variability was performed to explore impact of anthracnose (Colletotrichum truncatum) on the soybean yield in northern Brazil (Dias *et al.*, 2016). Higher Pearson´s r correlations coefficients were observed for the latter pathosystems: - 0.61 for soybean rust; - 0.76 for with mold; and -0.81 for anthracnose. The lower correlation coefficient of target spot is expected since many factors can intermediate up to the yield build-up: the closest the symptom to the harvesting organ, the higher the correlation is expected between disease symptom and grain yield.

Damage coefficients of 0.6 to 0.73 $\%^{-1}$ were estimated for soybean rust and 0.49 $\%^{-1}$ for white mold. Considering the overall damage coefficients, target spot could be classified a disease of intermediate importance: setting a potential soybean yield of 3500 kg ha^{-1}, a yield reduction of 168 kg ha^{-1}, 172 kg ha^{-1} or 212 kg ha^{-1} would be expected for each 10% increments of target spot severity, white mold incidence or Asian rust severity, respectively. However considering cultivar BMX Potência RR or M9144RR this yield reduction would be predicted to be 77 kg ha^{-1} and 294 kg ha^{-1}, respectively. Damage coefficient itself is not enough to describe or compare the relevance of the most prevalent diseases for a crop. Meanwhile it is feasible to find soybean rust severities at field levels close to 100%, white mold incidence barely overcome 40% and target spot rather present a whole plant mean severity of 50%. For this maximum disease levels, yield losses of 73% can be expected due to Asian rust, 20% to white mold, or 24% to target spot (11% for cultivar BMX Potência RR or 42% for M9144RR).

The wide variability of *C. cassiicola* populations (Dixon *et al.*, 2009); the continuous cultivation of susceptible varieties in no till systems and the use of low efficient fungicides as carbendazim to control the disease (Xavier *et al.*, 2013), provide favorable conditions for continuous multiplication of *C. cassiicola* accompanied by selection of more aggressive strains in different soybean production environments.

Cultivar selection was confirmed to be a key component in the setting of an integrated management strategy for target spot. Further studies should be performed with several cultivars under the same environmental condition to assess correctly cultivar tolerance effect on yield, using healthy reference plots with fungicides without physiological effects. The latter were some limitations of the present study. The environment component remains as a clear research priority for a fully understanding of how target spot epidemics can result in yield losses and provide a full insight for growers using fungicides as a profit tool in sustainable agro-systems.

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3.6 Supporting information

Table S1 Selected studies specifications

Study	Year[1]	State[2]	Cultivar	GH[3]	DS Chk[4] (%)	Yield Chk[5] (kg ha^{-1})	Yield EFP[6] (kg ha^{-1})	DP[7]	BY[8]	YR[9]	r[10]	n[11]
1	2012	MT	TMG803	D	42.5	2640	2858	H	L	L	-0.18	36
2	2012	MT	TMG803	D	36.2	2996	3655	H	H	H	-0.52	36
3	2012	MT	TMG803	D	41.2	2366	2907	H	L	H	-0.35	36
4	2012	MS	5G830	D	30.6	3347	3159	L	L	L	-0.19	36
5	2012	MS	BMX Potência RR	I	27.0	3971	4090	L	H	L	-0.19	45
6	2012	MT	TMG803	D	44.8	3230	3588	H	H	H	-0.49	36
7	2012	GO	M8336	D	47.8	3178	3564	H	H	H	-0.30	36
8	2013	MT	5G830	D	48.5	2687	3479	H	H	H	-0.78	40
9	2013	PR	BMX Potência RR	I	45.7	2690	3714	H	H	H	-0.63	40
10	2013	GO	Syn1180	I	26.5	2501	3440	L	H	H	-0.74	40
11	2014	MS	NA5909	I	28.1	3641	3730	L	H	L	-0.55	32
12	2014	MT	TMG1179	D	51.2	3298	3426	H	H	L	-0.32	36
13	2014	MT	TMG1179	D	22.5	3546	3436	L	H	L	0.25	36
14	2014	MT	TMG803	D	33.2	3618	3740	L	H	L	-0.25	36
15	2014	MS	BMX Potência RR	I	15.8	4252	4401	L	H	L	-0.22	36
16	2014	GO	BRSGO8151	I	12.5	3750	4211	L	H	H	-0.64	36
17	2014	GO	BRSGO9160	D	20.1	3750	4252	L	H	H	-0.69	35
18	2014	TO	M9144RR	D	41.0	1618	2218	H	L	H	-0.79	36
19	2014	MT	BRSGO8661	D	11.0	2152	2599	L	L	H	-0.47	36

20	2014	MT	M9144RR	D	18.0	1486	2134	L	L	H	-0.69	36
21	2014	PR	NA 5909 RG	I	14.5	2946	2874	L	L	L	0.12	36
22	2014	PR	BMX Ativa	D	29.4	2234	3023	L	L	H	-0.63	36
23	2015	MS	NA 5909 RG	I	17.0	3219	3878	L	H	H	-0.59	40
24	2015	MT	M9144RR	D	36.2	2530	2872	H	L	H	-0.67	40
25	2015	MS	BMX Potência RR	I	17.8	4228	4378	L	H	L	-0.11	40
26	2015	MS	BMX Potência RR	I	31.0	3488	3612	L	H	L	-0.18	40
27	2015	GO	AS3730	I	14.0	3577	3858	L	H	L	-0.59	40
28	2015	MT	TMG803	D	25.1	3742	4094	L	H	L	-0.53	40
29	2015	TO	M9144RR	D	45.0	3031	3867	H	H	H	-0.68	40
30	2015	MT	TMG1180	S	22.5	3488	3566	L	H	L	0.15	40
31	2015	MT	TMG1180	S	46.2	2968	2867	H	L	L	-0.10	40
32	2015	TO	M9144RR	D	52.0	1588	2440	H	L	H	-0.85	40
33	2016	MT	M8210	D	22.5	3170	3537	L	H	H	-0.45	36
34	2016	TO	M9144RR	D	49.9	2084	2567	H	L	H	-0.58	36
35	2016	GO	TMG2281	D	27.0	3431	4279	L	H	H	-0.58	36
36	2016	MT	TMG803	D	45.5	3534	4039	H	H	H	-0.48	36
37	2016	MT	NA5909	I	16.2	3458	3584	L	H	L	-0.21	36
38	2016	GO	M9144RR	D	12.4	1533	2331	L	L	H	-0.70	36
39	2016	MS	BMX Potência RR	I	32.7	3348	3500	L	H	L	-0.49	45
40	2016	MS	BMX Potência RR	I	14.4	3236	3224	L	L	L	-0.01	45
41	2016	TO	M9144RR	D	52.6	2001	2465	H	L	H	-0.54	36

[1] Year of harvest; [2]PR = Paraná, GO = Goiás, MS = Mato Grosso do Sul, MT = Mato Grosso, TO = Tocantins; [3]Growth habit (D=determinate; I=indeterminate, S=semideterminate); [4]Mean disease severity at untreated check; [5]Mean grain yield at untreated check; [6]Mean yield at reference fungicide (Mixture: Epoxiconazole + Fluxapiroxad + Pyraclostrobin); [7]Disease pressure class (Low < 35% < High); [8]Baseline yield (based on Yield_EFP: Low < 3300 kg ha^{-1} < High); [9]Yield response (100*(Yield_EFP/Yield_Check): Low < 10% < High); [10]Pearson´s *r* correlation coefficient; [11]Sample size (number of plots).

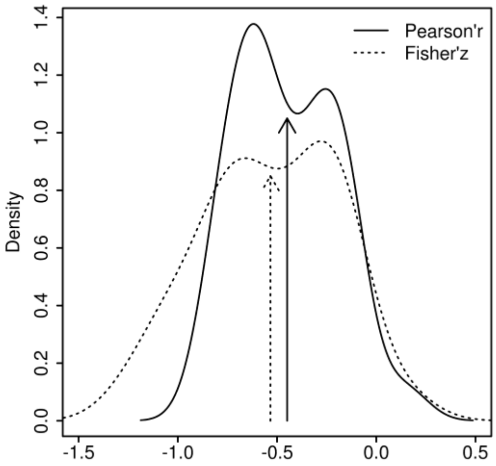

Figure S1 Density plot of Pearson´s *r* correlation coefficients and its Fisher´s *z* transformation for the 41 select studies.

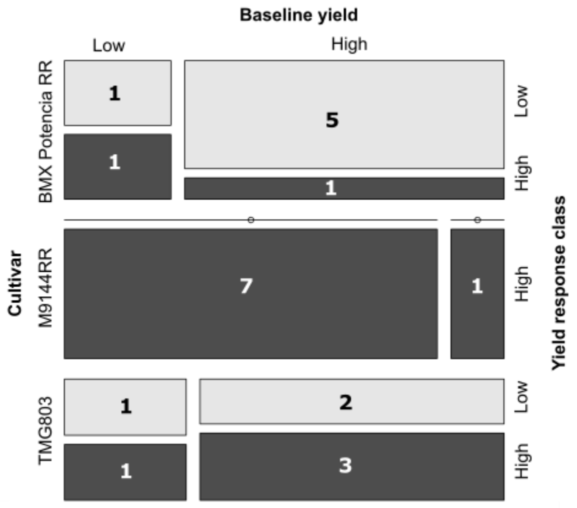

Figure S2 Frequency of cultivars BMX Potência RR, M9144RR and TMG803 at each factors combination (Baseline yield - Yield response)

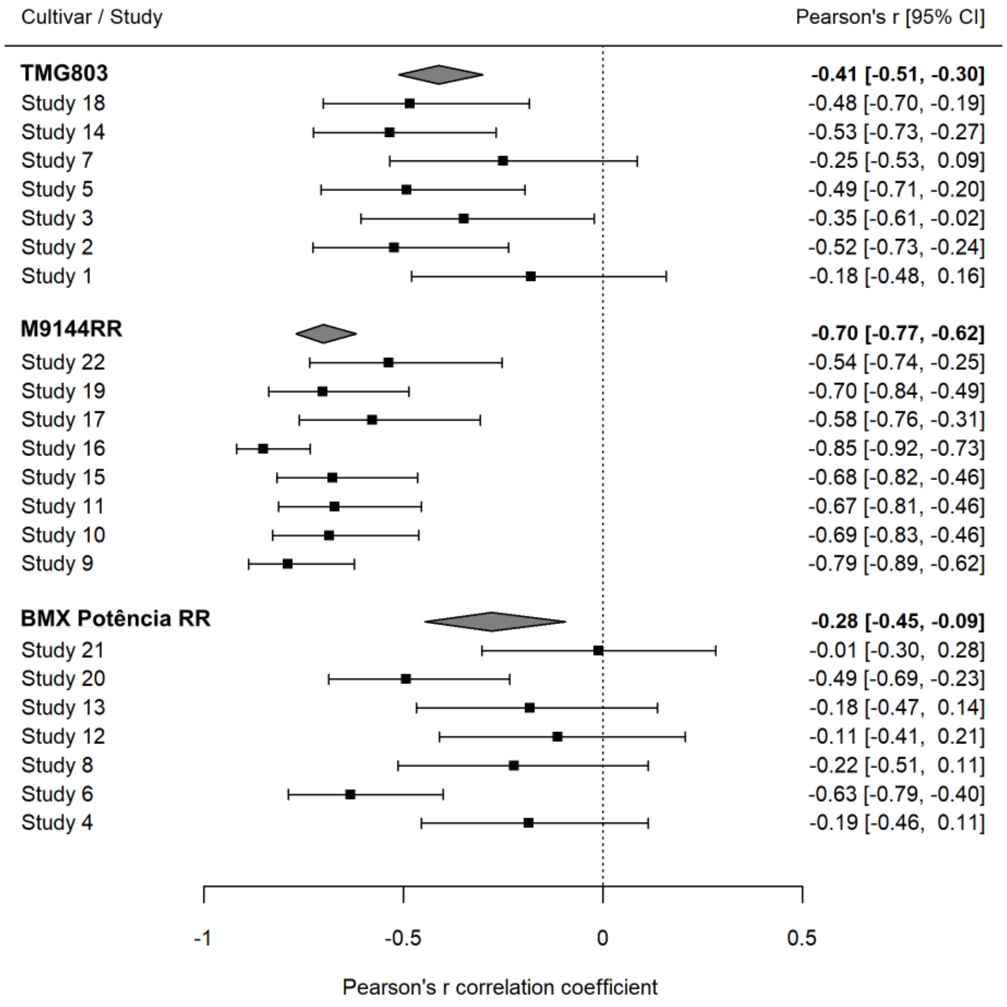

Figure S3. Forest plot of Pearson´s *r* coefficient for the reduced dataset containing only cultivar BMX Potência RR, M9144RR and TMG803. Grey diamonds are the cultivar mean estimates (fixed effects).

4 SCREENING OF SOYBEAN GENOTYPES RESISTANCE TO TARGET SPOT AND EFFECT OF CULTIVAR AND ISOLATE ORIGIN ON EPIDEMIC COMPONENTS

Abstract

Soybean target spot, a foliar disease caused by *Corynespora cassiicola*, has re-emerged in Brazil in the last decade probably due to the massive adoption of susceptible cultivars under no-till farming systems. Genetic resistance to target spot would be the most suitable technique for an integrated disease management program. The objectives of this study were: i) to screen a set of soybean cultivars adapted to the Southern Brazilian agricultural region for target spot resistance and ii) to explore the pathogen-host interaction effects on the epidemics. We individually inoculated three soybean cultivars of known relative resistance level to target spot with three *C. cassiicola* isolates from different regions of Brazil to evaluate the interaction between soybean cultivar and pathogen isolate. We observed high variability on the genetic resistance in the tested germplasm: cultivars with very low target spot intensity (BRS360) to highly susceptible cultivars. A single-point disease severity assessment was not as reliably as an integrative three-point assessment, which had no difference with a less time-consuming two-point disease assessment. The incubation period, disease severity, lesion density and lesion size at 14 days after inoculation, were influenced by the cultivar and no effect of the *C. cassiicola* isolate geographical origin was observed. Lesion size had the same pattern than disease severity in pairwise comparisons after variance analysis.

Key words: *Glycine max*; *Corynespora cassiicola;* genetic resistance; germplasm screening

4.1 Introduction

Target spot of soybean (*Glycine max* [L.] Merril), caused by *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei, was first reported in South America in 1976 in Brazil (Almeida *et al.*, 1976). Since then it was considered a minor soybean disease, however in the last decade it has established as an endemic disease, probably due to the massive adoption of no-till cultivation practices, increase of soybean monoculture or short-term soybean rotation systems, sowing of susceptible cultivars and a decreased sensitivity of the pathogen to single-site fungicides (Xavier *et al.*, 2013).

Foliar symptoms of target spot are reddish-brown rounded to irregularly shaped lesions with concentric rings of necrotic dead tissue, ranging in diameter from 10 to 15 mm and often surrounded by yellow halo. First symptoms are commonly observed in lower strata, moving up to middle and upper canopy (Almeida *et al.*, 2005) predisposing susceptible cultivars to premature defoliation (Sinclair, 1999) and yield losses. Soybean yield losses due to target spot in Brazil were estimated from 11 to 42% depending on the cultivar tolerance.

Fungicide applications on soybeans in Brazil are mainly based on the *Phakopsora pachyrhizi* presence, the causal agent of Asian rust, an endemic disease that can reduce yield up to 90% (Godoy *et al.*, 2016). The fungicide active ingredients for soybean spraying protection are mainly selected for their performance against this pathogen. On the other hand, genetic resistance has been used as an efficient mean for managing leaf spot diseases in soybean crops, as frogeye leaf spot (Mian *et al.*, 2003; Grau *et al.*, 2004) or brown spot (Almeida, 2001). Therefore, the selection for *C. cassiicola* resistance at breeding soybean native programs may contribute to local growers for a sustainable global soybean diseases management. A suitable screening method for disease resistance should be low time consuming and reliable enough to detect differences between genotypes. In the particular case of foliar diseases, it should also take into account some important epidemiological aspects as incubation period or infectious period that can make a difference between genotypes. Screening disease resistance under greenhouse environment, in comparison to field screenings, has the advantage that it is easier to regulate environmental factors, such as temperature and relative humidity, to favor infection and disease development. In the particular case of *C. cassiicola* infections are highly dependent of a period of at least 48 h of high relative humid.

Variability in the *C. cassiicola* populations' aggressiveness, defined as the quantitative component of pathogenicity (Pariaud *et al.*, 2009), has been reported for cucumber (Bezerra & Bentes, 2015) or sweet pepper (Shimomoto *et al.*, 2011). Teramoto *et al.*, (2013) also reported *C. cassiicola* isolate – soybean cultivar significant interaction, what would difficult resistance screening due to the differential genotypes rankings depending on the isolate employed in the inoculations.

Number of lesions and lesion size can be considered important epidemic components that represent a measure of the host-pathogen reaction for the processes of penetration and colonization (probably due to the cultivar susceptibility to the toxin cassiicolin). Variability was observed between cultivars in artificial inoculations of *C. cassiicola* for both components: ranging from 16 to 40 lesions.mm^{-2} and 1.7 – 6.6 mm (Ferreira Filho, 2012) or 1.6 - 2.7 mm diameter (Muliterno de Melo, 2009).

More studies are needed to explore the available soybean genetic resistance against *C. cassiicola* in Brazil and for a better understanding of the disease epidemic components. Based on this lack of information, the present study had as objectives: i) to conduct a resistance screening of a set of soybean cultivars adapted to the main Brazilian agricultural regions and verify the less time-consuming methodology for doing it; and ii) to explore the pathogen-host interaction effects on the epidemic development.

4.2 Material and methods

Two studies were performed to address the present objectives: i) Screening of soybean genotypes for target spot resistance; and ii) evaluation of *C. cassiicola* isolates and soybean cultivar effects on epidemic components. All experiments were carried out at the Plant Pathology and Nematology Department of the "Luiz de Queiroz" College of Agriculture (São Paulo University, Piracicaba – Brazil) facilities during August to December 2015 following a basic common protocol, as detailed below.

4.2.1 Inoculum and host preparation

Monosporic isolates of *Corynespora cassiicola* were obtained from soybean foliar symptoms collected in fields located at three different Brazilian regions: South (Paraná State), West (Mato Grosso State), and North (Tocantins State). *C. cassiicola* isolates stored on filter paper in freezer (-20 °C) were transferred into 9 cm diameter Petri dishes containing BDA medium with streptomycin (0.1%) and kept at 25°C and continuous light (fluorescent tubes at a distance of 30 cm). After 15-20 days of incubation, the pathogen colonies presented abundant sporulation and was used as inoculum. The germination rate of pathogen conidia was 70-90%. Five soybean seeds were sown in plastic pots (2 L) containing a mixture 2:1 of sterilized soil and sand. Each pot (experimental unit) was constituted by the three best-developed soybean plants (sub-samples) that were sprayed with *C. cassiicola* spores at the inoculation.

4.2.2 Inoculation, pre and post-inoculation conditions.

In order to stimulate stomata opening, 24 h prior to inoculation, soybean plants were transferred to a chamber with high relative humidity and kept in total darkness. Spore suspensions of the fungus were calibrated with aid of hemocytometer at a concentration of 10^4 spores' mL^{-1} and sprayed on the plants with a volume of 5 ml per pot at the V4 phenological stage, i.e. 3 developed trifoliates (Fehr & Caviness, 1977). Immediately after inoculation, plants were covered with plastic bags under the dark following Seaman *et al.* (1965). After 48 h, bags were removed, and as soon as the leaves dried, plants were returned to the greenhouse. Pots soil humidity was maintained by individual drip irrigation and air moisture was kept high by means of micro sprinkler programmed to give pulses of 15 seconds every 30 min between 8 and 18 h. Greenhouse temperature was maintained at 25 ± 2 °C.

4.2.3 Experimental design.

The experimental design for all experiments was a randomized complete block with three (for the cultivar screening trials) or four (for *C. cassiicola* isolates - soybean cultivars interaction trials) replications. All disease assessments were recorded at the central leaflet of 2° and 3° trifoliates since they were fully expanded at inoculation.

4.2.4 Screening of soybean cultivar resistance to *Corynespora cassiicola*.

Nineteen soybean cultivars were screened for target spot resistance at the greenhouse with artificial inoculations of *C. cassiicola* (Table 1).

With exception of cultivar M9144RR that is a maturity group 9 (adapted for the Northern Brazilian region), all tested genotypes are well adapted to southern sub-region, with maturity groups from 6 to 7.

Soybean plants were inoculated with a *C. cassiicola* isolate from Porto Nacional (Tocantins state, Brazil) which presented abundant sporulation *in vitro* and developed high values of disease severity in previous experiments. Target spot severity was assessed at 7, 14 and 21 days after inoculations (DAI) with aid of a diagrammatic scale (Soares *et al.*, 2009). Area under the disease severity progress curve (AUDPC, defined as the amount of disease integrated between two times of interest; for our purposes we considered $y = 0$ from time $x = 1$ DAI) was calculated with linear interpolation (trapezoidal method) using “auc” function of MESS R package (Ekstrøm *et al.*, 2017).

Analysis of the AUDPC variance (ANOVA) was performed to test the effect of cultivar. A mixed effect model was fitted to the square root transformation of AUDPC (for improving normality of residuals) with cultivar as fixed effects, and experiment and blocks as random effects. Model was fitted using lmer function from “lme4” R package (Bates *et al.*, 2015) and post-hoc Tukey's test mean comparisons with emmeans and R package (Lenth, 2017). To test whether a reduced data set (less time-consuming in further bigger screening works) was as effective as the full data assessments, we analyzed each single-point disease severity evaluations (at 7, 14 or 21 DAI) and calculated the AUDPC for combinations of two assessments (7-14, 7-21 or 14-21 DAI, from now on identified as $AUDPC_{t1-t2}$). In case of no differences among the variables, we confirmed the performance of each one by comparing it with the full AUDPC reference assessment by ranking the cultivars with Kruskal-Wallis algorithm and performing a Spearman correlation analysis between them.

Table 1 List of the tested soybean cultivars, its origin and maturity group.

Cod	Cultivar	Company	Maturity group
1	NA 5909 RG	Nidera	6.2
2	BMX Potência RR	Brasmax	6.7
3	BRS 388RR	Embrapa	6.4
4	BRS 399RR	Embrapa	6
5	BRS 359RR	Embrapa	6
6	BRS 360RR	Embrapa	6.2
7	DM 6563	Don Mario	6.3
8	M 6410 IPRO	Monsanto	6.4
9	BRS 1002IPRO	Embrapa	5.9
10	BRS 1004IPRO	Embrapa	5.8
11	BRS 1010IPRO	Embrapa	6.1
12	BRS 1005IPRO	Embrapa	6.2
13	BRS 1007IPRO	Embrapa	6.0
14	BRS 1003IPRO	Embrapa	6.4
15	BRS 1001IPRO	Embrapa	6.3
16	BRS 1006IPRO	Embrapa	6.5
17	BRS 397CV	Embrapa	6.2
18	BRS 391	Embrapa	6.3
19	M9144RR	Monsanto	9.1

4.2.5 *Corynespora cassiicola* isolate and soybean cultivar effects on epidemic components.

In order to test the significance of the *C. cassiicola* isolates – soybean cultivar interaction on the target spot epidemic components, two replicates of an experiment were carried out. Three soybean varieties with different levels of resistance (observed in the previous study): BRS360 (Moderately resistant), NA 5909 RG (Susceptible) and BMX Potência

(Moderately susceptible), were individually inoculated using three isolates with known aggressiveness from different regions of Brazil: Mauá da Serra – Paraná (South, PR), Porto Nacional – Tocantins (North, TO) and Sorriso – Mato Grosso (West, MT). Four epidemic metrics were assessed for testing the pathogen isolate – soybean cultivar interaction.

Defined as the period of time from inoculation to the appearance of the 50% of lesions, the incubation period (IP) was estimated by recording of the number of lesions at four moments (6, 10, 14 and 18 DAI) at each experimental leaflet (central leaflets of 2° and 3° trifoliate leaves). The final leaf spot number was fixed to its value assessed at 18 DAI, since it is known that affected leaves abscises prematurely around 20 DAI (EM). To estimate the day at y = 0.5 (50% of visualized lesions) a generalized linear model with “probit” link function was fitted for the lesion appearance curve progress for each replication (mean of 3 plants) (Fig. 1). The model with the log transformation of “days” as predictor variable had better fit than the model without it: presented lower residual deviance overall, and avoid over-estimation of the predicted day for the 50% of lesion appearance (day at y = 0.5) for most of the plants (data not shown). A generalized linear mixed-effects model for the negative binomial family was fitted to the estimated incubation period at pot level.

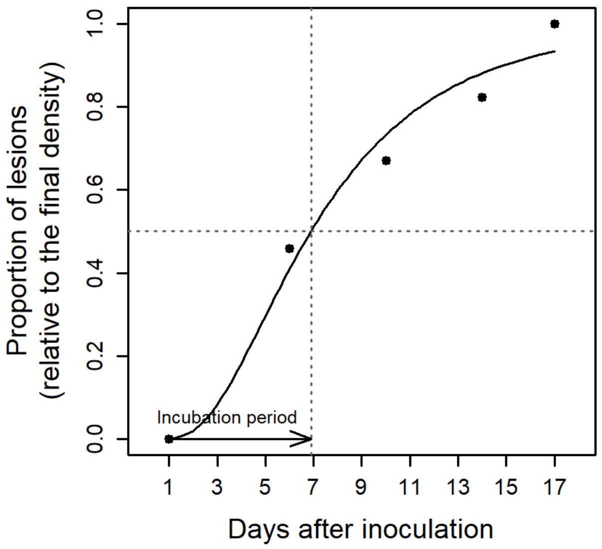

Figure 1 Example (cultivar BMX Potência RR, *C. cassiicola* isolate from Paraná state, pot 1) of the fitted model and prediction of time to reach the 50% of lesion appearance in a soybean leaf. Black points are the registered amount of target spot lesions, the black filled line is the fitted model and dashed lines indicates the cross-section for the predicted x value (days, back-transformed from the probit model) when y = 0.5.

Disease severity was visually assessed on 7 and 21 DAI. At day 14, each experimental leaflet was photographed and the total leaf area was estimated. The number of typical target spot was counted and their widest section of each lesion was measured by the image processing software ImageJ (Rasband, 2012). An average value of the total lesion diameter was recorded for each leaflet and the mean value was considered for each pot. The logit transformation of disease severity was analyzed in a linear mixed model. Lesion density was analyzed using Poisson regression techniques with GLM and lesion size with Gaussian linear mixed model. The interaction term between cultivar and isolate was tested using likelihood ratio test between a model containing the interaction effects and a model without it by using the drop1 function from "lme4" R package. (Bates *et al.*, 2015)

4.3 Results

4.3.1 Screening of soybean cultivar resistance to *Corynespora cassiicola*.

Variability was observed among the tested cultivars: from very low presence of disease symptoms along the assessment period up to more than 20% of target spot severity (Fig. 2). Target spot epidemic stabilized before the end of assessment period in some cultivars and it continued increasing its severity in other ones. Cultivar had significant effect ($P < 0.001$) on the AUDPC and soybean genotypes were grouped into three resistance groups upon the Tukey´s post-hoc test (Fig. 3). Nine cultivars were considered moderately resistant (MR), three cultivars were classified as moderately susceptible (MS) and seven cultivars as susceptible (S).

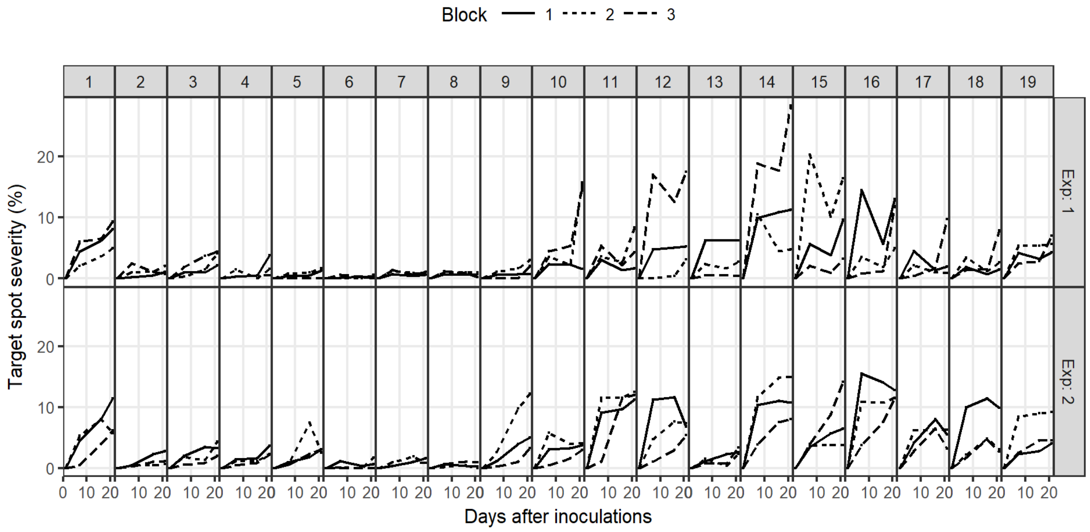

Figure 2 Target spot severity progress curves for the 19 tested soybean cultivars inoculated with a *Corynespora cassiicola* isolate from Porto Nacional (Tocantins state). Cultivar codes:

1 - NA 5909 RG, 2 – BMX Potência RR, 3 - BRS 388RR, 4 - BRS 399RR, 5 - BRS 359RR, 6 - BRS 360RR, 7 - DM 6563, 8 - M 6410 IPRO, 9 - BRS 1002IPRO, 10 -BRS 1004 IPRO, 11 - BRS 1010 IPRO, 12 - BRS 1005 IPRO, 13 - BRS 1007 IPRO, 14 - BRS 1003 IPRO, 15 - BRS 1001 IPRO, 16 - BRS 1006 IPRO, 17 - BRS 397 CV, 18 - BRS 391, 19 - M9144RR.

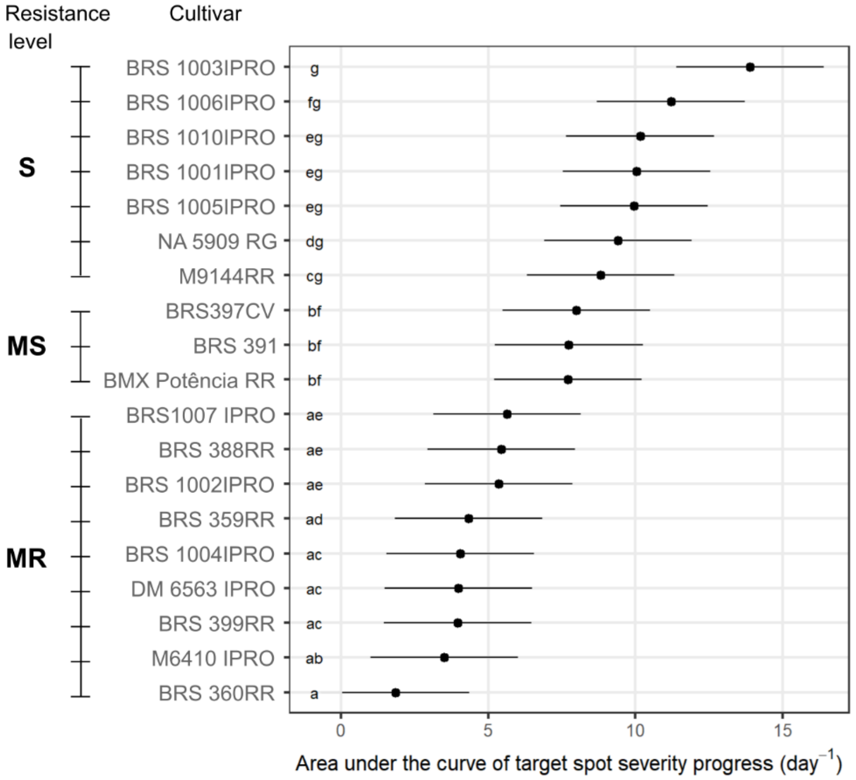

Figure 3 Predicted values (and 95% CI) estimated by the mixed model. Resistance levels are listed at the left vertical axis considering that same letters inside the plot do not differ by the Tukey test at 5% of probability.

The strongest Spearman correlation ($\rho = 0.959$) between AUDPC and a single-point assessment was observed at 14 DAI. Disease severity assessment at 7 and 21 DAI resulted in lower correlation with AUDPC ($\rho = 0.91$ and $\rho = 0.87$, respectively). However, the variance analysis of target spot severity (logit transformation) assessed at 14 DAI generated only two resistance groups: eleven cultivars were classified as MR and eight cultivars as S (Table S1)

The variance analysis of the AUDPC calculated with two disease severity assessments yielded the same resistance groups than the full AUDPC: MR, MS and S, and the cultivars were grouped within the same classes (Table S2) as using the full AUDPC. The Spearman correlation of the ranking of cultivars obtained by each two-point AUDPC against the full three-point AUDPC was highest for the AUDPC$_{7-21}$ (Fig. S1). We observed for this latter variable that six cultivars were ranked in differently than the full AUDPC, in other words, 13 cultivars were similarly ranked. Eight cultivars were ranked in different order when using AUPDC$_{7-14}$ and 10 cultivars when using AUPDC$_{14-21}$.

4.3.2 *Corynespora cassiicola* isolate and soybean cultivar effects on epidemic components

The progress of target spot lesions appearance along the time showed different shapes depending on the cultivar: BMX Potência RR presented a rapid increase since inoculation with maximum final amount of lesions of around 38 lesions per leaflet (Fig. 4). On the other hand, the lowest progress of lesion accumulation was observed on cultivar BRS360 with a mean maximum of 12 lesions per leaflet. S-shape like curves were observed on cultivar NA 5909 RG with a maximum mean value of 44 target spot lesion per leaflet.

The incubation period for the three cultivars ranged from 5.3 to 14.2 days with a median value of 7.7 days (Fig. 5).

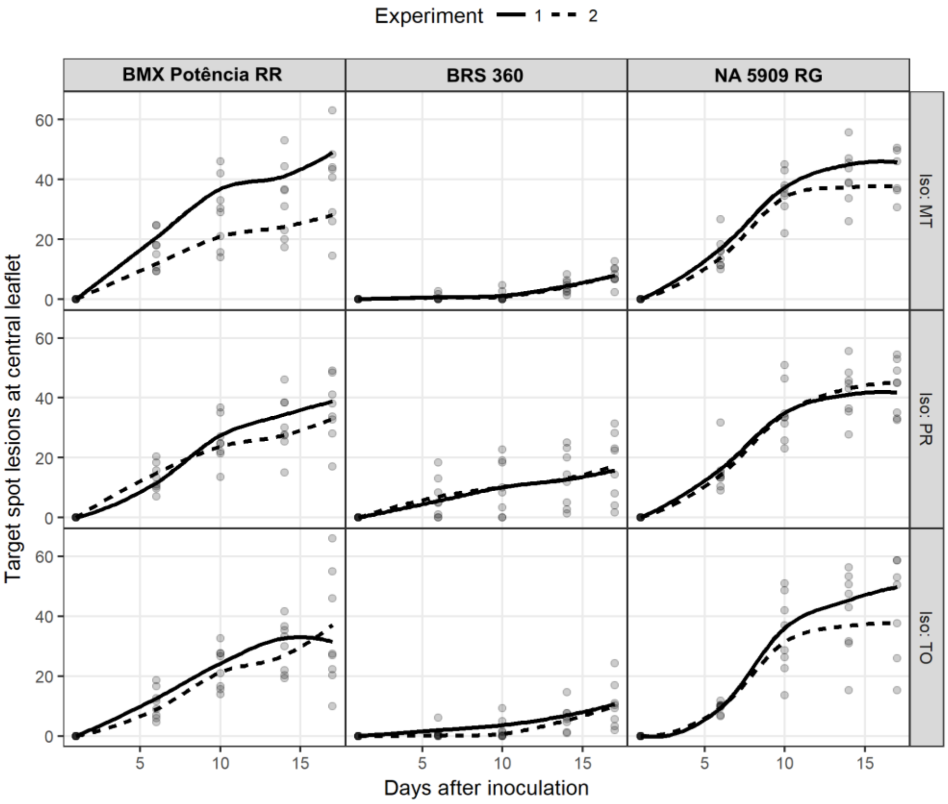

Figure 4 Number of target spot lesion progress at central leaflet in cultivars BMX Potência RR, BRS360, and NA 5909 RG, inoculated with *Corynespora cassiicola* isolates from Sorriso (MT), Mauá da Serra (PR) and Porto Nacional (TO).

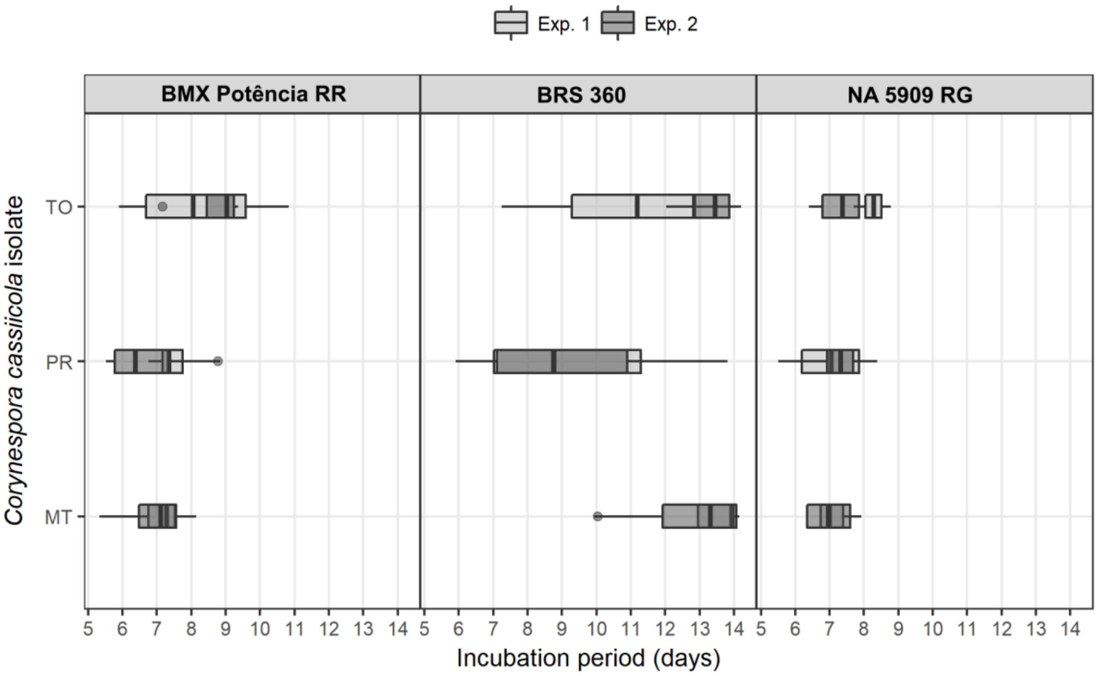

Figure 5 Estimated incubation periods in cultivars BMX Potência RR, BRS360, and NA 5909 RG inoculated with *Corynespora cassiicola* isolates from Sorriso (MT), Mauá da Serra (PR) and Porto Nacional (TO).

No interaction effect between cultivar and fungi isolate origin was observed to model the incubation period data (*P* = 0.5587). Only soybean cultivar was significantly included in the model (*P* < 0.001); *C. cassiicola* isolate origin (*P* = 0.1888). Pairwise comparison showed that cultivar BRS360 presented the longest incubation period and no differences were observed between BMX Potência RR and NA 5909 RG (Table 3)

Table 3 Marginal means of soybean cultivars and *Corynespora cassiicola* isolates levels estimated by the model with both simple additive factors. Tukey post-hoc comparisons and 95% confidence intervals.

Factor	Levels	IP*		CI95%
Cultivar	NA 5909 RG	7.3	a	6.3 - 8.47
	BMX Potência RR	7.5	a	6.5 - 8.7
	BRS360	11.4	b	10.1 - 12.8
Isolate	PR	7.7		6.7 - 8.9
	MT	8.8		7.7 – 10
	TO	9.2		8.1 - 10.5

* Incubation period. Means in the same column with different letters were significantly different (*P* < 0.05, ANOVA, Tukey-HSD)

Single assessment of disease severity at 14 DAI presented a strongly right skewed distribution with high frequency of values close to 0 and a right tail of values between 0.2 and 0.35 (Fig. 6). Lesion density presented a bimodal distribution with one of the peaks close to zero and the other one around 1.3 lesions.cm^{-2}. Lesion diameter had a symmetric distribution around the overall mean 0.28 cm.

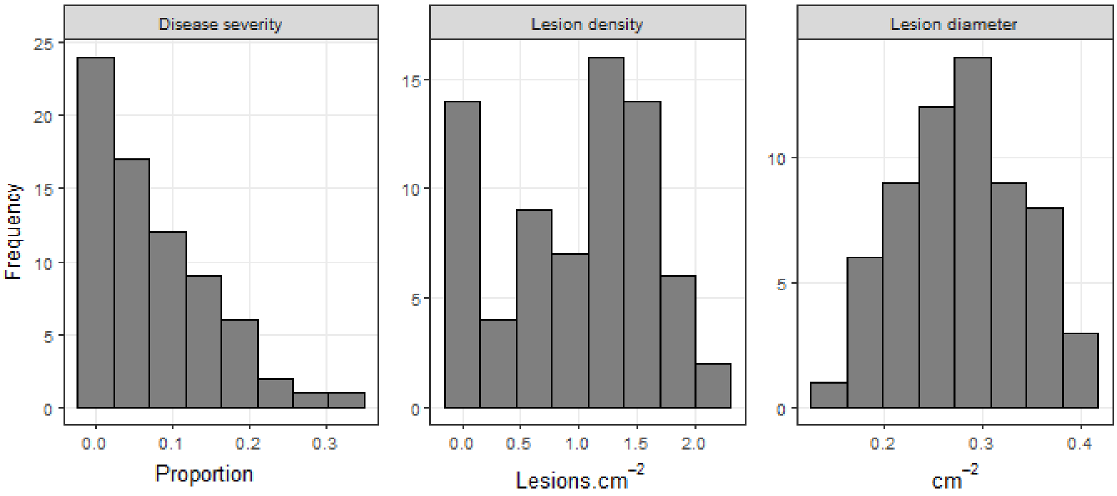

Figure 6 Histograms of target spot severity, lesion density and lesion diameter assessed on cultivars BMX Potência RR, BRS360, or NA 5909 RG 14 days after inoculation with *Corynespora cassiicola* isolates from Sorriso (MT), Mauá da Serra (PR) and Porto Nacional (TO).

No significant cultivar - isolate interaction was observed for any of the three tested variables, thus the final models were constituted by the simple effects of cultivar and isolate factors. Only factor cultivar was significant to model the three variables: disease severity ($P < 0.001$), lesion density ($P < 0.001$) and lesion diameter ($P < 0.001$). Cultivar BRS360 developed the lowest target spot severity, BMX Potência RR intermediate and NA 5909 RG the highest (Table 4). Lesion density was similar for BMX Potência RR and NA 5909 RG, and lowest for BRS360. Lesion diameter had the similar pattern than disease severity: lowest in BRS360, highest in NA 5909 RG, and intermediate in BMX Potência RR.

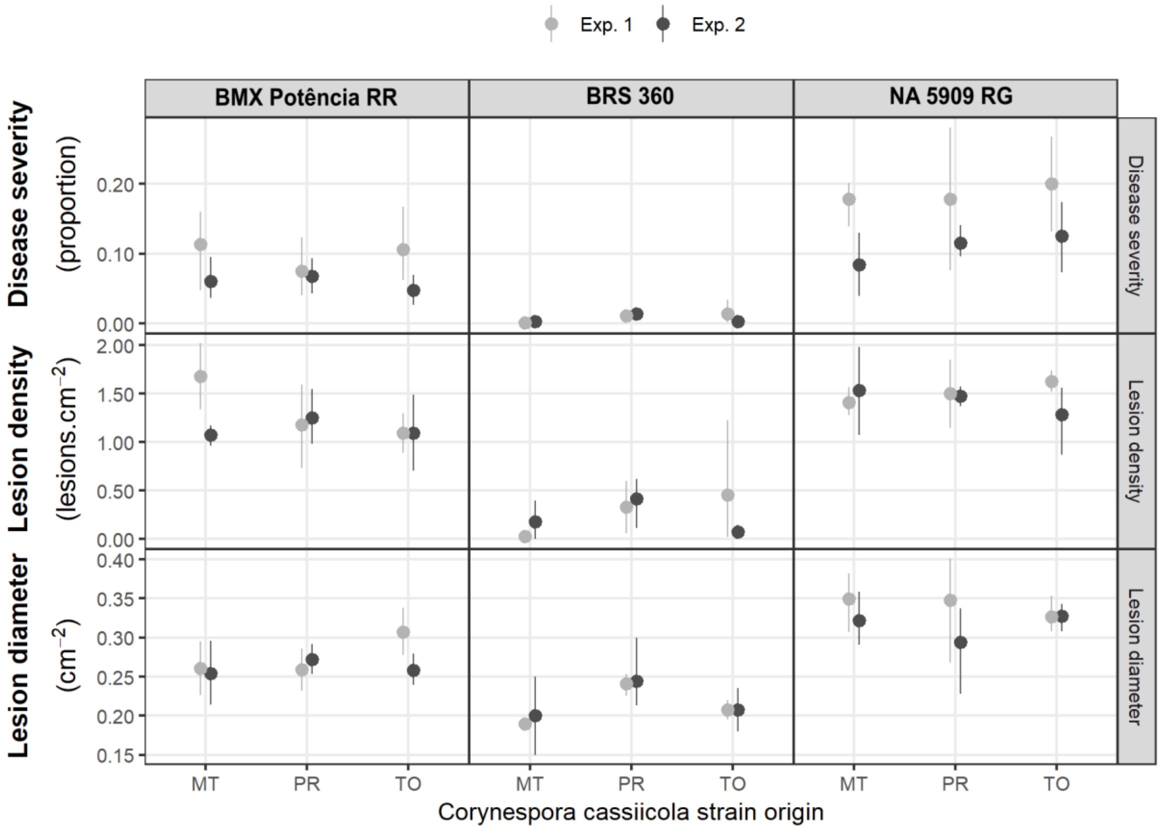

Figure 7 Mean values (and 95% confidence intervals) of target spot severity, lesion density and lesion diameter assessed on cultivars BMX Potência RR, BRS360, or NA 5909 RG, 14 days after inoculation with *Corynespora cassiicola* isolates from Sorriso (MT), Mauá da Serra (PR) and Porto Nacional (TO)

Table 4 Marginal means of target spot severity, lesion density and lesion diameter assessed on cultivars BMX Potência RR, BRS360, or NA 5909 RG 14 days after inoculation with *Corynespora cassiicola* isolates from Sorriso (MT), Mauá da Serra (PR) and Porto Nacional (TO).

Factor		Disease severity (proportion)	SE	Lesion density (lesions.cm^{-2})	SE	Lesion diameter (cm)	SE
Cultivar	BRS360	0.03 a	0.005	0.247 a	0.101	0.219 a	0.013
	BMX Potência RR	0.09 b	0.014	1.228 b	0.225	0.268 b	0.011
	NA 5909 RG	0.15 c	0.022	1.470 b	0.247	0.328 c	0.010
C. cassiicola isolate origin	MT	0.071	0.011	0.73	0.172	0.266	0.011
	TO	0.079	0.012	0.77	0.177	0.274	0.010
	PR	0.079	0.012	0.79	0.182	0.275	0.012

Means in the same column with different letters were significantly different ($P < 0.05$, ANOVA, Tukey-HSD)

4.4 Discussion

Resistance screening of soybean cultivars to *C. cassiicola* was performed initially with an unique aggressive isolate and the less resources-consuming screening methodology was determined. In a second step, three soybean cultivars varying in their resistance level were inoculated with three aggressive isolates obtained from different regions of Brazil.

We observed variability among the tested cultivars: from moderately resistant to susceptible cultivars. One of them (BRS360RR) developed low levels of target spot severity. This cultivar may be taken into account to be used under integrated disease soybean management programs for the Brazilian agricultural regions, as proposed by the main soybean target spot research authors (Snow & Berggren, 1989; Sinclair, 1999; Almeida *et al.*, 2005). It may also be used as resistant parent in breeding programs not only for its adaptation region (Southern) but for all the Brazilian soybean regions, where the disease is even more important (Western and Central). Further studies should be performed at field experiments with the best genotypes including grain yield since a desired characteristic of soybean cultivars for minimizing yield losses due to target spot epidemics is the disease tolerance. Meta-analysis of a net uniform fungicide trials showed that exist variability among cultivars in the yield stability in presence of target spot (Edwards Molina, *unpublished*). Thus, some high yielding cultivars with moderate resistance to *C. cassiicola* may be preferred by growers in some regions, as cultivar BMX Potência RR or NA 5909 RG, which were classified as MS and S respectively in the present work.

We observed that a single disease severity assessment was not as good as the AUDPC to perform a cultivar resistance screening for target spot, because even with strong correlation between both assessments a unique evaluation did not separate resistance groups as the integrate AUDPC did. For this reason, we suggest using AUDPC for greenhouse screening of soybean cultivars resistance to *C. cassiicola*. One possible reason is the difference in incubation period of the cultivars, which in short period of assessment (~ 20 days) can represent important differences in the results. However, at field conditions, a single disease severity assessment (at R5) was good enough to predict yield losses due to target spot. Screening soybean cultivars to target spot routines can be feasible with at least two assessments, at 7 and 21 DAI. This information could afford resources, without losing precision in the cultivar classification. Similar results were observed in a resistance screening with rice - *Pyricularia grisea* pathosystem, where a two data points AUDPC (initiation and end of the epidemic) provided similar information than the AUDPC obtained from 12 assessment points (Mukherjee *et al.*, 2010).

No interaction between *C. cassiicola* isolates and soybean cultivars was observed in our experiments for incubation period, disease severity, lesion density or lesion size. Our results did not agree with the observed by Teramoto *et al.*, (2013a) that reported significant interaction for six Brazilian *C. cassiicola* isolates and twelve soybean cultivars in a single assessment of disease severity. The high variability reported for the fungus aggressiveness (Dixon *et al.*, 2009; Teramoto *et al.*, 2013b) and in the host resistance did not implied an interaction effect for the present experimental conditions.

The incubation period (estimated at greenhouse) depended on the cultivar delaying in ~ 4 days in the resistant cultivar BRS360RR in comparison to the most susceptible one. This result were also observed by Seaman *et al.* (1965) that reported that when inoculated soybean plants in greenhouse and free moisture on the foliage was maintained, typical symptoms appeared within 7-10 days.

Disease severity and lesion size at 14 DAI presented the similar trend in the post-hoc comparisons among the three cultivars and the lesion density only could differentiate two groups. Wen *et al.*, (2015) found correlations between target spot candidate gene expression and the lesion size on the infected leaves of cucumber. The lesion size was already reported for other leaf necrotrophic spots – hosts as an indicator of partial resistance, for example: gray leaf spot of maize (Ward *et al.*, 1999), Cercospora leaf spot in sugar beet (Rossi *et al.*, 1999). Target spot lesion size may depend on the genotypes quantitative resistance that involves the cassiicolin host reaction (Fortunato *et al.*, 2015).

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4.6 Supporting Information

Table S1 Resistance level ranking upon disease severity assessment of 14 DAI of soybean cultivars inoculated with a *C. cassiicola* isolate from the Brazilian state Tocantins.

Id	Cultivar	Disease severity at 14 DAI (Logit)	CI$_{\text{Low}}$	CI$_{\text{Upper}}$	Cluster*	Resist. Class
6	BRS 360	-3.61	-4.33	-2.89	a	MR
8	M 6410 IPRO	-3.42	-4.14	-2.70	ab	MR
4	BRS 399 RR	-3.41	-4.13	-2.70	ab	MR
7	DM 6563	-3.35	-4.07	-2.63	ac	MR
2	BMX Potência RR	-3.32	-4.04	-2.60	ac	MR
13	BRS 1007IPRO	-3.16	-3.88	-2.44	ad	MR
5	BRS 359RR	-3.14	-3.86	-2.42	ad	MR
3	BRS 388RR	-3.11	-3.83	-2.39	ad	MR
9	BRS 1002IPRO	-3.05	-3.76	-2.33	ad	MR
10	BRS 1004IPRO	-2.88	-3.60	-2.16	ad	MR
18	BRS 391CV	-2.83	-3.54	-2.11	ad	MR
17	BRS 397 CV	-2.78	-3.50	-2.06	be	S
19	M9144RR	-2.64	-3.36	-1.92	be	S
15	BRS 1001IPRO	-2.56	-3.28	-1.84	ce	S
11	BRS 1010IPRO	-2.49	-3.21	-1.78	de	S
12	BRS 1005IPRO	-2.46	-3.18	-1.75	de	S
16	BRS 1006IPRO	-2.45	-3.17	-1.73	de	S
1	NA 5909 RG	-2.43	-3.15	-1.71	de	S
14	BRS 1003IPRO	-1.96	--2.67	-1.24	e	S

*Means followed by different letters at the column were significantly different ($P < 0.05$, ANOVA, Tukey-HSD)

Table S2 Tukey´s post – hoc comparisons for four AUDPC variables

Cultivar	$AUDPC_{7\text{-}14\text{-}21}$	$AUDPC_{7\text{-}14}$	$AUDPC_{7\text{-}21}$	$AUDPC_{14\text{-}21}$
BRS 360	a	a	a	a
M 6410 IPRO	ab	ab	ab	ab
DM 6563	abc	ab	abc	abc
BRS 1004IPRO	abc	ab	abcd	abc
BRS 399 RR	abc	ab	abcde	abc
BRS 359 RR	abc	ab	abcd	abcd
BRS 388 RR	abcd	abc	abcdef	abcde
BRS 1002IPRO	abcd	abc	abcdef	abcde
BRS 1007IPRO	abcd	abcd	abcdef	abcd
BRS 391 CV	bcde	bcd	bcdefg	bcde
BMX Potência RR	bcde	bcd	cdefg	bcde
BRS 397 CV	bcde	bcd	bcdefg	bcde
M9144RR	cdef	bcde	defgh	cdef
NA 5909 RG	def	bcde	efgh	def
BRS 1005IPRO	def	cde	fgh	def
BRS 1001IPRO	def	cde	fgh	def
BRS 1010IPRO	def	cde	fgh	def
BRS 1006IPRO	ef	de	gh	ef
BRS 1003IPRO	f	e	h	f

*Means followed by different letters at the column were significantly different ($P < 0.05$, ANOVA, Tukey-HSD)

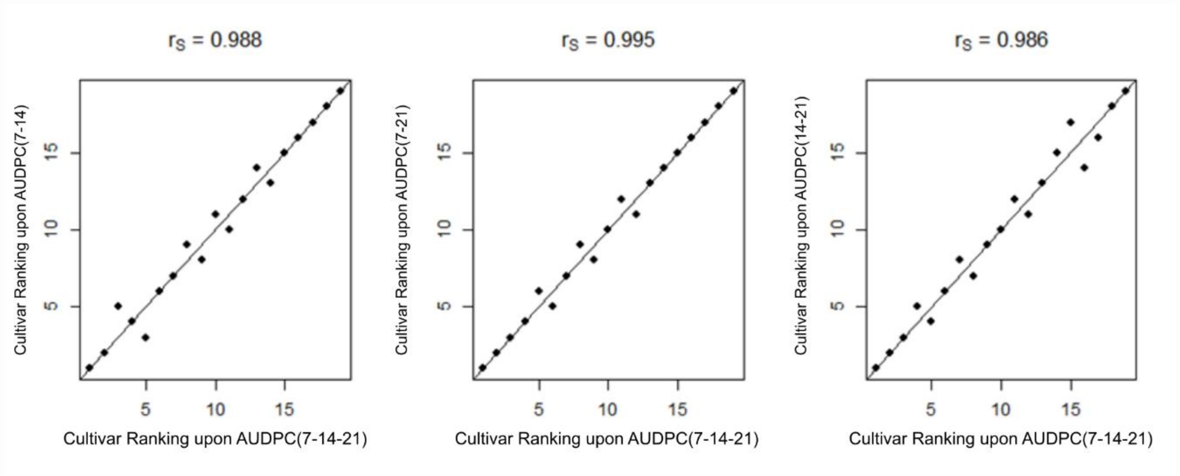


Figure S1 Ranking of cultivars obtained by Kruskal-Wallis algorithm for the AUDPC $_{7\text{-}14\text{-}21}$ on abscissa and two-disease assessments AUDPC on the ordinate axis. Points on the diagonal line represent exact concordance of ranking for both methods of classification.

5 RELATIVE IMPORTANCE OF SOYBEAN CANOPY POSITION AFFECTED BY TARGET SPOT ON GRAIN YIELD

Abstract

Foliar diseases that induce defoliation either by leaf senescence or by leaf abscission as soybean target spot can generate discrepancies in disease assessments. If defoliation is not considered the cumulative disease severity can be underestimated in single-point assessments at end of reproductive stages. For soybean, an important aspect to consider during disease assessment is at which canopy strata or leaf position where the green leaf area is reduced. Two field experiments were carried out in Southern Brazil to assess the relationship between the reduction of green leaf area throughout the reproductive stages at bottom, middle and top canopy sections with soybean yield. Using disease severity coupled with defoliation assessments throughout the reproductive growth stages, we performed correlations between grain yield and the leaf area injury at different plant positions – growth stages. The best correlation was observed with the assessments at R5.5 for injuries at middle canopy section. Area under the progress curve of defoliation or target spot severity individually from R2 to R5.5 or R6 were not significantly correlated with soybean yield.

Keywords: *Glycine max*; *Corynespora cassiicola;* critical-point assessment; yield losses

5.1 Introduction

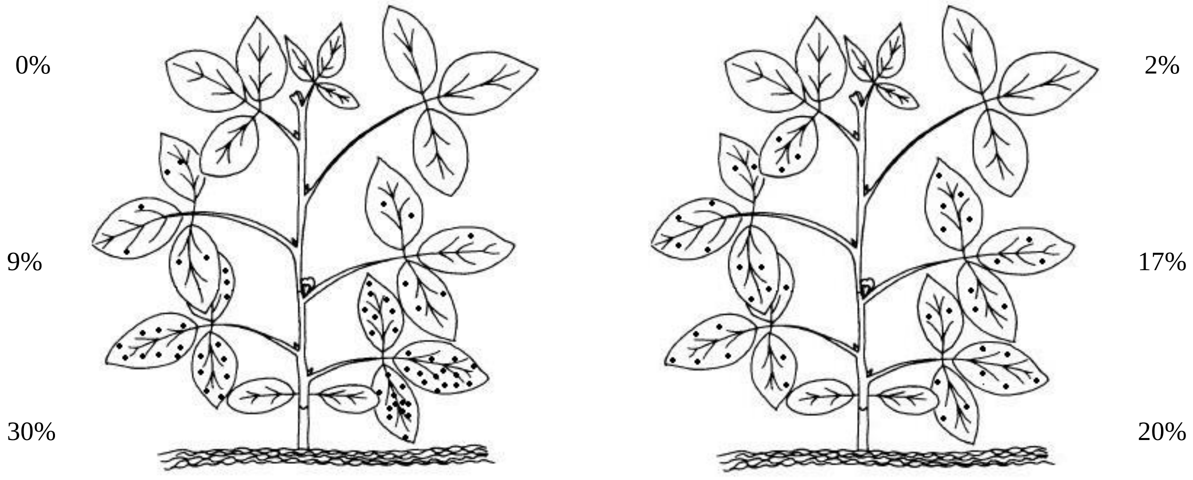
Target spot caused by *Corynespora cassiicola* has re-emerged in the United States, Brazil and Argentina in last decade. The disease is a typical representative of the light stealer and leaf senescence enhancer disease groups (Boote *et al.*, 1983) since it reduces the soybean photosynthetic leaf area by its symptoms and also by accelerating the natural senescence process (Snow & Berggren, 1989).

Disease-induced defoliation, caused either by leaf senescence or leaf abscission, usually complicates disease assessment (Nutter Jr *et al.*, 1991) because disease severity assessed at certain sampling time may not reflect the cumulative pathogen injury if the diseased dropped leaves are not accounted during assessment (Kranz, 1988).

Ideally, disease assessments should be obtained quickly and be reproducible. For damage and losses estimations it is also desirable that disease severity can correlate with yield (Campbell & Madden, 1990). Single-point or critical point assessments are measurements of the pathogen injury at one particular crop growth stage. Many critical-point models were established for cereals. For example, scald of barley (*Rhynchosporium secalis*), an individual assessment of the top two leaves when the grain is in "milky ripe" stage of growth provides a reliable estimate of yield are not accounted during assessment (James, 1974). Levy & Leonard (1990) showed that the leaves above and contiguous to the ear in the maize plant would contribute the most to grain yield than other leaves in the plant canopy. The mechanical removal of these leaves reduced corn yield by 32%. Adipala *et al.* (1993) observed that Northern leaf blight severity estimated on the ear leaf was well correlated with corn yield at tasseling growth stage. The best critical-point model to predict peanut yield loss due to *Cercosporidium personatum* spots was established when the crop was assessed on the whole plant 2–3 weeks prior to digging (Nutter & Littrell, 1996).

It has been reported that not all soybean leaves have the same contribution to grain yield, in reference to their position in the canopy. A greater yield loss was observed from manual defoliation of the upper plant canopy portion compared with the bottom canopy during R3 and R5 stages, but no difference between canopy position where detected during R6 stage (Owen *et al.*, 2013). Most of the classical soybean yield losses studies (McAlister & Krober, 1958; Begum & Eden, 1965; Turnipseed, 1972) agreed that yield reductions from defoliation were higher when pods are forming (R3 to R6) than earlier, at vegetative growth stages, or later, when beans have filled pods.

This physiological information may contribute to design a rational weighting system to account for the relative importance of symptoms on different plant organs (Zadoks, 1972). Considering that initial symptoms of target spot can be commonly detected at the bottom soybean canopy, we hypothesize that the cumulative injury, which includes disease symptoms and consequent defoliation, at the bottom part of the plant may be less damaging for the crop than injuries at the upper canopy. Leaves at the upper part of soybean plants intercept more photosynthetically active radiation than leaves at the middle or the bottom parts of the plant. For example, an overall 13% disease severity in the entire plant may depend on the vertical distribution of the symptoms to account for yield reduction. A strong bottom - middle - top gradient of disease severity (Fig. 1A: 30% - 9% - 0%) would be less damaging to yield than a weak vertical gradient (Fig. 1B: 20% - 17% - 2%).



A) Overall disease severity = 13%

B) Overall disease severity = 13%

Figure 1 Hypothetical situations of vertical distribution of target spot symptoms on bottom – middle – top canopy strata.

The overall goal of the current study was to compare the sensitiveness of the canopy strata injured by *C. cassiicola* to reduce grain yield.

5.2 Material and methods

Two field experiments were done to address the proposed objectives. The first experiment was carried out in Londrina (Paraná state, 23.1918° S, 51.1827° W) and the second one in Piracicaba (São Paulo state, 22.7152° S, 47.6298° W), Brazil. Seeds of cultivar NA 5909 RG were sown on December 4, 2014 and October 23, 2015 to a no tilled or tilled seedbed for experiments 1 and 2, respectively, at a rate of 20 seeds m^{-1}. Experimental plots had 6 m long × 4 rows 50 cm apart. The objective of using such early planting date relative to the optimum for the region was to minimize the risk of *Phakopsora pachyrhizi* (Asian soybean rust) infections that could potentially affect disease - yield relationship. To achieve a wide range of target spot severity soybean plots were inoculated with a mix of five *C. cassiicola* isolates obtained from soybean crops in Paraná and Mato Grosso states. *C. cassiicola* colonies were grown in PDA plates during 20 days under continue fluorescent light were harvested and mixed in a blender, then filtrated into a 10 liters solution of tap water and tween 80 (0.1%). Suspension concentration was adjusted with aid of hemocytometer to 10^{5} conidia or mycelium fragments ml^{-1} and sprayed on plots with a manual backpack sprayer.

With the aim of generating a wide range of target spot symptoms, plots were inoculated at R1 stage (Fehr *et al.*, 1971) with different volumes rates of the inoculum suspension: 100, 200, 300 or 400 L ha^{-1}. Non-inoculated plots were considered as reference controls. All treatments (conidia suspension volumes) had three replicated plots. For the second experiment, plots were irrigated daily during the following 5 days after inoculations in order to ensure the pathogen infection.

5.2.1 Injury assessments.

Target spot severity (TS) was assessed using a diagrammatic scale (Soares *et al.*, 2009) at bottom, middle or top canopy. Senesced leaves proportion was estimated visually. Senesced leaves values were visually estimated in a percentage scale as a proportion of senesced leaves over the total of leaves at each canopy strata. For the first experiment canopy strata were considered subjectively by picking leaves from the bottom, middle or top plant vertical profile to do the assessments. For the second experiment, three plants were established at each plot to register the number of senesced trifoliate leaves and assessing target spot severity. A plastic ring was fixed to the fifth node (without considering the unifoliate node), and to the ninth node, in order to differentiate the three portions of the canopy: bottom canopy < first mark ≤ middle canopy < second mark ≤ top canopy. Senesced leaves were considered when at least two single leaflet where fully chlorotic or the three leaflets were equal or higher than 50% chlorotic leaf area. Soybean yield was estimated by mechanically collecting soybean plants along 4 m of 2 inner plot rows in the first experiment and by manually harvesting and weighting pods and seeds of 10 plants in the 2nd experiment. Fresh grain biomass was extrapolated to kg ha^{-1} adjusted to 13% humidity. No insect’s injury or presence of Asian rust or other diseases were observed. Observed defoliation was considered as natural or induced by target spot effect.

5.2.2 Disease index calculations.

As a first step we defined six canopy strata weight distributions ($WD_{1 \text{ to } 6}$), detailed in Table 1.

Table 1 Six relative weights distribution (WD_i) for each canopy leaves strata

Canopy strata	WD_1	WD_2	WD_3	WD_4	WD_5	WD_6
Bottom	1	0	0.33	0.5	0.3	0.2
Middle	0	1	0.33	0.5	0.5	0.7
Top	0	0	0.33	0	0.2	0.1

Each weight distribution has a physiological meaning in relationship with hypothetical yield contributions. WD_1 and WD_2 assumes that bottom or middle canopy respectively are the unique portions that contributes to yield built; WD_3 assumes that the whole plant leaf area has the same impact on yield; for WD_4 bottom and middle canopy contribute the same to yield and top canopy does not contribute at all; WD_5 and WD_6 middle canopy contributes most (50% and 70% respectively), and bottom and top canopy contributes upon the weights detailed in Table 1. With WD_i indices, we can now calculate each disease index (DI) as follows including target severity and defoliation assessments:

$$DI_{i(GS)} = \sum_{j=1}^{3}\left[Def_j + \left(1 - Def_j\right) * TS_j\right] * w_j \qquad (1)$$

$$DI_{i(GS)} = \sum_{j=1}^{3} W_j \qquad (2)$$

where $DI_{i(GS)}$ is the disease index (in proportion scale) for the i-th relative weights distribution at growth stage GS; Def_j or TS_j are the mean proportion of defoliation or target spot severity at j-th canopy portion; w_j is the relative weight of the j-th canopy portion. All the right-hand equation side in 1 can be summarized by the sum of each canopy weighted leaf area reduction (by defoliation and TS) W_j at 2.

Following, we introduce a real example of leaf area reduction progress considering an inoculated field plot (Fig. 2):

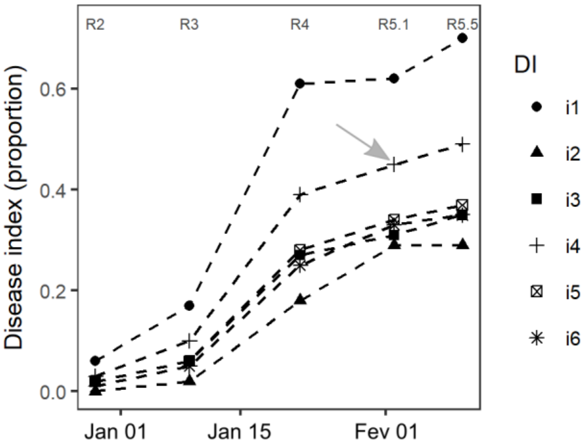


Figure 2 Temporal progress of target spot indexes at an experimental soybean plot inoculated with 10^5 *C. cassiicola* conidia or mycelium fragments ml^{-1}. Grey arrow is pointing at the $DI_{4(R5.1)}$ calculated in the illustrative example. The DIs represent the leaf area reduction (defoliation + target spot symptoms) in: 1) bottom canopy; 2) middle canopy; 3) overall plant; 4) mean of bottom – middle portions; 5 and 6) proportional weights distributions (bottom – middle – top) 0.3-0.5-0.2 and 0.2-0.7-0.1 respectively.

Using the weights distribution in Table 1 and equation 1 and we estimated each of the points plotted in Fig. 2. Below there are calculations for the $DI_{4(R5.1)}$ (weights 0.5-0.5-0, at R5.1 growth stage):

$$W_{bottom} = [0.45 + (1 - 0.45) * 0.3] * 0.5 = 0.3075$$

$$W_{middle} = [0.2 + (1 - 0.2) * 0.281] * 0.5 = 0.147$$

$$W_{top} = [0 + (1 - 0) * 0.02] * 0 = 0$$

$$DI_{4(R5.1)} = W_{botton} + W_{middle} + W_{top} = 0.375 + 0.147 + 0 = 0.52$$

Using all six target spot indexes we can calculate their area under the curve progress through the time (AUC) as well for the individual variables (defoliation and TS) with linear interpolation (trapezoidal method) using "auc" function of MESS R package (Ekstrøm *et al.*, 2017). Finally, we performed Pearson´s *r* correlations (*r*) between all the disease variables (single-point or AUC) and soybean yield. This coefficient *r*, also called the linear correlation coefficient, measures the strength and the direction of a linear relationship between two variables as it is the case for soybean yield and proportion of injury.

5.3 Results

Soybean yield ranged from 2586 to 3780.2 kg ha^{-1} (median = 3415 kg ha^{-1}) and from 1961 to 2572 kg ha^{-1} (median = 2317 kg ha^{-1}) for the first and second experiment respectively. Target spot severity (proportion) ranges throughout all the assessed growing stages for bottom, middle, and top canopy were, respectively, 0 - 0.6 / 0 - 0.39 / 0 - 0.08 for the first experiment and 0 - 0.27 / 0 - 0.25 / 0 - 0.1 for the second experiment. Maximum levels of defoliation for bottom, middle, and top canopy were, respectively, 0.65 / 0.07 / 0 for the first experiment and 0.6 / 0.15 / 0, for the second experiment.

The problematic situation reported by Kranz (1988) concerning assessments of defoliation-induced diseases was be observed in our experiments. the progress curves of disease severity, defoliation and total leaf area removed form plants in two plots from experiment 1, a non – inoculated plot and an inoculated with 10^5 *C. cassiicola* conidia or mycelium fragments ml^{-1} are described at Fig. S1. In inoculated plots, target spot severity increase up to some point when the leaf starts to get chlorotic and finally die. At R5.5 stage, target spot severity levels were similar in inoculated and non-inoculated plots, however the senescence was higher in inoculated plots, masking the effect of the disease (Fig. 3)

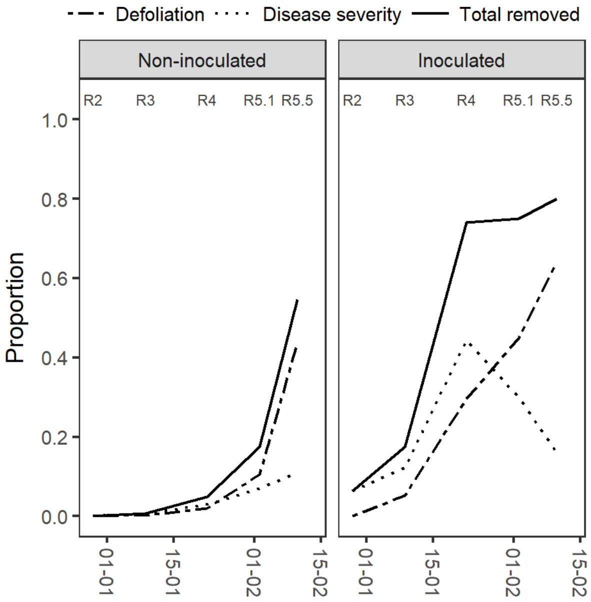


Figure 3 Temporal progress of defoliation (senesced or abscised leaves, dashed lines), target spot severity (dotted lines) and leaf area reduction (defoliation + disease severity, solid line) in bottom canopy leaves at non-inoculated and inoculated plot with 10^5 *C. cassiicola* conidia or mycelium fragments ml^{-1}.

We observed a wide range of Pearson´s *r* coefficients among single – point relationships for both experiments, ranging from -0.774 to 0.178 and from -0.755 to -0.172 for experiments 1 and 2 respectively. At experiment 1 only four disease indexes (DI) at growth stage (GS) R5.5 were significantly correlated with yield (Fig. 4).The highest *r* value (-0.774, $R^2 = 59.9\%$) was observed for DI_2. At experiment 2, several growth stages presented significant correlation with yield at R4, R5.5 and R6, however the higher *r* values were observed at R5.5, ranging from -0.648 to -0.692, corresponding to R^2 values from 42% to 48%.

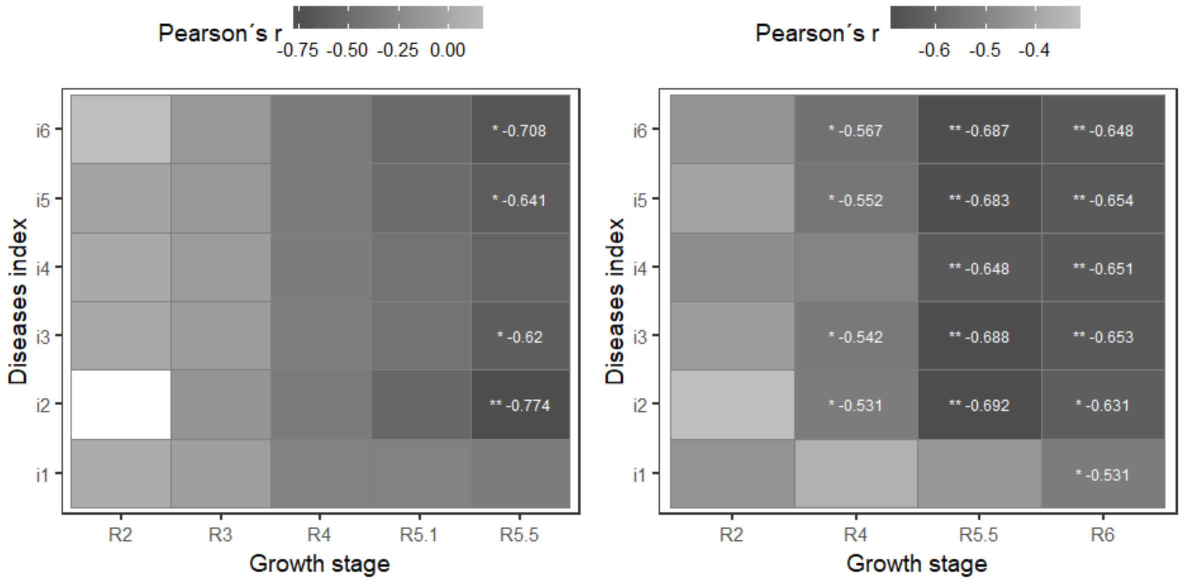


Figure 4 Pearson´s *r* correlation coefficients ($0.001 < P < 0.01$) for the six target spot disease indexes and soybean yield for experiment 1 (left) and 2 (right) performed in Londrina (Paraná state) and Piracicaba (São Paulo state) during 2014/2015 and 2015/2016 respectively. White squares represent situation with DI=0, then correlation was no estimated.

Both variables defoliation and TS severity individually had lower maximum *r* values in comparison to the DIs (Table 2). The correlation between total plant defoliation and soybean yield was significant only at R6 in the second experiment. The AUC of target spot severity progress did not correlate with soybean at any of both experiments. The AUC of DIs progress had significant correlations with soybean yield only at the second experiment with a maximum value of $r = 0.687$ for I_2 and I_6.

Table 2 Correlation analysis between total defoliation, AUC of disease severity or disease index progress and soybean yield

Experiment	Variable	Pearson´s *r*	*P* value
1	Def. at R2	-	-
	Def. at R3	-0.537	0.089
	Def. at R4	-0.451	0.164
	Def. at R5.1	-0.505	0.113
	Def. at R5.5	-0.544	0.084
2	Def. at R2	-0.357	0.191
	Def. at R4	-0.321	0.243
	Def. at R5.5	-0.508	0.053
	Def. at R6	**-0.606**	**0.017**
1	AUC target spot severity	-0.345	0.298
2	AUC target spot severity	-0.266	0.429
1	AUC i1	-0.250	0.458
	AUC i2	-0.491	0.125
	AUC i3	-0.334	0.315
	AUC i4	-0.328	0.324
	AUC i5	-0.364	0.271
	AUC i6	-0.408	0.213
2	AUC i1	**-0.535**	**0.040**
	AUC i2	**-0.687**	**0.005**
	AUC i3	**-0.676**	**0.006**
	AUC i4	**-0.656**	**0.008**
	AUC i5	**-0.674**	**0.006**
	AUC i6	**-0.687**	**0.005**

5.4 Discussion

In agreement with other reported studies of similar pathosystems affecting soybean yield, the total leaf area affected by target spot severity and defoliation, had a higher correlation coefficient with losses in yield than with defoliation or disease severity evaluated independently. Samborski & Peturson (1960) also demonstrated the significance of recording chlorosis or necrosis when they reported substantial losses in yield of wheat cultivars hypersensitive to leaf rust

We observed that assessments performed at growth stage R5.5 were the best correlated to soybean yield as reported by the cited authors (McAlister & Krober, 1958; Begum & Eden, 1965; Turnipseed, 1972) that observed better correlations between soybean defoliations and yield between R3 and R6. We observed that leaves at middle canopy section were the most sensitive to produce variations in grain yield (DI2) as previously reported by Owen *et al.* (2013). Other DIs including the bottom and top canopy sections showed low variation terms of correlations levels. One possible reason of the non-significance of the top canopy could be that too little disease was observed for this particular portion at both experiments.

The target spot disease indexes tested here present lower r values in comparison with brown spot (Septoria glycines). Brown spot severity or its AUC best correlated with soybean yield reduction at R6 ($r > -0.75$) (Lim, 1980). For frogeye leaf spot (Cercospora sojina) r values were –0.52 and –0.59 (Mengistu *et al.*, 2014). The second experiment presented lower Pearson r coefficients, but they were significant for a wider range of growth stages.

Although significant correlations were observed for the proposed disease indexes, the coefficient of determination was low. This could indicate that probably critical-point assessment is not the best method to predict yield loss accurately, and as stated by Waggoner & Berger (1987), yield is likely to be more determined by the healthy leaf area duration, or more precisely, by the absorption of insolation during a season by this healthy area. However, an important information was obtained from this study, since in both experiments the most sensitive canopy strata to *C. cassiicola* infections to affect yield of was the middle one. The simple presence of target spot symptoms at low canopy strata may not affect yield as well as at middle strata. Then disease scouting and fungicide protections should be focused on this particular plant section.

5.5 References

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