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In vitro sensitivity assessment of late season soybean pathogens to fungicide mixtures

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Abstract Late season diseases cause yield reductions to soybean grown worldwide. In Argentina, fungicide mixtures composed of quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs), and the newly introduced succinate dehydrogenase inhibitors (SDHIs), have been effective in managing these diseases. Nevertheless, the risk of selecting strains with resistance to these classes of fungicides is considered to be high. This preliminary study was carried out to determine in vitro sensitivities as determined by the effective concentration that inhibited 50% of radial mycelial growth (EC_{50} values) of *Cercospora kikuchii*, *Colletotrichum truncatum* and *Phomopsis phaseoli* to selected QoI and DMI fungicide mixtures. The results indicated that EC_{50} values ranged from 0.0065 to 0.0402 μg/ml for *C. kikuchii*, from 0.0344 to 0.1744 μg/ml for *C. truncatum* and from 0.0001 to 0.1974 μg/ml for *P. phaseoli*. To better study the possible resistance against these pathogens, future tests should consider several isolates for each pathogen from different production areas and different fungicide active ingredients.

Keywords EC_{50} · *Cercospora kikuchii* · *Colletotrichum truncatum* · *Phomopsis phaseoli* · Fungicide resistance

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Late season soybean diseases (LSDs) are a combination of various diseases that cause premature senescence and reduce grain yield and seed quality worldwide (Carmona et al. 2015). The main LSDs are caused by *Septoria glycines*, *Cercospora kikuchii*, *Colletotrichum truncatum* and *Phomopsis phaseoli* (Hartman et al. 2015). Severity of LSDs has increased in recent years in Argentina, mainly due to soybean monocropping and conservation tillage (Wrather et al. 2010). This has led to increased fungicide use to reduce LSDs damage (Carmona et al. 2011). In Argentina, fungicide mixtures composed of quinone outside inhibitors (QoI) and demethylation inhibitors (DMI) have been effective in managing LSDs (Carmona et al. 2015). Recently, succinate dehydrogenase inhibitors (SDHI) have been introduced to the Argentine market, in mixtures with QoI or QoI + DMI. Unfortunately, the risk of resistance to QoI and SDHI is considered high (FRAC Code List© 2017).

Given the reliance on fungicides for managing Argentine LSDs and the risk of potential resistance in these fungicide classes it is critical to develop a better understanding of baseline sensitivities (Russell 2004). Thus, the objective of the current study was to determine the in vitro sensitivity of LSDs pathogens to QoI + DMI fungicide mixtures and one QoI + DMI + SDHI mixture currently in use in Argentine soybeans.

In the present study, five commercial fungicides were tested in vitro against soybean pathogens *C. kikuchii*, *C. truncatum* and *P. phaseoli*, for inhibition of mycelial growth. Active ingredients are listed in Table 1. One isolate of each pathogen was isolated during the 2015 growing season from symptomatic plants of soybean crops located in the Pergamino area. Colletotrichum waxy acervuli and Phomopsis pycnidia were carefully broken and removed from the surface of symptomatic leaves and placed in test tubes with sterile water. In the case of Cercospora symptomatic leaf sections of aprox 2 cm^2 were cut and placed in sterile test tubes

Table 1 Fungicide active ingredients used in the in vitro tests

QoI active ingredient	DMI active ingredient	SDHI active ingredient	Manufacturing company
azoxystrobin 20%	cyproconazole 8%		Syngenta Agro S.A.
picoxystrobin 20%	cyproconazole 8%		DuPont Argentina S.R.L.
trifloxystrobin 37.5%	cyproconazole 16%		Bayer S.A.
pyraclostrobin 13.3%	epoxiconazole 5%		BASF Argentina S.A.
pyraclostrobin 13.3%	epoxiconazole 5%	fluxapyroxad 5%	BASF Argentina S.A.

with sterile distilled water (3 ml). All tubes were shaken vigorously. Sixteen squares were marked on the bottom of water agar (1.5%) plates. The prepared homogeneous spore suspension was then transferred with a sterile pipette, onto the surface of the water agar plate, with a drop placed above each of the drawn squares. In the case of *C. kikuchii*, few drops of the spore suspension were placed on a glass slide and observed with a stereomicroscope for conidia. Then, single spores were transferred using fine glass needle to water agar. Water agar pates were amended with streptomycin sulfate (180 μg/ml). Afterwards, plates were allowed to incubate at 24 °C+/−1 °C for 7 days. Colonies exhibiting growth characteristics consistent with *C. kikuchii*, *C. truncatum* and *P. phaseoli* were selected, transferred via hyphal tips to V8 agar (20% juice), and maintained at 25 °C with a 12 h light:dark cycle. *C. kikuchii*, *C. truncatum* and *P. phaseoli* isolates were maintained in the fungal bank of the Reference Center for Mycology (CEREMIC) from the Rosario National University (UNR, Argentina) under accession numbers Ck_2015_01, Ct_2015_01 and Pp_2015_01.

Because of scant sporulation in culture, radial growth assays for assessing inhibition for fungicide mixtures were utilized instead of spore germination assays as the method for determining fungicide sensitivity. Serial dilutions of the fungicides were amended to potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) at 0.001, 0.01, 0.1, 1, 10 and 20 μg ml^{-1}. All amendments were added to sterilized, molten PDA (~50 °C), and aseptically dispensed into sterile petri dishes (15 × 90 mm, ~20 ml/dish). Mycelial discs (6 mm diameter) were cut from the margins of 5-day-old stock cultures of each isolate actively growing on PDA using a #3 cork borer, inverted, and transferred to amended PDA. Petri dishes were incubated under 12 h fluorescent and black light/12 h darkness cycle at 24 °C+/−1. Non-fungicide-amended PDA served as a control. After incubation for 7 days, colony diameter was measured twice using a digital caliper (DIGIMESS, No. 1304I, China). Each experiment was conducted using a randomized complete block design with incubator shelves serving as blocks. There were four replicates per fungicide concentration. Each experiment was repeated twice.

Statistical analysis was performed using R Statistical Software version 3.2.5 (R Core Team 2013). Nonlinear regression analysis of natural log concentration by relative mycelial growth was performed for each fungicide-pathogen combination. Relative growth (colony diameter of treated plates/colony diameter of control) was used as the response variable. Four models were fitted and their derived parameters estimated: the three-parameter log-logistic model (LL.3), the four-parameter log-logistic model (LL.4), the asymmetric Weibull type I model (W1.4) and the generalized five-parameter log-logistic model (LL.5). Function *drm()* from R package *drc* (Analysis of Dose-Response Curves) version 2.5–12 (Ritz et al. 2005; 2015) was used for dose-response analysis to fit models W1.4 and LL.5. Function *nls()* from the R package *stats* was used to fit models LL.3 and LL.4. The equations for each dose-response model are shown in Table 2. Estimation of parameters was based on the maximum likelihood principle, which under the assumption of normally distributed response values simplifies to nonlinear least squares. The Gauss–Newton algorithm was used to solve non-linear least squares. In addition, hypothesis testing for parameters were conducted to verify the significance of the parameters. Residual standard errors (RSE) and the Akaike Information Criterion (AIC) were calculated. The effective fungicide concentration to inhibit 50% of fungal radial growth (EC_{50}) was estimated for each treatment-pathogen combination using the *ED()* function of the *drc* package. Estimated effective doses were obtained by inserting parameter estimates and solving the equation: $(ED100\alpha,\beta) = (1-\alpha)\lim_{x\to 0} f\ (x,\beta) + \alpha \lim_{x\to\infty} f\ (x,\beta) = (1-\alpha)c + \alpha d$. Mean EC_{50} values and their 95% confidence intervals were estimated for each fungicide and isolate by combining data from the two experiments.

Table 2 Equations adjusted by nonlinear regression for each model: three-parameter log-logistic model (LL.3), four-parameter log-logistic model (LL.4), asymmetric Weibull type I model (W1.4) and five-parameter log-logistic model (LL.5)

Model	Equation
LL.3	$y = \frac{a}{1+e^{\frac{b-\log(x)}{c}}}$
LL.4	$y = a + \frac{b-a}{1+e^{\frac{c-\log(x)}{d}}}$
W1.4	$y = b + (c-b)e^{-e^{(a(\log(x)-\log(d)))}}$
LL.5	$y = b + \frac{c-b}{(1+e^{a(\log(x)-\log(d))})^e}$

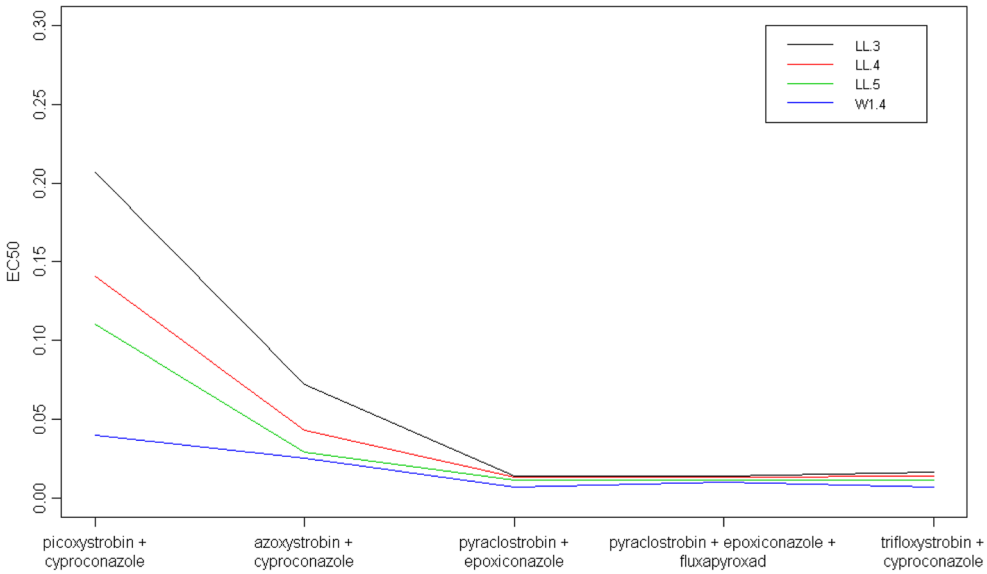

Fig. 1 EC_{50} values for five fungicides in *C. kikuchii* according to each model fitted. W1.4 gives the smallest estimate values for all fungicides

To evaluate each of the proposed mathematical models, the variable EC_{50} was compared using fungicide as a blocking variable. The three fungi were studied separately. For each pathogen the nonparametric Quade test was applied with a significance level of 5% and, if significant differences were found, multiple comparison tests were performed and the Bonferroni correction was applied.

For *C. kikuchii* significant differences were found between EC_{50} estimates in the proposed mathematical models ($F_{3,12} = 18$, p-value < 0.0001)). The posteriori multiple comparison tests showed that the W1.4 model differed from the LL.4 model (p-value <0.0003) and LL.3 model (p-value <0.00001). Differences were also found between the LL.3 and LL.5 models. For *C. truncatum* significant differences were also found between estimates of EC_{50} in the proposed mathematical models (F3,12 = 7.8, p-value < 0.0037). The model for W1.4 differed from the LL.5 model (p-value <0.003) and LL.5 differed from the LL.3 model (p-value <0.0006). For *P. phaseoli* no significant differences were found between estimates of EC_{50} from the proposed mathematical models ($F_{3,12} = 0.48$, p-value < 0.69). According to Fig. 1, the choice of a particular model does not depend on the fungicide. Similar trends were observed for each mathematical model for each pathogen (e.g. W1.4 gives the smallest estimate values for all fungicides). Additionally, the model that obtained more similar results of EC_{50} independently of the fungicide was W1.4. There were no significant differences between models for RSE and AIC values.

Although the fit of the models for each data combination was similar in all models, W1.4 was selected because the mean EC_{50} values obtained with this model were close to zero and had the lowest relative variation (C:V:= 0.83). These EC_{50} values are more likely to occur biologically and are similar to previous reports from the literature (Price et al. 2015; Batzer et al. 2016). In the present study, for two of the three fungi analyzed (*C. kikuchii* and *P. phaseoli*), the W1.4 model provided the lowest EC_{50}. In the case of *C. truncatum*, the EC_{50} means obtained from W1.4, although the lowest, were not significantly different from those estimated from models LL.3 and LL.4. The EC_{50} values estimated for each pathogen-fungicide combination with the W1.4 are shown in Table 3.

Table 3 EC_{50} and 95% confidence interval estimates for each pathogen-fungicide combination using the Weibull type I model (W1.4)

Mixture of Fungicide	*C. kikuchii*			*C. truncatum*			*P. phaseoli*		
	EC_{50}	LL[a]	UP[b]	$EC_{50.}$	LL	UP	EC_{50}	LL	UP
azoxystrobin + cyproconazole	0.0253	0.0134	0.0371	0.1604	0.0464	0.2744	0.1974	−0.0650	0.4599
picoxystrobin + cyproconazole	0.0402	−0.0013	0.0817	0.1744	0.0059	0.3428	0.0270	−0.0060	0.0599
trifloxystrobin + cyproconazole	0.0070	0.0019	0.0120	0.0635	−0.0192	0.1480	0.0001	−0.0002	0.0003
pyraclostrobin + epoxiconazole	0.0065	0.0010	0.0120	0.0526	0.0073	0.0978	0.0094	−0.0132	0.0321
pyraclostrobin + epoxiconazole + fluxapyroxad	0.0098	0.0060	0.0136	0.0344	0.0093	0.0595	0.1670	0.0303	0.3036

[a] LL = lower limit

[b] UP = upper limit

According to the scale proposed by Edgington et al. (1971), all mixture of fungicides controlled all tested pathogen isolates with an $EC_{50} < 1$ ppm, indicating that the isolates tested were sensitive to these molecules. Nevertheless, this is a preliminary study and future tests should consider several isolates for each pathogen from different production areas and different fungicide active ingredients. It is necessary to create a fungicide sensitivity-testing program in Argentina due to the numerous cases of fungicide resistance that have been already reported worldwide in soybean. In the United States, *C. kikuchii* isolates exhibiting multiple resistance to QoI and benzimidazole fungicides have already been confirmed and reported (Price et al. 2015).

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