**EFFICACY OF FUNGICIDES AGAINST PEANUT SMUT IN ARGENTINA**

**Abstract**

Peanut smut, caused by *Thecaphora frezii*, increased its incidence and prevalence in the main growing region of Argentina in the last decade, becoming the main peanut disease. Despite this fact, growers continue producing peanut without any disease management strategy, which is leading to a local accumulation of *T. frezii* inoculum. The goal of the present study was to assess the efficacy of 12 fungicides in controlling *T. frezii*. For this purpose, *in vitro* assays, pot, and field experiments were performed during 2014/15 and 2015/16. *In vitro* assays classified the fungicides regarding their capacity to inhibit *T. frezii* mycelium growth as: thiophanate-methyl – not fungitoxic (EC50 > 100); mancozeb – moderately fungitoxic (EC50 = 6.28); difenoconazole, propiconazole, cyproconazole, tebuconazole, picoxystrobin, azoxystrobin, kresoxim-methyl, pyraclostrobin, penthiopyrad, and chlorothalonil – highly fungitoxic (EC50 < 0.1). Azoxystrobin showed the highest control levels in pot experiments: 58.9% (2014/15) and 92% (2015/16). The greatest control efficiency in field experiments was observed for cyproconazole in 2015 and azoxystrobin in 2016, reducing peanut smut by 47.7% and 39.5%, respectively. Based on our results, chemical control can be considered as a moderately efficient practice which may complement the cultivar genetic resistance and cultural practices in an integrated approach for managing peanut smut.

**1. Introduction**

Peanut (*Arachis hypogaea* L.) is an important crop that is cultivated worldwide, with its center of origin in northwestern Argentina and southeastern Bolivia (Hammons et al., 2016). Argentina produces a high-quality peanut in an area of 350,000 has, concentrating more than 90% in the province of Córdoba located at the center region of the country. More than 95% of peanut production is exported and was previously processed *in situ* by the industry established in the same growing region (Agüero, 2017; Calzada and Rozadilla, 2018).

Peanut smut, caused by the soil-borne fungi *Thecaphora frezii* Carranza and Lindquist, is an endemic and yield reducing disease for the main Argentine growing region (Marinelli et al., 2008, 2010; Rago et al., 2014). The pathogen is a biotrophic fungus that produces sori consisting of a powdery mass of teliospores which colonize the seed tissue partially or totally, with potential total damage of the plant pods (Astiz Gasso et al., 2008). The infection process occurs during the crop pegging stage, when flower gynophores penetrate the soil surface and their exudate stimulates spore germination (Marinelli et al., 2008).

Currently, no management strategies for reducing *T. frezii* infections are adopted by Argentine growers, which is leading to inter-annual inoculum accumulation (Paredes et al., 2017). Granoleico peanut cultivar (highly susceptibility to the smut) is planted in more than 80% of the growing region (Cazón et al., 2018; Cignetti et al., 2010; Rago et al., 2017). Breeding programs with the aim of incorporating wild resistance genes have recently been initiated (Bressano et al., 2019). However, for the industry characteristics (maintain the same cultivar in process) and growers’ production, new genotypes are not expected to be adopted in the short term.

Smut spores have a survival structure which can remain viable for new infections up to four years (Cazón et al., 2016b) which implies that a short peanut crop rotation is an inefficient practice to reduce disease pressure in the current Argentine production system. Preliminary research suggested a wide range of control efficacies with fungicide applications while higher doses of DMI + QoI fungicide mixtures were reported as providing the best control. Fifty eight percent smut control was observed by spraying a mixture of azoxystrobin and cyproconazole at a rate of 1000 cm³/ha, which is twice and half the recommended dose for late peanut leaf spot management caused by *Nothopassalora personata* (syn. *Cercosporidium personatum*) (Cazón et al., 2013; Paredes et al., 2015a; Paredes et al., 2015b). In addition, the effect of time of the day on fungicide smut control efficacy was compared: fungicide applications during the night had higher control efficacy than sprayings during the day (Paredes et al., 2015c). The application timing effect may be due to the fact that peanut plants fold their leaves at night and open them in the daytime (a process known as nyctinasty), allowing for more spraying drops to reach the soil surface.

No fungicide sensitivity studies of local *T. frezii* populations are registered in Argentina, and this information is essential for setting up chemical control. The lack of studies examining a wide set of fungicides to control peanut smut led us to perform this study. The objectives of this work were: i) to assess the fungicides’ efficacy against *T. frezii* in *in vitro* conditions; and ii) to test the field performance of fungicides against peanut smut.

**2. Materials and Methods**

**2.1. *In vitro* sensitivity of fungicides based** **on a mycelial growth inhibition test**

To measure the *in vitro* sensitivity of fungicides, a mycelial growth test was carried out. A mixture of 10 *T. frezii* isolates obtained from smutted pods in 2015 in General Deheza (Córdoba province, Argentina) were used for the fungicide sensitivity test. Smut teliospores (100 mg) were suspended in aqueous solution of 0.63% NaOCl, agitated for 5 minutes, rinsed twice with sterile distilled water, and resuspended in 2 ml sterile distilled water. Disinfected teliospores were placed in potato dextrose agar made up of broth extract of healthy grains at a rate of 50 g/l (Potato Peanut Dextrose Agar, PPDA). Plates were incubated at 25±1º C in darkness (Astiz Gasso and Wojszko, 2011). After 7–10 days, spores of *T. frezii* were germinated and colonies were transferred to a culture medium of 2% potato dextrose agar to obtain pure *T. frezii* colonies. The identities of all isolates were confirmed with specific primers for *T. frezii* (Cazón et al., 2016a).

A total of 12 fungicide products (isolated active ingredient and adjuvant) varying in their mode of action were included in the study (Table 1). Five concentrations of each active ingredient were analyzed: 0.01, 0.10, 1.00, 10.00, and 100.00 μg a.i./ml medium. Petri plates without fungicides were used as an experimental control. Each experimental unit was represented by a plate (55 mm diameter), with five repetitions per treatment.

Mycelial discs (6 mm in diameter) were extracted from actively growing culture isolates of *T. frezii* (8-day-old) and transferred to the center of Petri plates containing PDA amended medium. Five replicated plates were used for each fungicide concentration incubated at 25 ºC in darkness.

The radial growth (colony diameter) of each isolate was measured with a digital caliper in two perpendicular directions, and it was subtracted to the original mycelial disc diameter (6 mm). The measurements were taken at the moment in which the mycelium of the control plates reached their plate margins. The inhibition of mycelial growth relative to the treatment without fungicide was calculated as:

GIi = (CDcheck − CDi)/CDcheck × 100 (1)

where GIi is the inhibition of i-th colony growth; CD is the mean colony diameter for the control without fungicide (check) or for the *i*-th fungicide. A linear model regression was fitted to the GI obtained along the exponential–transformed doses, and the EC50 was estimated by replacing components of the fitted equations.

The fungitoxicity of fungicides was classified according to Tonin et al. (2015): EC50 < 1 µg/ml – highly fungitoxic; between 1–50 µg/ml – moderately fungitoxic; EC50 > 50 µg/ml – non-toxic.

**2.2.** **Fungicides’ efficacy in controlling peanut smut trials**

Two sets of replicated experiments were conducted during the growing seasons 2014/2015 (from now on “2015”) and 2015/2016 (from now on “2016”). The peanut cultivar, Granoleico, was used in all experiments due to its known susceptibility to smut (Oddino et al., 2013). Twelve fungicides labeled for peanut leaf spot were tested (Table 1). A non-treated control was included in each experiment by spraying only water. We used one and a half fold the active ingredient dose recommended for peanut leaf spot control. One set of trials was conducted in pots and the other in field plots.



2.2.1. Field experiment

Field trials were conducted at the Aceitera General Deheza (AGD) experimental station in General Deheza (province of Córdoba, Argentina – 32° 45’ 20.53” S, 63° 46’ 56.5” W), located in the main peanut producing region with high *T. frezii* inoculum field pressure (Paredes, 2017). The inoculum concentration in the experimental area was higher than 4500 smut teliospores/g of soil. Treatments were distributed in a randomized complete block design with four replications. Plots consisted of four rows of peanut seedlings at 0.7 m spacing and 8 m long. Fungicides were applied at night (between 9–11 pm) considering the soil as the spraying target, with a CO2 pressurized backpack and handheld boom equipped with four nozzles (hollow cone, ALBUS ATR 80), 50-cm spacing, delivering 120 L/ha at a pressure of 310 kPa and 0.9 m/s spray walking speed. The first application was done seven days after the R2 stage (beginning peg) (Boote, 1982) and the second one ten days after the first one.

2.2.2. Pot experiments

Two experiments with peanut plants growing in pots were conducted at the INTA research facilities in Córdoba city (−31° 28’ 3.8” S, −64° 8’ 50.4” W). Single peanut plants were grown in 10 L pots containing a substrate composed of a 3:1 mixture of sterilized soil-vermiculite and *T. frezii* teliospores. Inoculum was added at a rate of 10,000 teliospores/g of substrate by drenching of a *T. frezii* spore suspension. *T. frezii* spores and soil used to fill the pots were obtained from the experimental area of the field trials. A completely randomized design, with five (2014/15, from now on 2015) and eight (2015/16, from now on 2016) repetitions was used for each treatment. Treatments consisted of two fungicide sprayings: at 7–10 days after R2 (beginning peg) and ten days after the first one. Each fungicide application consisted of three manual sprays (approximately 2.5 ml), directed to the plant base and pegs.

**2.3. Disease assessment**

Smut intensity assessment was performed at the physiological mature crop stage (R8) (Boote, 1982; Rago et al., 2017). Severity was visually estimated using a five-class scale (Astiz Gasso et al., 2008) which takes into account both grains’ disease status within the pod (Figure 1). Disease assessment in the field plot experiment was performed by collecting 1 m² from the two central grooves of each plot (and all pods produced in that area were evaluated) and in the pot experiment, all the pods contained in the pots were evaluated.

For the purposes of this analysis, we used the proportion of severely damaged pods (SDP, disease class 3 and 4) as the response variable. Disease in these classes is considered to have an impact on inoculum increase as they are lost at harvest and produce is discarded by the industry because of quality issues (Morichetti S. personal communication). SDP was calculated as:

SDP = (n3 + n4) / N           (2)

where n3 and n4 are the number of pods corresponding to disease severity classes 3 and 4, respectively and N is the plot sample size.

Then, for graphical displaying of the results, the smut control efficiency (CE) was calculated using the following formula:

CE = (1 − (Treatment SDP / untreated check SDP)) \* 100)         (3)

**2.4. Data statistical analyses**

Univariate generalized linear models (GLMs) with binomial variance and logit link function were used to analyze SDP. The full model included the year–treatment interaction, which resulted in significance of P < 0.01, then a separated model was fitted to each year. Treatment marginal means were estimated with the “emmeans” function from the emmeans R package (Lenth et al., 2018) and post hoc comparisons among them were conducted with Tukey’s test at a significance level of 5%.

Multivariate analysis was performed to integrate all the efficacy tests (Deepak et al., 2006): principal component analysis (PCA) was conducted to the scaled variables, as: (x − mean(x)) / sd(x), where x is each variable, and the scaling is done by dividing the (centered) vectors of x by their standard deviations. CE% were obtained from the field plots and peanut plants grown in pot trials (2015 and 2016). We obtained the correlation coefficients for each variable with the first and second principal components to explain the variability explained by the first and second principal components. Each fungicide (individuals, represented by points) and their performance in each trial (variables, represented by vectors) are displayed together on a biplot.

**3. Results**

**3.1.** ***In vitro* sensitivity**

The growth of *T. frezii* isolates was 4.76 (± 0.14) mm per day in non-treated control plates. A wide range of sensitivity of *T. frezii* isolates was observed to the evaluated fungicides: with EC50 values ranging from0.001 to >100. The highest mycelial growth inhibitions were observed with DMI’s fungicides, QoI’s pyraclostrobin and azoxystrobin, and penthiopyrad, EC50 < 0.1 (Figure 2). Moderate sensitivity was observed with mancozeb (EC50 = 6.28). The lowest sensitivity of *T. frezii* was observed with thiophanate-methyl (EC50 > 100 µg/ml). According to the EC50, only thiophanate-methyl was classified as non-toxic, and mancozeb as moderately fungitoxic, the rest of the a.i. were classified as highly fungitoxic.

**3.2.** **Control efficacy experiments**

Accumulated precipitations and mean temperature registered in General Deheza during the peanut cropping season (December to April) in 2014/15, 2015/16 are presented in Table S1. A normal crop development (without severe hydric stress) was observed in both field experiments. A total precipitation of 465 and 417 mm was recorded during the pegging phenological stage (January to March) in 2015 and 2016, respectively.

Smut pressure in pot experiments, indicated by the mean SDP at the non-treated check plants, was higher in 2015 (0.73) than 2016 (0.52). Azoxystrobin resulted in the lowest SDP values in both years: 0.3 and 0.04 in 2015 and 2016, respectively. The latter values represented 59% and 92% control efficacy relative to their corresponding non-treated controls. No significant differences in smut control were observed between azoxystrobin and difenoconazole (0.43 SDP, 41% control efficiency) in 2015 or from cyproconazole (0.09 SPD, 82% control efficiency) or tebuconazole (0.13 SPD, 74% control efficiency) in 2016. Intermediate smut control was observed with tebuconazole, picoxystrobin, and cyproconazole in 2015, and picoxystrobin, propiconazole, mancozeb, difenoconazole, and kresoxim-methyl in 2016. On the other hand, the mean SDP for chlorothalonil, penthiopyrad, thiophanate-methyl, and pyraclostrobin did not differ from the non-treated check in both years (Table 2; Figure 3).







In the field experiments, mean SDP in the non-treated control was 0.39 (2015) and 0.43 (2016). Cyproconazole and azoxystrobin, in 2015, presented the lowest SDP values: 0.21 and 0.25 (control efficacy of 47% and 37%, respectively) (P < 0.05). Azoxystrobin was the most efficient fungicide in 2016 to control smut, with the lowest SPD: 0.26. No significant differences of SPD with the non-treated control were observed for kresoxim-methyl, thiophanate-methyl, tebuconazole, penthiopyrad, and chlorothalonil for both years (Table 3; Figure 4).

**3.3. Multivariate analysis**

The multivariate analysis allowed us to integrate all the trial results: two independent principal components (PC) accounted for 76.5% of the total variation (56.2% and 20.3% for PC1 and PC2, respectively). PC1 (horizontal axe) represented the control efficacy values in the field plot trials (with correlation coefficients of 0.86 and 0.77, in 2015 and 2016, respectively) and pot experiments (correlation coefficients were 0.83 and 0.73, in 2015 and 2016, respectively). The further to the right on the PC1-axis, the higher the efficacy control of the fungicide (Figure 5). PC2 on the y-axis represented the EC50 values: the higher in the axis, the lower the smut sensitivity to the fungicide. Considering this coordinate meaning, we may select as the best fungicides those located in the right quadrants, and the lower as possibly: azoxystrobin and picoxystrobin (QoI group), and cyproconazole and difenoconazole (DMI group).

We observed that control efficacy in 2015 followed a similar trend in both types of experiments (plants grown in pots and field plots), but a weak correlation among both experimental types was observed in 2016.

**4. Discussion**

Since its re-emergence in 2006, peanut smut has continued increasing its prevalence and incidence in the main Argentine peanut-growing region, without good perspectives for the future due to the current absence of adoption of management practices for controlling smut (Rago et al., 2017). By means of the present two-year fungicide-screening study, we observed variability in the available set of commercial fungicides, including highly efficacious active ingredients to non-effective ones. To the best of our knowledge, this is a first study assessing chemical management as a technique to maintain peanut smut at low intensity levels.

*In vitro* mycelial growth inhibition tests showed a generally good performance of the DMI group, QoI’s azoxystrobin and pyraclostrobin, and SDHI’s penthiopyrad. The latter two chemical groups are powerful inhibitors of spore germination (Fungicide Resistance Action Committee [FRAC] group 11 and 7), a process that was not evaluated in the *in vitro* tests. This fact could have a potential sub-estimation effect of the evaluated active ingredients since smut spore germination is a fundamental first step in epidemic onset after the gynophore exudates’ stimulation (Astiz Gasso et al., 2008; Marinelli et al., 2008). In relation to both fungi cycle processes, Kosiada (2011) observed a weak correlation between mycelial growth and germination of teliospores and basidiospores according to the sensitivity of some fungicides for head smut of corn (*Sphacelotheca reiliana)*.

Another weak correlation was reported between results of laboratory and field condition experiments (Hollowell et al., 2003), which led us to perform pot and field trials for assessing fungicide efficacy to control peanut smut. However, in both years of the study, we observed that azoxystrobin was the fungicide with the highest smut control efficacy levels in pot or field experiments.

Pavez Badilla et al. (2013) showed different levels of sensitivity *in vitro* for pyraclostrobin and kresoxim-methyl (QoI) in front of the *Venturia inaequalis*, nevertheless they assure that it is very difficult to extrapolate this type of investigation to field conditions due to the mode of action. Differences in control levels for peanut smut were observed compared to *in vitro* results. The DMIs a.i. showed a lower EC50 than the rest of the chemical groups. Cyproconazole had the highest EC50 comparatively with other DMIs; however, it is one of the a.i. that shows the highest control efficiency in field and pot experiments. For their part, chlorothalonil and penthiopyrad (SDHI) had an EC50 similar to QoI, but did not demonstrate disease control efficiency. Azoxystrobin, picoxystrobin, cyproconazole, and tebuconazole were the best a.i. responses demonstrated in the assessment; however, they were not the lowest EC50within the chemical groups to which they belong.

The lack of consistency of the observed results between pot and field experiments may be due to differences in soil properties in both experimental conditions: soil humidity content may lead to different fungicide degradation rates or particle absorption. Further studies are required for a better understanding of the latter process and consequently, a more efficient use of fungicides against *T. frezii* infections. Paredes et al. (data not published) observed, in growing chamber experiments, that the lower the soil humidity, the higher the incidence of smut in peanut plants.

QoI, SDHI, and DMI are the most common fungicides used to control peanut diseases in crops. These fungicide groups performed with less than 40–50% efficacy to control peanut smut in the field, even when used at higher doses compared to leaf spot (Rago et al., 2017). Mutations in genes encoding fungicide targets of pathogenic fungi are often the cause of developed resistance to QoI, SDHI, and DMI fungicides (Sierotzki and Scalliet, 2013). Nevertheless, research on the molecular basis of *T. frezii* fungicide resistance has not been possible due to the lack of genetic information. Recently, Arias et al. (2019) reported the complete mitogenome of *T. frezii* and provided molecular tools to study fungicide target genes and suggested potential resistance to strobilurin and carboxamide fungicides.

Individual performances of cyproconazole and azoxystrobin had the highest control efficacy in the present study. However, higher performances were observed for mixture formulations of the fungicides in previous field experiments under the same disease pressure (4400 teliospores/g of soil): azoxystrobin + cyproconazole and azoxystrobin + picoxystrobin reduced smut severity by 58% and 47%, respectively relative to non-treated controls (Paredes et al., 2015a, 2015b). This higher performance of the fungicides as mixtures could be due to a “potentiation” effect (Cid, 2014).