In vitro and greenhouse trials of the fungicides Chlorothalonil and Tebuconazole

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ABSTRACT

Phakopsora pachyrhizi **urediniospores were exposed to dilutions of fungicide formulations: Chlorothalonil: technical (tech.) and Echo 720®, Tebuconazole: tech. and 3.6 F. Germtube and appressoria inhibition and the effect on appressoria formation were evaluated. Echo 720® and Chlorothalonil tech. were the most effective fungicides. Echo 720® reduced germtube formation in all dilutions tested. Chlorothalonil tech. was not as effective at the dilutions of 10-3 and 10-4. Tebuconazole tech. and 3.6 F were marginal to poor in germtube inhibition and fair for appressoria inhibition. Under greenhouse conditions, soybean plants were sprayed with concentrations of fungicides that performed satisfactorily** ***in vitro.*** **Three days prior to inoculation with** ***P. pachyrhizi.*** **Urediniospores were applied to the upper and lower surfaces of the leaves (3 ml of a 40.000 urediniospores/ml suspension), followed by a period of 18 hours in a humidity chamber. Evaluations were performed 30 and 60 days after inoculation (DAI). Chlorothalonil tech. did not affect the rust. Tebuconazole (tech. and 3.6F) apparently protected plants, but resulted in reduced plant growth. Echo 720® was considered the best fungicide formulation because it significantly reduced rust to the levels verified on non-inoculated controls. Thirty five-day-old soybean plants were inoculated (14 days later than the first greenhouse trial) and lesions observed at 30 DAI. No plant growth inhibition was verified in any of the treatments and no statistical differences were found among the two best concentrations for each fungicide as determined by the** ***in vitro*** **tests. We have demonstrated that growth inhibition only occurs during early stages of plant development. The conclusion is that both Tebuconazole and Chlorothalonil can be used for soybean rust control in the concentrations 1.0X and 0.1X tested in the present work. Coverage efficiency and plant age should be taken into consideration**

MATERIALS & METHODS

Fungicide Toxicity Experiments. Two experiments were carried out to determine the influence of fungicides on: a) the inhibition of *P. pachyrhizi* urediniospores germination and b) inhibition of appressoria formation (**Figure 1**). Fungicides, at rates specified in **Table 1**, were added to Difco potato dextrose agar medium (PDA), after it had been autoclaved and cooled to approximately 50 °C.

Germtube inhibition – ***Germtube inhibition during fungicide exposure***: Four fungicide were added to PDA plates in 5 concentrations (1, 1/10, 1/100, 1/1000, 1/10000 of the original formulation –**Table 1**), for each treatment. Methodology was based on the work published by Mueller et al, 2005. Regular (not amended) PDA plates were used as controls. Three replicates of each fungicide concentration were tested. A volume of 50 µL of a spore suspension 1 X 10^5 urediniospores per milliliter was pipetted directly on the surface of PDA fungicide amended plates. Plates were incubated overnight at room temperature in the dark. Percentage of inhibition was calculated based on the ratio of urediniospores counted: non-germinated/ (total germinated + non germinated). A minimum of 100 urediniospores were counted for each evaluation (**Figure 2A**).

Appressoria Inhibition – ***Appressoria Inhibition after fungicide exposure***: The same formulation dilutions (**Table 1**) were used for the appressoria formation. A volume of 50 µL of a spore suspension of 1 X 10^5 urediniospores per mL was pipetted directly on the surface of polystyrene covered slides to induce appressoria formation (Leite & Nicholson, 1992). Polystyrene covered slides were incubated overnight at room temperature under constant fluorescent light for 24 hours. The number of appressoria formed was the average of appressoria found in 3 microscope fields observed (200X magnification) (**Figure 2B**).

Greenhouse Trial 1 – Experimental genotype *Glycine max* (MG VII) was evaluated at the NFREC-IFAS greenhouses. Single seeds were planted into each 15-cm-diameter plastic pot filled with planting mix (Basics Potting Soil: 77-87 aged pine bark, sand and Perlite®), with 6 replicates per treatment. Plants grew for 21 days in greenhouse before they were treated with fungicides and transferred to a high humidity chamber for inoculation.

The concentration of fungicides applied was based on *in vitro* test results (**Table 1**). Three days after the fungicide application, all plants were inoculated with soybean rust pathogen, *Phakopsora pachyrhizi*. Urediniospores were suspended in deionized water containing 0.01% tween 20 (vol/vol) at a concentration of 40,000 spores per ml, and 5 ml of inoculum was sprayed onto each plant using an atomizer (**Preval Sprayer**, Yonkers, NY). Inoculated plants were placed into a high humidity chamber, room temperature for 18 hours and moved back to greenhouse benches until evaluation of disease was performed. Measurements were recorded at 30 and 60 DAI. The number of sporulating lesions was counted within 1.5-cm-diameter circles on either side of the mid-rib of the center leaflets of 10 representative trifoliates of each plant. Data were statistically analyzed. This methodology was adapted from Feng et. al., 2005.

Chlorophyll Index - The Minolta SPAD – 502 Chlorophyll meter was used to investigate differences in chlorophyll levels. The meter performs instant on-site measurements without the need for taking samples. A total of 1080 leaflets were measured.

Greenhouse Trial 2 – The second greenhouse trial was carried out similarly to the first trial. The major differences were: 1) plants inoculated were 35-day-old (not 21-day-old), 2) we reduced the number of leaflets counted to 15 (5 trifoliates) and 3) two concentrations of each fungicide tested was used (1.0X and 0.1X). These concentrations are the same determined during the *in vitro* phase.

RESULTS

Table 1 – Fungicides used during these tests were prepared according the concentration parameters found bellow. Serial dilutions (10 fold) were used to facilitate the determination of the minimal inhibitory concentration (MIC). Chlorothalonil technical and Tebuconazole technical received 30 µL of Tween 20, since these technical grade active ingredients are practically insoluble in water.

GERM TUBE INHIBITION

Chlorothalonil					
Approximate full label rate vs. *P. pachyrhizi* (mg active ingredient per liter of water):	1500				
Dilution of full label rate:	10000	1000	100	10	1
		Test concentrations in agar (mg/L)			
Chlorothalonil technical 98 % (mg/L of agar)	0.153	1.53	15.31	153	1531
Echo 720 (mg/L of agar)	0.278	2.78	27.8	278	2778

Tebuconazole					
Approximate full label rate vs. *P. pachyrhizi* (mg active ingredient per liter of water).	150				
Dilution of full label rate:	10000	1000	100	10	1
		Test concentrations in agar (mg/L)			
Tebuconazole technical 97%	0.0155	0.155	1.55	15.5	155
Tebuconazole 3.6 F	0.0388	0.388	3.88	38.8	388

APPRESSORIA INHIBITION

Same concentrations were used

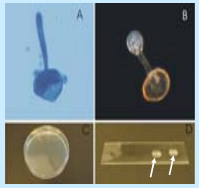

Figure 1 – A) Typical urediniospore of *Phakopsora pachyrhizi* germinating on the surface of a PDA plate. In PDA urediniospores will form long germtubes rather than appressoria, B) Urediniospore forming an appressorium on the surface of a polystyrene covered slide (PCS), C) PDA plate where germtube formation is stimulated, D) On PCS spore will preferentially form appressoria. Notice that drops of water on the right side (arrows) are more contained due to hydrophobic nature of polystyrene.

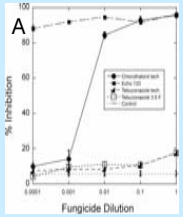

Figure 2 A

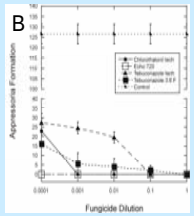

Figure 2 B

Figure 2A – *Phakopsora pachyrhizi* germtube inhibition after exposure to four fungicide formulations: Chlorothalonil technical, Echo®, Tebuconazole technical and Tebuconazole 3.6 F. Control line represents the % germination in deionized water. Germtubes recorded in all fungicide treatments were on average 1/10 of the germtube length observed in the control. None of the test substances provided 100% inhibition (or 0% inhibition), possibly because leaves with pustules were placed in a moist chamber for 48 hours prior to the experiment, therefore some spores may have germinated before they were exposed to test substances. Echo 720® and Chlorothalonil technical were the most inhibitory fungicide preparations.

Figure 2B – Number of *Phakopsora pachyrhizi* appressoria on hydrophobic polystyrene coated slides after exposure to four fungicide formulations: Chlorothalonil technical, Echo® , Tebuconazole technical and Tebuconazole 3.6 F. Control line (…◣…) represents the number of appressoria formed in deionized water. Undiluted fungicide solutions (1.0X) were difficult to count. On polystyrene coated slides the absorption of excess particles is not possible. In this graph zero appressoria formation was recorded. It is important to emphasize that an appressorium is an advanced stage in the infection process, compared to the formation of germtubes. Echo 720®and Chlorothalonil technical again exhibited superior inhibitory capacity compared to Tebuconazole.

Figure 3

Figure 4

Figure 3 - Soybean plants have been inoculated with *Phakopsora pachyrhizi* for the second phase of this fungicide trial, according to the methodology proposed by Feng et al., 2005. Plants were placed in a high humidity chamber for 18 hours (**A**). Plants are were monitored for the presence of sporulating lesions (pustules) (**B**).

Figure 4 - These pictures were taken 30 days after inoculation. The number of sporulating lesions was not sufficient, at that point for any fungicide comparison. However, we have noticed that plants treated with Tebuconazole formulations were almost half of the size of the control and Chlorothalonil treated plants. In addition, Tebuconazole treated plants were greener. Tebuconazole seemed to influence or interfere in the normal soybean development. Plants were evaluated again at 60 DAI. Each treatment was composed of a total of six plants.

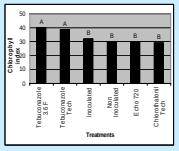

Figure 5

Figure 6

Figure 5 – Typical sporulating pustule observed at the end of the greenhouse trial. Whenever there was any doubt, the presence of typical urediniospores was confirmed by light microscopy.

Figure 6 – Greener plants observed in **Figure 4**, which correspond to Tebuconazole treatments, were confirmed to have a higher chlorophyll index. In addition, these plants exhibited differences in surface characteristics. Leaves were smaller, thicker and rough. The abnormal development resulted in a reduction in the number of sporulating *Phakopsora pachyrhizi* lesions. It is our assessment that this effect is not a result of the fungicide activity per se, but rather a growth regulation influence.

Table 2 – Number of lesions counted in 30 leaflets (10 trifoliates) chosen as representative of the plant condition. Notice that for Tebuconazole treatments regardless of the fact that lesions were present, no sporulating pustules were detected. Numbers presented here are the counts performed 60 DAI.

Treatment	Mean	N (# of leaflets)	Total # of lesions counted	Duncan Grouping
Inoculated	1.83	30	55	A
Chlorothalonil Tech. 98 %	0.80	30	24	B
Non Inoculated	0.1	30	3	C
Echo 720®	0.07	30	2	C
Tebuconazole 3.6 F	0	30	0	C
Tebuconazole Tech. 97%	0	30	0	C

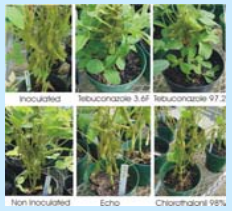

Figure 7 – Typical appearance of soybean plants 60 days after inoculation. Pictures were taking immediately after trifoliates were collected for evaluation.

Table 3 – Differences between inoculated plants and treatments was highly significant (P<0.0001). Despite the fact that there are only two Duncan groups (A and B), being Duncan A grouping represented by the inoculated control. the only treatment that was identical to the non-inoculated control was Echo 720® 1 (153mg/L).

Treatment	Mean	N	Total # of lesions	Duncan Grouping
Inoculated	3.2	15	48	A
Tebuconazole Tech. 97% 1	0.93	15	14	B
Tebuconazole 3.6 F 1/10	0.33	15	5	B
Chlorothalonil Tech. 98 % - 1	0.20	15	3	B
Tebuconazole Tech. 97% 1	0.20	15	3	B
Tebuconazole 3.6 F - 1	0.20	15	3	B
Chlorothalonil Tech. 98 % - 0.1	0.13	15	2	B
Echo 720® - 0.1	0.13	15	2	B
Echo 720® -1	0.00	15	0	B
Non Inoculated	0.00	15	0	B

In vitro **tests**

The *in vitro* effect of fungicides has been classically evaluated by their ability to inhibit germination of fungal spores. In this study we also assessed the influence of fungicides on appressoria formation. Targeting these two important infection steps one can possibly foresee the *in planta* performance of tested substances. Different fungi have specific requirements to achieve germination. In general, obligate parasites require only oxygen and water. Saprophytic fungi will require oxygen, water, inorganic salts and carbon sources. *P. pachyrhizi*, as an obligate parasite, requires a film of water and oxygen to germinate. In this scenario, where nutrient availability is secondary, testing of fungicides on a rich medium (Potato Dextrose Agar) may be unnecessary. We are in the process of optimizing an alternative method which utilizes polystyrene coated slides (PCS). Polystyrene provides a hard hydrophobic surface that physically stimulates the formation of appressoria. The PCS bioassay could save time and resources during the screening of a large number of fungicides against soybean rust and other fungi. We have observed that *in vitro* studies were highly interrelated with the greenhouse results.

Greenhouse evaluation – Trial 1 / 21-day-old plants were Inoculated

Greenhouse trial confirmed the superiority of Chlorothalonil (Echo 720®) compared to Chlorothalonil technical 98% or Tebuconazole preparations (technical 97% or 3.6F). Our results indicate that further studies under field conditions should continue with Echo 720® for the following reasons: a) Echo 720® is readily water soluble, in contrast Chlorothalonil Tech 98% required Tween 20 0.01% (vol/vol) to make the product adequately homogeneous for application, b) Echo 720® was the most effective fungicide formulation against *Phakopsora pachyrhizi* in our tests performed *in vitro* and *in planta*, c) No reduction in yield, measured by fresh weight/plant was verified in plants treated with Echo 720®, d) No influence of Echo 720® was verified on plants normal development.

Greenhouse evaluation – Trial 2 / 35-day-old plants were Inoculated

Two concentrations of fungicides tested. Concentration 1.0X and 0.1X where chosen because they were the best according to the *in vitro* phase. No plant growth inhibition was observed this time in any treatment, indicating that the Tebuconazole plant growth inhibitory effect is plant age dependent. Differences between inoculated plants and treatments are highly significant (P<0.0001). Despite the fact that there are only two Duncan groups (A and B), with Duncan A grouping represented by the inoculated control, the only treatment that was identical to the non-inoculated control was Echo 720® 1.0 (1531mg/L). We hypothesize that the two concentrations tested (1.0X and 0.1X) performed satisfactorily under greenhouse conditions due to the fact that an atomizer was used to spread leaves with the fungicides. Therefore, the use of reduced amounts of the product combined with efficient applications may result in treatment cost reductions.

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