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IN VITRO INFLUENCE OF SELECTED FUNGICIDES ON *SPHACELOTHECA REILIANA* AND *USTILAGO MAYDIS*

*Tomasz Kosiada**

Poznań University of Life Sciences, Department of Phytopathology
Dąbrowskiego 159, 60–594 Poznań, Poland

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Abstract: The study evaluates the effects of selected fungicides on radial mycelial growth and the germination of teliospores and sporidia in two species. The influence of fungicides on the growth of the same developmental forms of both fungi varied at times. Germination of teliospores *Ustilago maydis* in all used concentrations completely inhibited the fungicides: benomyl, carboxin and thiram, flusilazole.

Complete inhibition the germination of teliospores and sporidia *Sphacelotheca reiliana* caused grapefruit extract, benomyl, carboxin and thiram at concentrations of 10,000 to 100 mg/ml and azoxystrobin, flusilazole at concentrations of 10,000 to 1,000 mg/ml. Inhibition of mycelial growth of both species by the fungicides used was weaker than the inhibition the germination of teliospores and sporidia.

Key words: S*phacelotheca reiliana, Ustilago maydis,* fungicide

INTRODUCTION

Regardless of the growing method and crop destination, corn is affected by two species of smut fungi: *Sphacelotheca reiliana* (J. G. Kühn) G. P. Clinton, which causes head smut, and *Ustilago maydis* (DC.) Corda, which causes corn smut. While symptoms on corn plants may be, to some extent, similar, the development of both pathogens is quite different. Both diseases can originate from mycelium, teliospores, or sporidia. In the case of head smut (*S. reiliana*), however, infection occurs in the very early stages of growth. During germination, while in the seedling phase, the plant is affected systemically. The corn smut fungi (*U. maydis*) infects all the intensively growing parts of plants above ground, and only a local infection occurs (Shurtleff 1973). Since both species can have multiple developmental forms, research was undertaken to study the effects of fungicides on the growth of mycelium and germinating of teliospores, as well as sporidia.

MATERIALS AND METHODS

Substances used

Various substances were used in the study that are known to inhibit fungal growth, either directly or indirectly. Substances of natural origin have been used: chitosan – (2% active substance (a.s.) in Biochikol 020 PC) and grapefruit extract (33% a.s. in Biosept 33 SL). Some synthesized substances were also used: azoxystrobin (25% a.s. in Amistar 250 SC), benomyl (50% a.s. in Benlate 50 WP), carboxin and thiram (37.5% each in Zaprawa Oxafun T 75 DS), flusilazol (40% a.s. in Punch Bis 400 EC), and metalaxyl (35% a.s. in Apron XL 350 ES). All the combinations were used in the concentrations: 10,000, 1,000, 100, 10, and 1 μg/ml of a single active substance or a combination of both active substances present in the formulation.

Experimental procedure

Fungal growth was determined as the radial growth, by a daily increment in growth between days 7 and 2. The test was carried out by placing Merck Potato Dextrose Agar (PDA) disks overgrown with the mycelium of both species, on a 90 mm wide Petri dish with PDA. The cultures were grown from a few dozen teliospores of both species. A suspension of teliospores containing 200 μg/ml of streptomycin had been sprinkled onto Petri dishes with an appropriate amount of fungicides. The germination of teliospores was observed through the mycelial growth originating in the germinating teliospores. A similar procedure was applied with regard to sporidia. The suspension of sporidia was prepared by mixing a suspension of teliospores in glucose agar (5 g of glucose, 1 g of yeast extract made up to 1 l with distilled water) with 200 μg/ml of streptomycin for two days. The growth of the mycelium originating in teliospores and sporidia was observed after two days of suspension sprinkling. A percentage of dish coverage by the mycelium growing from teliospores and sporidia, respectively, was calculated.

*Corresponding address:
kosiada@up.poznan.pl

Data analysis

Analysis of variance (ANOVA)

Calculations were made using Statistica software version 8. The measured values: linear growth (mm) and percentage of Petri dish coverage by fungus (%), were juxtaposed with control values so that all types of fungal growth could be compared with each other. The resultant values were subjected to a single-factor analysis of variance (factor 1: formulation in various concentrations). This analysis was done separately for both species and for the multiple developmental forms: mycelium, germinating sporidia and teliospores. The separate analysis of variance was also done for the development of the mycelium grown from them. Homogeneous groups were identified using the Newman-Keuls test (Tables 1, 2). A double-factor analysis of variance was also conducted in which the primary factor was the species of fungus and the secondary factor was the fungicide applied. Separate analyses of variance were performed for the multiple developmental forms: mycelium, germinating sporidia and teliospores, as well as for the development of the mycelium grown from them (Figs. 1, 2, 3).

Principal component analysis (PCA)

Statgraphics Plus software version 2.1 was used to calculate a biplot of correlations. A matrix (observations x measured values) was created (Gabriel 1971). The observations were: radial mycelial growth, growth of the mycelium originating in U. maydis teliospores and sporidia, and radial mycelial growth, growth of the mycelium originating in S. reiliana teliospores and sporidia. They are shown in the figure as vectors. The measured values of growth coverage, controlled by the relevant developmental phase of fungus, are shown in the figure as points.

RESULTS

Table 1 shows how the formulations used, affected radial mycelial growth, the germination of teliospores and sporidia, and the growth of the resultant mycelium of *U. maydis*. All the values are presented as a percentage of growth compared with the control. Growth on PDA agar (with no fungicides added) was 4.0 mm/day for mycelium, with the mycelium grown from germinating teliospores covering 63.8% of the dish and that grown from sporidia covering 82.5% of the dish. Most of the combinations, in the concentrations used, inhibited the growth of *U. maydis*, regardless of their developmental form. This was true except for the fungicide metalaxyl, which inhibited mycelial growth and the germination of teliospores for a concentration of 100 μg/ml, and the germination of sporidia for a concentration of 1,000 μg/ml. The germination of teliospores, at all the concentrations applied, was totally inhibited by the following fungicides: benomyl, carboxin, thiram, and flusilazol. Only grapefruit extract in a concentration of 1,000 μg/ml and benomyl from 10,000 to 10 μg/ml brought a complete inhibition for mycelial growth. Azoxystrobin, in all its concentrations, similarly reduced mycelial growth to 60–70% of the control.

Table 2 shows how the formulations applied, influenced radial mycelial growth, the germination of teliospores and sporidia, and the growth of the resultant mycelium of *S. reiliana*. All values are reported as a percentage of growth compared with the control. Growth on PDA agar (with no fungicides added) was 4.2 mm/day for mycelium, with the mycelium grown from teliospores covering 45.8% of the dish and that grown from sporidia covering 50.0% of the dish. The germination of sporidia and teliospores was completely inhibited by grapefruit extract, benomyl, carboxin and thiram in concentrations ranging from 10,000 to 100 μg/ml, and azoxystrobin and flusilazol in concentrations ranging from 10,000 to 1,000 μg/ml. Azoxystrobin, in all its concentrations, similarly reduced mycelial growth to approx. 95% compared to the control. Mycelial growth on dishes with chitosan was similar to that on dishes without chitosan, or for some concentrations, it was even substantially higher. A mycelial growth considerably higher than in the control was recorded for the fungicides carboxin and thiram in concentrations of 10 and 1 μg/ml.

Although the radial mycelial growth of *U. maydis* and *S. reiliana* was similar (4.0 and 4.1 mm/day, respectively), several formulations were found to be different from each other. For chitosan, the mycelium of *S. reiliana* grew substantially faster than that of *U. maydis*, as was also the case with carboxin and thiram, and azoxystrobin (Fig. 1). Things were different with the germination of sporidia, where, for metalaxyl and benomyl, *U. maydis* was found to grow faster than *S. reiliana* (Fig. 2).

The substances used were found to have different effects on how the teliospores of both species germinated. For grapefruit extract, the highest growth rate was recorded in *U. maydis*, whereas for chitosan and flusilazol, the highest growth rate was recorded for *S. reiliana* (Fig. 3).

All the tested features and resultant outcomes are summarized in a biplot (Fig. 4). The formulations used in the experiment are shown as points, whereas the tested features as vectors (Gabriel 1971). Vector lengths reflect standard deviations of variables, whereas the cosine of the angle between vectors provides an approximation of a correlation coefficient between variables (Aitchison and Greenacre 2002). A high correlation is observed between germination of *U. maydis* teliospores and sporidia, and between germination of *S. reiliana* teliospores and sporidia. Mycelial growth is less correlated with the germination of teliospores and sporidia within a species.

Projections of points represent the formulations. Vectors represent the individual developmental forms of both species. With the projection of points onto vectors, growth rate for each form in the presence of a particular fungicide can be determined. The more distant a projection is from the origin of the coordinate system, the bigger is the value. Distances between points (fungicides) show that the tested fungi responded to fungicides similarly. Accordingly, the highest growth could be observed among the fungi growing on the control medium and those on agar with chitosan. Response to benomyl, grapefruit extracts, and flusilazol was visibly similar (closely spaced points). With these fungicides, the fungal growth

of *S. reilaian* and the mycelial growth of *U. maydis* were slow. The fungi responded to azoxystrobin, carboxin, and thiram similarly. *U. maydis* teliospores and sporidia germinated particularly slowly.

Table 1. *In vitro* growth of *Ustilago maydis* mycelium, sporidia and teliospores

Fungicide	Concentration of fungicide [µg/ml]	Radial growth of mycelium [% of check]	Germination of sporidia [% of check]	Germination of teliospores [% of check]
Chitosan	10,000	65.0 kl	0 a	1.5 a
	1,000	35.0 h	47.0 f	6.6 a
	100	87.5 n	63.6 g	19.2 ab
	10	105.0 op	86.4 i	60.5 def
	1	100.0 o	101.5 j	68.3 ef
Grapefruit extract	10,000	0 a	0 a	5.3 abc
	1,000	7.5 bc	0 a	0 a
	100	27.5 g	0 a	0 a
	10	42.5 i	87.9 i	48.3 cde
	1	80.0 m	98.5 j	126.1 g
Azoxystrobin	10,000	60.0 k	0 a	0 a
	1,000	67.5 l	12.1 b	0 a
	100	65.0 kl	18.2 c	0 a
	10	62.5 kl	37.9 e	22.6 abc
	1	65.0 kl	47.0 f	39.5 bcd
Benomyl	10,000	0 a	0 a	0 a
	1,000	0 a	0 a	0 a
	100	0 a	75.8 h	0 a
	10	0 a	83.3 i	0 a
	1	50.0 j	100.0 j	0 a
Carboxin and thiram	10,000	10 cd	0 a	0 a
	1,000	2.5 ab	0 a	0 a
	100	17.5 ef	0 a	0 a
	10	67.5 l	0 a	0 a
	1	107.5 p	5.2 a	0 a
Flusilazole	10,000	15.0 def	0 a	0 a
	1,000	12.5 cde	1.5 a	0 a
	100	22.5 f	0 a	0 a
	10	37.5 hi	28.8 d	0 a
	1	57.5 k	89.4 i	0 a
Metalaxyl	10,000	17.5 def	0 a	0 a
	1,000	52.5 j	86.4 i	34.4 abc
	100	85.0 mn	97.0 j	74.9 f
	10	102.5 op	100.0 j	82.7 fg
	1	122.5 r	100.0 j	101.3 g
Control		100.0 op	100.0 j	100.0 g

Each value in the column is the average of four replications. Means followed by the same letter are not significantly different at 0.05 according to the Newman-Keuls test

Table 2. *In vitro* growth of *Sphacelotheca reiliana* mycelium, sporidia and teliospores

Fungicide (comercial name)	Concentration of fungicide [µg/ml]	Radial growth of mycelium [% of check]	Germination of sporidia [% of check]	Germination of teliospores [% of check]
Chitosan	10,000	103.8 g	10.0 a	0.9 a
	1,000	85.7 def	72.5 d	33.5 a
	100	95.2 efg	82.5 e	64.2 b
	10	202.4 i	100.0 g	93.1 c
	1	190.5 i	125.0 h	102.2 c
Grapefruit extract	10,000	0 a	0 a	0 a
	1,000	4.8 a	0 a	0 a
	100	19.0 ab	0 a	0 a
	10	40.5 bc	9.5 a	0 a
	1	69.0 de	90.0 ef	58.3 b
Azoxystrobin	10,000	85.7 def	0 a	0 a
	1,000	101.5 fg	0 a	0 a
	100	92.7 efg	2.0 a	1.1 a
	10	93.1 efg	8.0 a	1.4 a
	1	95.2 efg	11.5 a	7.7 a
Benomyl	10,000	0 a	0 a	0 a
	1,000	0 a	0 a	0 a
	100	0 a	0 a	0 a
	10	0 a	3.0 a	0 a
	1	107.1 fg	12.5 a	4.2 a
Carboxin and thiram	10,000	0 a	0 a	0 a
	1,000	9.5 a	0 a	0 a
	100	42.9 bc	0 a	0 a
	10	101.9 fg	0 a	0 a
	1	102.4 fg	97.5 fg	100.2 c
Flusilazole	10,000	0 a	0 a	0 a
	1,000	0 a	0 a	0 a
	100	0 a	0 a	0.4 a
	10	11.9 a	25.0 b	38.2 ab
	1	38.1 bc	45.0 c	102.1 c
Metalaxyl	10,000	11.9 a	0 a	0 a
	1,000	59.5 cd	8 a	9.8 a
	100	73.8 de	27.5 b	64.5 b
	10	83.3 def	45.0 c	102.6 c
	1	85.7 def	72.5 d	101.1 c
Control		100.0 efg	100 g	100.0 c

Each value in the column is the average of four replications. Means followed by the same letter are not significantly different at 0.05 according to the Newman-Keuls test

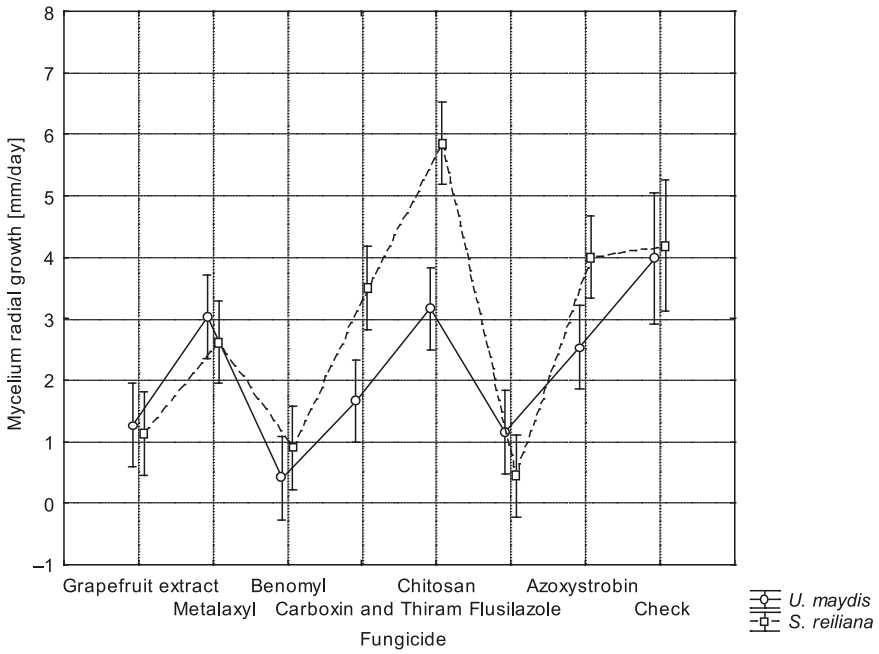

Fig. 1. Radial growth of *U. maydis* and *S. reiliana* mycelium depending on the fungicides applied

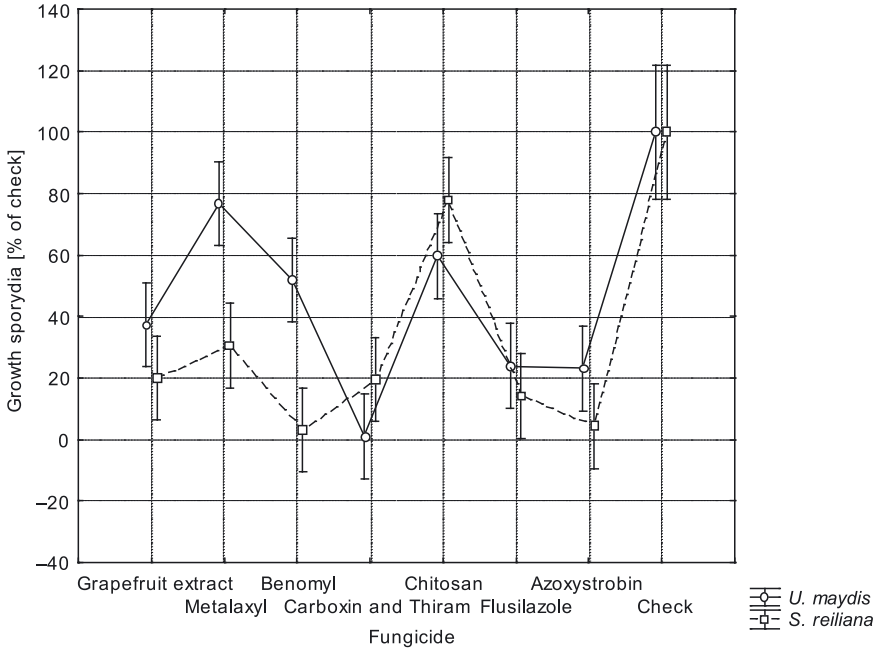

Fig. 2. Germination of *U. maydis* and *S. reiliana* sporidia depending on the fungicides used

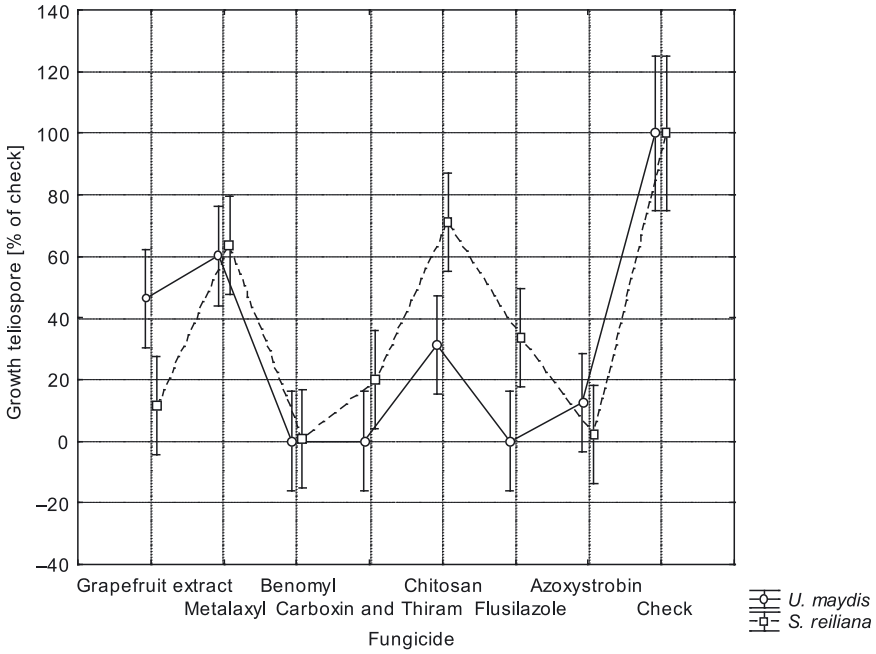

Fig. 3. Germination of *U. maydis* and *S. reiliana* teliospores depending on the fungicides used

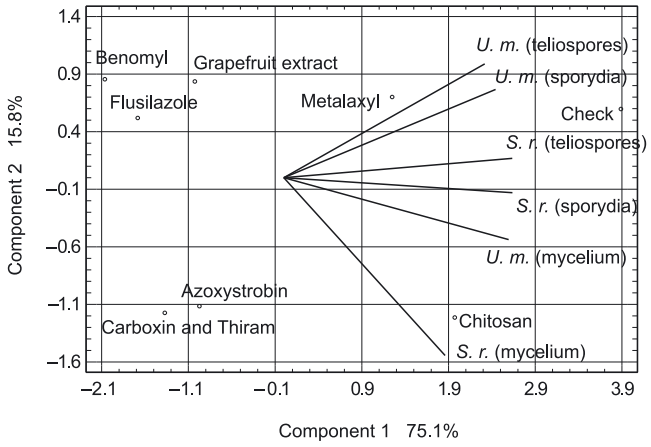

Fig. 4. Biplot the correlation between the growth of *U. maydis* (*U. m.*) and *S. reiliana* (*S. r.*) and the fungicides applied

DISCUSSION

Sensitivity of *U. maydis* to azoxystrobin is lower among wild types than among isolates previously exposed to mutagenic agents. For wild types, EC_{50} in liquid culture was 0.01 µg/ml, and for mutant isolates, it was 1–10 µg/ml (Ziogas *et al*. 2002). A unique relationship was observed for the fungicide azoxystrobin. There was a lack of correlation between the radial mycelial growth of *U. maydis* and the concentration of azoxystrobin, mostly ranging from 1 to 1,000 µg/ml. Growth reduction amounted to 62–67% of the control here. The reduction in the mycelial growth of *S. reiliana* under the same conditions was even lower, amounting to 92–101% of the control. However, the influence of azoxystrobin on germination, and then on the mycelium originating in teliospores and sporidia, was more inhibitory. A total lack of growth in *S. reiliana* originating in sporidia and teliospores could be observed for a concentration of 100–1,000 µg/ml (Table 2). A total lack of growth in *U. maydis* originating in sporidia and teliospores could be observed for a concentration of 1,000 µg/ml (Table1). This demonstrates that azoxystorobin is markedly more potent in inhibiting spore germination than mycelial growth. Other research also confirms that azoxystrobin is better in inhibiting the germination of fungal spores (*B. cinerea*). EC_{50} inhibiting spore germination amounted to 0.32 µg/ml and EC_{50} inhibiting mycelial growth already amounted to 3.1 µg/ml. Whereas the same parameters, were reversed for carbendazim, amounting to >25 µg/ml for spore germination and 0.045 µg/ml for mycelial growth (Slawecki *et al*. 2002). For the germination and growth of the mycelium of *Alternaria alternata* in the presence of azoxystobin, however, EC_{50} was similar, amounting to 72 µg/ml for germination and 80 µg/ml for mycelial growth (Reuveni and Sheglov 2002).

Differences in the growth rates of the wild types and the resistant mutants of *U. maydis* could be observed for other fungicides as well. For other fungicides, the differences between both types approached 100% and the fungicides being tested for concentrations ranged from 4 to 32 μg/ml (Tillman and Sisler 1973).

Out of all the formulations tested, chitosan was least inhibitory to fungal growth. For 1,000 μg/ml, mycelial growth accounted for 35.0% of the control, spore germination for 47.0% of the control, and the germination of *U. maydis* teliospores for 6.6% (Table 1). It was even less inhibitory to the growth of *S. reiliana*: 85.7% (mycelium), 72.5% (sporidia), and 33.5% (teliospores). At low concentrations of 1,10 μg/ml, chitosan stimulated mycelial growth (Table 2). With its inhibitor index amounting to 78.35%, the modified chitosan used in the NCSCA formulation was substantially more effective against *S. reilian* at a concentration of 1,000 μg/ml (16.8 mm diameter colony, check 77.6 mm). The inhibitor index of the traditional fungicide AMULET under the same conditions was 57.79% (32.5 mm diameter colony, 77.0 mm control) (Zeng *et al*. 2010), (100% of the control = inhibitor index 0%, 0% of the control = inhibitor index 100%).

Test results under field conditions may differ from those observed in vitro; corn grains were less affected by head smut when treated with azoxysrobin than with carboxin+thiram (Wrighta *et al*. 2006). Under *in vitro* conditions, growth of *S. reiliana* was more readily inhibited with carboxin and thiram than with azoxystrobin. In other field studies, a better or comparable efficacy was recorded when 2.5 kg of carboxin per ha of soil was applied, compared with 1.6 and 3.2 kg of benomyl per ha of soil used to inhibit head smut (Fullerton *et al*. 1974).

Flusilazole inhibited the germination of *U. maydis* teliospores for all concentrations used, while almost completely inhibiting the germination of sporidia within a range from 100 to 1,000 μg/ml and poorly inhibiting mycelial growth. In other studies, EC_{50} amounted to 0.18 μg/ml (Markoglou and Ziogas 2000).

For some fungicides, especially at lower concentrations, an increase in fungal growth was observed that was comparable to or slightly higher than the control. These differences were not statistically significant in relation to the control. In other studies assessing the effect of benomyl on Ustilago striformis, it was observed that the fungicide, at concentrations ranging from $2x10^{-4}$ to $2x10^{-7}$, stimulated fungal growth (Robinson and Hodges 1973).

A weak correlation (reflected by the straight lines in the biplot – figure 4) between mycelial growth and the germination of teliospores and sporidia indicates that the effect of the formulations used was different on mycelial growth and different on the germination of teliospores and sporidia for both species of fungi.

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