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Note

ALTERNATIVE PRODUCTS IN THE "IN VITRO" INHIBITION OF *Sclerotinia sclerotiorum*

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ABSTRACT: The white mold, caused by *Sclerotinia sclerotiorum*, is a very important disease in tomato crops. The objective of this work was to study the effect of plant extracts, animal residues and industrial by-products extracts on the fungus *in vitro* growth. Treatments consisted of different concentrations of pyrolignous oil, neem oil, monosodium glutamate, sewage sludge and organic compost [coffee residue (50%) coal residue (10%), maize residue (25%), poultry waste (12.5%), poultry meal (2.5%)]. Positive control consisted of Petri dishes with PDA medium and negative control treatment consisted of PDA medium with procymidone. Fungus colonies were incubated at 22ºC and light intensity of 260 lux. Variables such as mycelium growth rate, sclerotia production, and viability 7 and 17 days after the transfer of mycelium disc to neon media were assessed. The extract of organic compost at 30% was effective in controlling mycelial growth and sclerotia production. This treatment, as well as neem oil at 0.5% increased soil respiration.
Key words: *Azadirachta indica*, sclerotinia white rot, alternative control, sewage sludge, soil respiration

PRODUTOS ALTERNATIVOS NA INIBIÇÃO DE *Sclerotinia sclerotiorum* "IN VITRO"

RESUMO: O mofo-branco, causado por *Sclerotinia sclerotiorum*, é uma doença importante na cultura do tomateiro. O objetivo do trabalho foi avaliar o efeito de extratos de plantas, resíduos animais e subprodutos industriais no desenvolvimento micelial e na produção de escleródios do fungo "in vitro". Os tratamentos testados foram diferentes concentrações de licor pirolenhoso, óleo de nim, glutamato monossódico, biossólido e composto orgânico [(borra de café (50%) cinza de carvão (10%), resíduo de milho (25%), esterco de aves (12,5%) e farinha de aves (2,5%)], além de duas testemunhas, sem adição de produtos ao meio e com adição do fungicida procimidone. O fungo submetido aos diferentes tratamentos foi incubado à temperatura de 22ºC e luminosidade constante de 260 lux. Foram avaliadas o crescimento micelial, a produção de escleródios e a viabilidade dos mesmos aos 7 e 17 dias após a repicagem do fungo para meio de neon. O composto orgânico a 30 % mostrou-se eficiente na inibição do crescimento micelial e na produção de escleródios. Este tratamento, assim como o óleo de nim a 0,5%, foi analisado com relação à sua influência na microbiota no solo por meio de uma análise de respirometria, que indicou que ambos os tratamentos foram degradados rapidamente, não causando assim malefícios à mesma.
Palavras-chave: *Azadirachta indica*, podridão de sclerotinia, controle alternativo, lodo de esgoto, respirometria

INTRODUCTION

Tomato crop (*Lycopersicon esculentum* Mill.) is one of the most important vegetable in Brazil (Lopes & Santos, 1994). There are several crop limiting factors, including its great number of diseases (Kurozawa & Pavan, 1997; Lopes & Santos, 1994).

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Sin. *Whetzelina sclerotiorum)* (Lib.) (Korf & Dumont) is a disease causal agent in more than 400 botanical species (Boland & Hall, 1994). This polyphagous fungus is able to remain active in the soil, as sclerotia, for long periods (Coley-Smith & Cooke, 1971). Usual disease control methods are soil solarization (Ferraz, 2001), crop rotation, and chemical control (Kurozawa & Pavan, 1997).

Today, phytosanitary handling of tomato, a *Sclerotinia* susceptible crop, requires extensive fungicide spray during the crop cycle. In an attempt to reduce environmental and human contamination, alternative food production methods have been employed. This new form of agricultural production can be classified in different ways, depending on the principles involved, but in general employs animal, vegetal, and low solubility mineral inputs to allow slow nutrient availability to the crop.

Among the alternative products to control pests and diseases are neem oil, pyroligneous liquor, bio-fertilizers and monosodium glutamate (Bettiol, 2001). Neem oil, usually extracted from seeds of *Azadirachta indica* tree A de Jussieu, has been reported to be an excellent insect repellent (Blaney & Simmonds, 1990) and effective in inhibiting "in vitro" *Tanatephorus cucumeris* growth (Lakshmanan et al., 1990). Pyroligneous liquor or acid, obtained from smoke condensation released from wood burning in coal bunkers is a by-product also used to control plant diseases and, according to Novaes et al. (2000), it is able to minimize losses caused by tobacco mosaic virus (TMV) when sprayed in low concentrations (20,000 ppm). Tratch (1996) observed that bio-fertilizers obtained from feedlot dairy cattle manure, mixed with potato-dextrose-agar (PDA) medium at 10% to 20%, inhibited mycelial growth of *Sclerotinia sclerotiorum, Rhizoctonia solani, Alternaria solani, Botrytis cinerea, Sclerotium rolfsii, Pythium aphanidermatum, Septoria lycopersici* and *Fusarium oxysporum* f.sp. *phaseoli*. I n the same work, the author verified that bio-fertilizers were also effective to control powdery mildew on pumpkins (*Sphaerotheca fuliginea*). Moreover, Lewis et al. (1992) observed that the biossolid (sewage sludge) was able to control damping-off in peas caused by *Rhizoctonia solani and Pythium ultimum.*

In this work, the following products were used to test their efficacy on the "in vitro" growth inhibition of *S. sclerotiorum*: neem oil, pyroligneous liquor, liquid residue based on monosodium glutamate, biossolid from the sewage treatment Station in Jundiaí (ETE) and commercial organic compost produced from the mixture of coffee residue (50%), coal residue (10%), corn residue (25%), poultry waste (12.5%), and poultry meal (2.5%). Treatments with the best results in fungus control were analyzed with respect to their biodegradability and influence on soil microbial activity.

MATERIAL AND METHODS

Preparation of culture medium and mycelium transfer

For treatments based on neem oil and pyroligneous liquor, PDA medium was prepared and mixed in a sterile chamber with the neem oil or pyroligneous liquor medium in 3 concentrations: 0.25, 0.50, and 2%. In addition, a positive control (PDA only) and a negative control (PDA added to the procymidone pesticide, at 1 g L^{-1} concentration) were also prepared.

Monosodium glutamate (20 mL/dL H_2O), sewage sludge (20 g dry matter/dL H_2O) and organic compost (20 g dry matter/dL H_2O) residues (Table 1) were initially incubated for a five day period at 25ºC with 12-hour photophase. After the incubation period, successive filtrations were carried out in plastic sieve and gauze and the extracts were centrifuged at 20,000 *g* for 30 minutes. Retained solids were filtered in Glass Microfibre Filter (GMF) and in millipore filter (0.22 mm pore), and stored. Sterilization of millipore filter was carried out in humid heat, and a new filtration in millipore filter was carried in sterile chamber to eliminate other contaminating agents. Extracts were mixed to the culture medium in 5, 10, and 30% concentrations.

Table 1 - Chemical composition of sewage sludge, monosodium glutamate and organic compost added to potato-dextrose-agar medium.

Nutrient	Sewage sludge	Monosodium glutamate	Organic compost
	---------- g kg^{-1} ----------		
N	14.26	49.71	18.41
C	231.97	130.5	398.66
P	4.85	1.31	8.14
K	1.2	5.57	7.16
Ca	65.06	0.43	57.65
Mg	3.83	0.49	6.25

Mycelium discs of *S. sclerotiorum* were collected using a 7 mm perforator from 7 day fungus colony [of an infected tomato crop at Jarinú, SP, Brazil (23º06'05''S; 46º43'42''W)]. Mycelium discs were collected from the circumference of the plate along a single row, so that the inoculum had the same age, thus decreasing variability in fungus growth. Disks were placed in the center of the plate over the culture medium prepared the prior day. These experiments were repeated twice, under the same conditions, at different times.

Assessments

Mycelia growth rate was determined, according to Nakasone (1998). The number of sclerotia was assessed 7 and 17 days after inoculation and its viability was determined in neon medium (Steadman et al., 1994). There were 5 replicates per treatment, in a completely randomized statistical design. Initially linear regression analyses were performed between each treatment and the positive control. Every time a significant difference ($P < 0.05$) was detected each concentration within each treatment was individually compared to both positive and negative controls. In the cases where initial regression analyses were non-significant concentrations within treatment were blocked and compared with both positive and negative controls. Statistical analysis was performed by statistical software STATISTICA (StarSoft, Tulsa, OK, USA).

Soil Respiration

Based on inhibition results of *S. sclerotiorum* from previous stages, the best treatments were used to study biodegradability and microbial activity, measured by CO_2 production using the soil respiration test.

Fallow Mollisol samples (50 g) were filtered into 1 L capacity flasks. Water was added to reach 60% of soil field capacity. Neem oil, compost extract (non-filtered in GFA and millipore) and procymidone fungicide were added with the water at concentrations of 0.5, 30%, and 1 g L^{-1}, respectively. An extra treatment consisted of adding solid compost to soil samples in amounts equivalent to 10 t ha^{-1} to mimic field conditions. Smaller flasks containing 10 mL NaOH 0.5 mol L^{-1} were placed over the soil samples, the larger flasks were sealed with plastic film, and placed in dark growth chambers (Conviron®). There were 5 replicates per treatment. Production of CO_2 was measured 24, 48, 72, and 144 hours of incubation by titration with HCl 0.5 molL^{-1}, according to Öhlinger (1996). Means were compared by Duncan test using STATISTICA (StatSoft, Tulsa-OK, U.S.A.).

RESULTS

Mycelial growth rate varied according to the product and concentration tested. Variation also occurred in sclerotia production both after 7 and 17 days (Table 2). Neem oil and organic compost reduced mycelial growth rate, as compared to the positive control (Table 2). Culture media containing monosodium glutamate (experiments 1 and 2) and sewage sludge (experiment 2) favored mycelial growth and sclerotia production when compared to the positive control, proving to be favorable to fungus development (Table 2). As glutamate concentration residue increased in the culture medium, mycelial growth rate and sclerotia production also increased (Table 2). On the other hand, culture media prepared with pyroligneous liquor allowed mycelial development and sclerotia production similar to the positive control, showing a non-significant difference for the majority of the contrast comparisons (Table 2).

Neem oil and organic compost effectively inhibited *in vitro* fungus growth. The former did not stimulate mycelial growth in both experiments when compared to the positive control (Table 2), whereas the latter did not stimulate mycelial growth at 5 and 10% concentrations and inhibited growth at a concentration of 30%. Sclerotia formation increased at 5 and 10% concentrations compared to the positive control, and at concentration of 30%, sclerotia formation was nill. All sclerotia produced were viable when submitted to the neon medium test.

Compost extract at 30% and neem oil at 0.5% were tested as soil CO_2 production inhibitors. Neem oil, organic compost, and non-filtered in millipore compost extract showed higher CO_2 production than the control, suggesting these products stimulate soil microbial activity (Table 3).

Table 2 - Mycelial growth rate and average number of sclerotia formed in Petri plate seven and seventeen days after inoculation into culture medium in experiments

Treatment	1st Experiment			2nd Experiment		
	Rate	Sclerotia		Rate	Sclerotia	
	mm day^{-1}	7 days	17 days	mm day^{-1}	7 days	17 days
Neem oil 0.25%	4.5 a B	0	6.8	1.2 a	0	0 a
Neem oil 0.5%	4.1 a B	0	4.4	0.0 a	0	0 a
Neem oil 2 %	3.9 a B	0	0.4	0.0 a	0	0 a
Pyroligneous liquor 0.25%	18.8 B	0	0.4	22.4 B	7.6	14.6 B
Pyroligneous liquor 0.5%	14.1 a B	0	0	19.7 B	1.2	16.4 B
Pyroligneous liquor 2%	13.0 a B	0	7.6	15.5 B	0.2	9.4 B
Compost 5%	14.7 a B	0	16.4 B	14.9 a B	4.8	22.2 a B
Compost 10%	9.9 a B	0	21.8 B	8.2	7.2	20.4 a B
Compost 30%	0 a	0	0	0 a	0	0 a
Sewage sludge 5%	24.0 B	0	22 a B	26.7 a B	3.8	22 a B
Sewage sludge 10%	21.3 B	11.8 ab	21.8 a B	21.8 a B	15.8 a B	26.6 a B
Sewage sludge 30%	19.5 B	12.4 ab	32.0 a B	22.6 a B	15.6 a B	23.0 a B
Glutamate 5%	28.6 a B	0	19.6 B	35.3 a B	0	13.4 a B
Glutamate 10%	29.1 a B	0	11.6	37.0 a B	0.6	9.0 a B
Glutamate 30%	30.3 a B	65.8 ab	66.2 a B	38.5 a B	30.2 a B	30.2 a
Positive Control	21.2	0	6.0	16.0	2.8	12.6
Negative Control	0	0	0	0	0	0

*Means within columns with letters 'a' do not differ from positive control by contrast analysis. Means within columns with letters 'B' do not differ from negative control by contrast analysis

Table 3 - Cumulative CO_2 production 24, 48, 72 and 144 hours of respiration test, for treatments that inhibited "in vitro" *S. sclerotiorum* growth only.

Treatments	CO_2 (mg)
Control	50.85 A
Procimidone 0.1%	53.92 A
Neem oil 0.5%	133.43 B
Organic compost	196.15 C
Compost extract	325.90 D

*Different letters denote means that differ, $P < 0.05$.

DISCUSSION

The liquid residue obtained from monosodium glutamate production has been used as a fertilizer in agriculture. This residue did not favor *Penicillium digitatum* development on orange fruit (*Citrus sp.*) during the post-harvest period (Franco & Bettiol, 2000). In this study, this product, perhaps due to its high nitrogen content (Table 1), stimulated mycelial growth. When it reached the Petri plate borders, nutrients of the culture medium were used up, thus stimulating large sclerotia production, and therefore differing from the behavior observed by Franco & Bettiol (2000) for *P. digitatum*.

Sewage sludge, used as an additional fertilizer (Berton et al., 1989) has been tested as a plant disease control. Possible theories for this action include direct competition between microorganisms and the presence of fungistatic substances, such as acetic acid produced by microorganisms present in the sewage sludge (Wildmer et al., 1998). In this experiment, the media prepared with sewage sludge extract favored mycelial growth and sclerotia production, meaning that this residue did not have fungistatic action over the *S. sclerotiorum*. Lumsden et al. (1983) observed in greenhouse tests that sewage sludge applications were effective to inhibit growth of *Aphanomyces solani, Rhizoctonia solani, Pythium myriotylum* and *Sclerotinia minor*, but unable for *Fusarium solani, Thielaviopsis basicola and Pythium ultimum*, suggesting the action would depend mostly upon the type of pathogen and the possible resistance mechanism activation in the host plant.

The pyroligneous liquor contains several chemical compounds including approximately 3% acetic acid. The results of this study differ from those found by Novaes et al. (2000) for the tobacco mosaic virus, where 2 mL L^{-1} concentration of this by-product controlled the viruses. Probably, the efficacy of this liquor varies according to the organism under study and on the likely induction of plant resistance.

The inhibition of mycelial development and the sclerotia production in the treatments with neem oil were similar as obtained by Lakshmanan et al. (1990) to control *Thanatephorus cucumeris* and by Mirza et al. (2000) to control *Phytophthora infestans*. As for *oomycetes*, neem in the form of raw oil and leaf extract inhibited mycelial growth and sporangium production and germination.

Cattle, equine and poultry waste is highly effective in the control of *S. sclerotiorum* in lettuce, decreasing the amount of sclerotia formed in the soil, number of diseased plants, and thus increasing final crop production (Asirifi et al., 1994). Organic compost at 5 and 10% concentrations showed greater formation of sclerotia than the positive control, probably a result of poor mycelial development in the culture media observed in these treatments. At 30% concentration, mycelial and therefore sclerotia development had not occurred, highly suggesting that the extract is very effective for pathogen inhibition.

Ferraz et al. (1999) observed that organic matter enhanced *Sclerotinia sclerotiorum* apothecia formation as the substrate organic compost concentration increased. The final chemical composition of the compost is one of the likely factors may explain different results, as the product used by those authors was based on mixtures of red latosol, vermiculite, cattle manure and lime, whereas the compost used in this study was a mixture of coffee residue, coal ash, corn residue, poultry waste and poultry meal.

Organic compost can be part of soil management practices to control *Sclerotinia sclerotiorum* for its effective "in vitro" growth inhibition and ineffectiveness on soil microbiota. Studies on field performance, application levels, economic feasibility and possible effects on the host plant of this organic compost are recommended.

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