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Biological Control of Lettuce White Mold with Cyanobacteria

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ABSTRACT

The aim of this work was to determine the activity of cyanobacterial extracts as control agents of *Sclerotinia sclerotiorum* (Lib.) de Bary infecting lettuce (*Lactuca sativa*). The bioactive substances from *Nostoc muscorum* (Cyanobacteria) were extracted from the biomass using methanol, distilled water and ether as solvents. Water and ether extracts were the most effective ones against the phypathogen in *in vitro* experiments. Methanolic extract and extracellular products showed no effect. Interaction of plant-pathogen-cyanobacterial substances were studied by treating infected plants with the cyanobacterial extracts. Ether extract inhibited fungus infection by 45.5% after 8 days of treatment. The effect increased to 72.8%, when the dose was two fold, with the advantage of lasting longer. In the field assays, *N. muscorum* ether extract was applied twice to a lettuce crop infected with *S. sclerotiorum*. At harvest, the increment of healthy plants was 25.5% with 14% increase of fresh weight, which represent a significant economical benefit. This cyanobacterial strain could be used as a bio-control agent to obtain higher yield in horticulture.

Key Words: Cyanobacteria; *Nostoc muscorum*; *Sclerotinia sclerotiorum*; Lettuce; Biological control

INTRODUCTION

The Cyanobacteria or blue-green algae are prokaryotic, photosynthetic microorganisms. They represent a continually renewable biomass source that can release to the environment soluble organic substances as extracellular products also known as secondary metabolites, which can be mineralized by the microflora. These substances can be vitamins, enzymes, carbohydrates, peptides, amino acids and growth promoters and/or inhibitors for other organisms of the environment i.e., plants, fungi, bacteria (Kulik, 1995; Zulpa *et al*., 2003).

The fungus *Sclerotinia sclerotiorum*, causes "white mold", which is one of the most polytheist plant pathogens, mostly affecting Compositae notably lettuce (*Lactuca sativa* L.) and other species of rosette plants. In Argentina, lettuce is third major crop among the cultured vegetables, following potato and tomato (Alcalá *et al*., 2004). The plants can be infected at any stage of their development. The pathogen totally invades the stem and rots it; the leaves above the wound wilt and die rather quickly. Tu (1997) reported that infection can be produced by ascospores or sclerotia. The latter remain viable for 3-5 years on the soil surface (Alexander & Stewart, 1994). Sclerotia fall from the infected plant or are incorporated to the soil together with the infected plant remains. Nutrients availability favours the direct germination of sclerotia, which produces a new mycelium, being a frequency higher than one sclerotium per 800 cm^3 of soil, enough to cause infection in plants (Venette, 2004).

Conventional control methods have been not quite efficient due to the survival of the reproductive structures in the soil. On the other hand, chemical agents inhibit root and stem growth (Nyporko *et al.*, 2002). However, two contact fungicides, iprodione and vinclozolin, have shown in the last two years successful results (Westerdijk, 2000). Information about biocontrol shows that most experiments have been performed in the laboratory and very few have been carried out in the field. Research on the effect caused by soil microorganisms and their metabolites has been scarce (Thaning *et al*., 2001). Recently, various antagonistic organisms have been identified for almost every life cycle stage of *S. sclerotiorum*. Antagonistic organisms can be fungi, bacteria and cyanobacteria (Yuen *et al*., 1994). Among Cyanobacteria, *Nostoc muscorum* has been shown to exert antifungal activity on soil fungi and especially those producing "damping off" (Caire *et al*., 1976, 87 & 1990; Mulé *et al*., 1977). *Nostoc* ATCC 53789, a known cryptophycin producer, is a source of natural pesticides against the fungi such as *S. sclerotiorum*, insects, nematodes, with cytotoxic effect (Biondi *et al*., 2004). *N. muscorum* also inhibit the growth of other fungi producing the "wood blue stain" (Zulpa *et al*., 2003).

The aim of this work was to study the antifungal effect of the Cyanobacteria in the interaction plant-pathogen-cyanobacterial substances when *Lactuca sativa* was grown

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in a culture-chamber and in a field with the soil contaminated by *Sclerotinia sclerotiorum*.

MATERIALS AND METHODS

Cyanobacterial strains. *Nostoc muscorum* (79a), *Tolypothrix tenuis* (40d), *Microchaete tenera* (13a), *Nostoc punctiforme* (40b) *Anabaena oryzae* (50) and *Scytonema hofmanni* (58) isolated from paddy fields of Argentina, belong to the culture collection of the Laboratory of Biology of Cyanobacteria, University of Buenos Aires. The strains were obtained in axenic condition by U.V. radiation (68 132A germicide lamp 253.7 nm, General Electric, Cleveland, Ohio 44112, USA) and are kept in modified Watanabe culture medium (Zulpa *et al*., 2003). For each strain, the inoculants were grown in Watanabe medium for 30 days, under 45µmol photon m^{-2} s^{-1} light intensity and 28±1°C. In order to characterize the inoculants, pH of the culture medium, optical density and chlorophyll *a* of the strains, were determined. To obtain the mass cultures, the strains were cultivated in the same conditions in Erlenmeyer with increasing volumes and with sterile bubbling air and kept at stationary phase.

Cyanobacterial extracts. The cyanobacterial biomass and the culture medium containing the extracellular products (EP) of each species were separated by centrifugation (8000 xg – for 25 min at -10ºC) under sterile condition. The cyanobacterial metabolites were extracted from the axenic biomass using water, ether and methanol as solvents. To prepare the ether extract, the fresh biomass was extracted with peroxide free ether (Caire *et al*., 1976) in a relation 1:10 (v/v), transferred to sterile distilled water after ether evaporation and sterilized by filtration through nitrocellulose membranes 0.22 µ pore diameter. Water and methanol extracts were obtained in the same way.

Fungal strain. *Sclerotinia sclerotiorum* (Lib) D. Bary, causing agent of white mold, was isolated from *Lactuca sativa* var. capitata, cv. gallega (lettuce) in Marcos Paz, Province of Buenos Aires, Argentina, were the field experiment took place. The fungus was grown in Petri dishes with potato glucose agar culture medium (PGA) and to avoid the pathogenicity loss, it was kept at 18ºC and weekly transferred to sterile rice. The plant infecting mycelium was obtained from sclerotia, isolated from the same field soil and sterilised in surface with 70% ethyl alcohol (2 min.) and 2% sodium hypochlorite solution (diluted Clorox), (2 min).

Lettuce seeds. The seeds used in the assays belong to var. capitata, cv. gallega cultivated in our country and originated in Province of San Juan, Argentina.

Soil. The soil for the assays in pots and in the field was classified as Vertic Argiudoll, with 20.0 g kg^{-1} oxidizable carbon (Nelson & Sommer, 1982), 2.2 g kg^{-1} total N, 102.5 mg kg^{-1} extractable P, 37.4 mg kg^{-1} $N\text{-}NO_3$, pH 7.3, electrical conductivity 0.3 dS m^{-1} (Nelson & Sommers, 1982) with clay silty loam texture. The soil was sterilised for pots experiment.

Selection of cyanobacteria with inhibitory effect against *S. sclerotiorum* growing *"in vitro"*. For each cyanobacterial strain, Petri dishes with 5 mL PGA plus 1.5 mL of the extract (water, ether or methanol) were prepared, with corresponding controls. A plug 2 mm diameter of *S. sclerotiorum* mycelium was placed in the Petri dish border in order to measure the fungus radial growth (mm) on an imaginary axle that coincides with the dish diameter direction. The dishes were incubated at 37°C for 6 days with a completely randomised experimental design (n=7).

Lettuce grown in culture chamber. In order to infect the plants, 0.7 g of mycelium were applied to the plant stem at a distance of 2.5 mm from the soil surface.

The lettuce seedlings growing in pots (350 cm^3) were kept in a culture chamber with 90% relative humidity, at 18±1°C, photoperiod 12:12 and 450 µ Einstein cm^{-2} seg^{-1} (Licor L- 1000 radiometer with data logger) light intensity. The soil was kept at field capacity by gravimetry. The plants were inoculated with *S. sclerotiorum* mycelium when they had 7-8 expanded leaves. Treatment with extracellular products and water and ether extracts were applied on the pathogen with a dosifying pipette. The treatments were, T1- control plants (no inoculation), T2 - plants inoculated with mycelium, T3 - plants inoculated with mycelium + 1.5 mL ether extract, T4 - plants inoculated with mycelium + 1.5 mL water extract, T5- plants inoculated with mycelium + 1.5 mL extracellular products. The pots were placed following a completely randomised experimental design, n=11. The same experiment was repeated doubling the dose of the most effective treatment. Both assays were observed at days 8 and 12 after the inoculation of the pathogen.

Field experiment. The plot with the highest lettuce plants infection, for the previous 3 years, was chosen to perform the field experiment in two successive crops. Before performing the experiments 15 soil samples taken at 10 cm depth were cultivated in PGA culture medium obtaining 14 viable sclerotia per 800 cm^3 of soil, a concentration that makes the plant infection sure (Nelson *et al*., 1989). Lettuce was sown with a density of 16 plants m^{-2}. The harvests took place 100 days after. The treatments with cyanobacterial substances were applied to the root-shoot transition zone using a spraying calibrated rucksack, FT1: plants sprayed with water (control); FT2, plants sprayed with 10 mL of *N. muscorum* ether extract 1:4 (v/v, extract/water). In the successive crops, 20 plots with 50 plants each were randomly defined in the crop rows, for each treatment. The treatments were performed at day 40, when the leaves covered the soil creating suitable conditions for the sclerotia germination and were repeated at day 64. The soil was kept at approximately 60% field capacity by irrigation. The number of infected plants was evaluated at days 52, 64, 76, 88 and at day 100 when the manual harvest took place and the stem biomass dry weight in each treatment was determined.

Statistical method. Analysis of Variance was performed using a program for PC. Homogeneity of variances was

previously determined by the test of Bartlett. In order to detect differences between means the test of Tukey ($p<0.05$) was used.

RESULTS AND DISCUSSION

Biomass water, methanol and ether extracts obtained from the cyanobacterial strains, *N. muscorum*, *N. punctiforme*, *A. oryzae*, *T. tenuis*, *M. tenera*, *S. hofmanni*, caused different effects in the "*in vitro*" growth of the phytopathogen *S. sclerotiorum*. The radial growth was measured at days 4 and 8 (Fig. 1a, 1b, 1c, 1d, 1e & 1f). Only the water and ether extracts from *N. muscorum* inhibited *S. sclerotiorum* not showing any difference between their effects. Both *N. muscorum* extracts produced a significant growth inhibition, 38 and 44% for water extract and 43 and 47% for ether extract at days 4 and 8, respectively. This result coincides with Caire *et al*. (1987) who worked with cyanobacterial extracts against a phytopathogen causing "damping off". The methanol extract produced no significant effect on the growth of the fungus. On the other hand, Mulé *et al*. (1991) demonstrated that methanol extracts and extracellular products from another axenic strain of *N. muscorum* inhibited the growth of *S. sclerotiorum* by 50% and this effect decreased as a function of time. The contradictory effects of the methanol extracts from two *N. muscorum* strains on *S. sclerotiorum* could be due to the treatment with UV radiation used to obtain the strains in an axenic condition, though no morphological differences between them were detected. Caire *et al*. (1990) also demonstrated that cyanobacterial products from *N. muscorum* inhibited the growth of *Rhizotocnia solanii*, another "damping off" causing agent.

In culture chamber, *N. muscorum* ether extract was the most effective treatment against *S. sclerotiorum*, inhibiting plant infection by 45.5% at day 8 (Table I). The inhibitory effect increased to 72.8% at day 8 when the dose was doubled (Table II). So the response depended on the dose and probably involved plant defence mechanisms because after 12 days the antifungal effect decreased probably, because of the exhaustion of the cyanobacterial active substances, which could act as elicitors. Mercier and Reeleder (1986) found in an *in vitro* culture with lettuce leaves that three mycoparasites inhibited *S. sclerotiorum* ascospore germination by 34 to 60%. In the field the effect of the treatment was corroborated at harvest (Table III).

Despite the fact that in the inhibition of the mycelium radial growth, ether and water extracts did not show a significant difference, in the interaction plant-pathogen-cyanobacterial substances no mycelium inhibition was produced by the water extract. This result could be due to a lesser activity of the extract when in contact with the plant, because of a different penetration of the active substances in the plant tissue.

In the field assays, there was no significant difference between the values obtained for all the variables determined in the two successive crops, so these values were considered as repetitions. *N. muscorum* ether extract was applied to the plants 40 and 64 days after sowing. The plants were evaluated at days 52, 64, 76 and 88 after sowing (Fig. 2a, 2b, 2c & 2d). At day 52 there were 4% more infected plants in the control than in the plot treated with cyanobacterial substances. In the second, third, and fourth measurement, the differences were 5.3, 8.8 and 16.7%, respectively.

The time required to reduce the infection with the cyanobacterial treatment was about the same as for the mycoparasite *Trichoderma koningii*, with the disadvantage for the latter of being effective only in the months when the medium temperature reaches 20-35°C (Trutmann & Keane, 1990). The treatment with cyanobacterial metabolites is independent from the temperature and the soil organic matter content that increases the sclerotia susceptibility to *Trichoderma viride* infection (Anas & Reeleder, 1988). In addition, the mycoparasite *Sporidesmium sclerotivorum* showed that infection by sclerotia is controlled by the environmental conditions (Adams & Ayers, 1982).

The treatment of the lettuce crop with cyanobacterial

Table I. Effect of cyanobacterial products on lettuce plants as a function of time, in a culture chamber experiment

Treatment	Infected plants (%)	
	Day 8	Day 12
Control (without mycelium)	0 cA	0 cA
Plant infected with mycelium	100 aA	100 aA
Plant infected with mycelium + 1.5 ml ether extract	54.5 bB	100 aA
Plant infected with mycelium + 1.5 ml water extract	100 aA	100 aA
Plant infected with mycelium +1.5 ml extracellular product	100 aA	100 aA

Small letters indicate significant differences ($p<0.05$) between treatments for each day. Capital letters indicate significant differences ($p<0.05$) between days, for each treatment.

Table II. Effect of cyanobacterial ether extract on lettuce plants exposed to inoculum as a function of time in a culture chamber experiment

Treatments	Infected plants (%)	
	Day 8	Day 12
Control	0 c A	0 c A
Plants exposed to inoculum	81.8 a A	90.9 a A
Plants exposed to inoculum + 3 ml ether extract	27.2 b B	63.6 b A

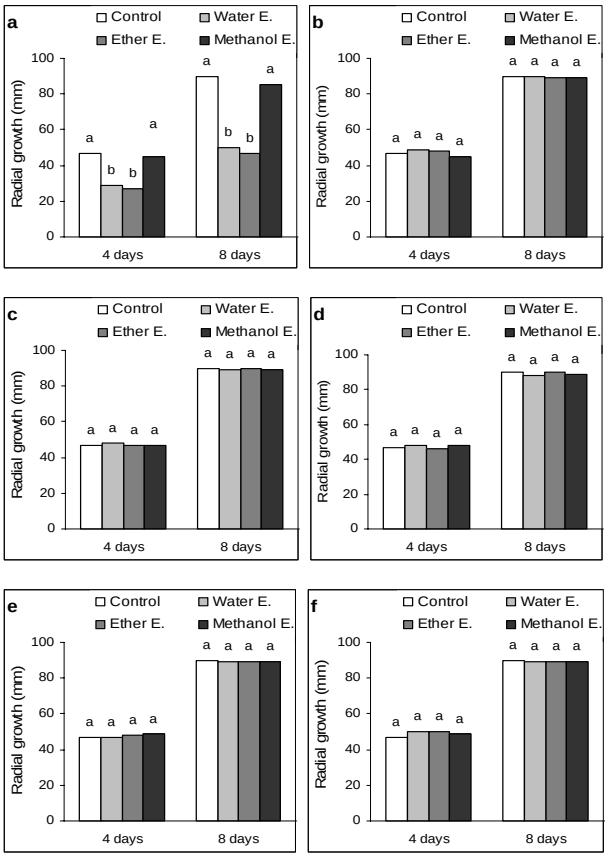
Small letters indicate significant differences ($p<0.05$) between treatments for each day. Capital letters indicate significant differences ($p<0.05$) between days for each treatment.

Table III. Enhancement in crop productivity of lettuce plants per 250 m^2 due to cyanobacterial biocontrol, at harvest

	Number of healthy plants	Plant fresh weight (g)	Plants per box	Number of boxes
Control	1436 b	295 b	42 a	34.2 b
Treated plants	1870 a	337 a	38 b	49.2 a

Different letters indicate significant differences ($p<0.05$) between control and treated plants for each column

Fig. 1. Radial growth of *Sclerotinia sclerotiorum* treated with cyanobacterial extracts. a *Nostoc muscorum* (79a), b *Nostoc punctiforme* (50), c *Anabaena oryzae* (40b), d *Tolypothrix tenuis* (40d), e *Microchaete tenera* (13a) y, f *Scytonema hofmanni* (58). E: extract. Different letters indicate significant differences between treatments for each time



extract inhibited the mycelial growth. The necessity of applying the extract a second time is probably due to the dilution of the active substances as the leaf surface increases and to the fact that the irrigation, the rain and the natural humidity produced by the crop washes it off. At harvest, there were 25% more healthy plants in the plot treated with cyanobacterial substances (Fig. 3). Mercier and Reeleder (1986) obtained similar results in a lettuce crop treated with mycoparasites.

During the assays the number of infected lettuce plants increased with time, because of the close proximity between plants, which favours the proliferation of the pathogen reproductive structures that cause secondary infection (Tu, 1997) and the canopy formed by the leaves that grow near the soil surface. The infection rate decreased in the plot treated with cyanobacterial metabolites (Fig. 4). At harvest the dry weight of the plants treated with cyanobacterial substances was highest, comparing with the not infected control (Fig. 5). The difference in weight could be due to bioactive substances in the *N. muscorum* extract such as

Fig. 2. Number of plants infected in the field by *Sclerotinia sclerotiorum* after treatment with *N. muscorum* ether extract (*n*=2000). a day 52, b day 64, c day 76 and d day 88 after sowing. Different letters indicate significant differences between control and treatment for each time

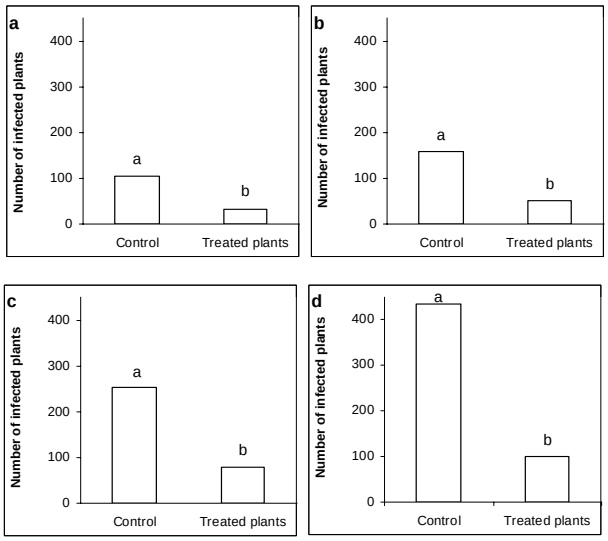
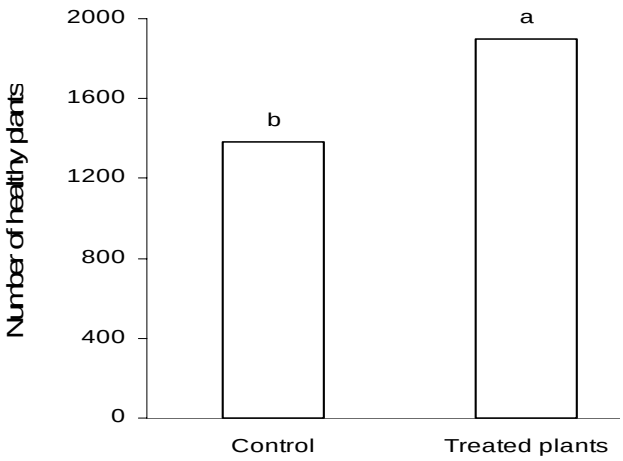


Fig. 3. Number of healthy plants in the field, at harvest (*n*=2000). Different letters indicate significant differences between control and treatment



vitamins, amino acids, peptides and various phytohormones (Caire, 1981).

The treatment of lettuce plants exposed to the infection by *S. sclerotiorum* with *N. muscorum* metabolites not only increased by 25% the number of healthy plants but also the plant fresh weight by 14% (Table III). This difference represent 10% decrease in the number of plants per box, increasing by this treatment the number of boxes by 44%, with a significant economic benefit for the producer Caire *et al.* (1979) found similar effects on rice plants dry weight treated with cyanobacterial substances.

Fig. 4. Difference in number of infected plants in the field between days 64 and 52 (1), 74 and 64 (2), 88 and 76 (3), from sowing, after treatment with *N. muscorum* ether extract. Different letters indicate significant differences between control and treatment

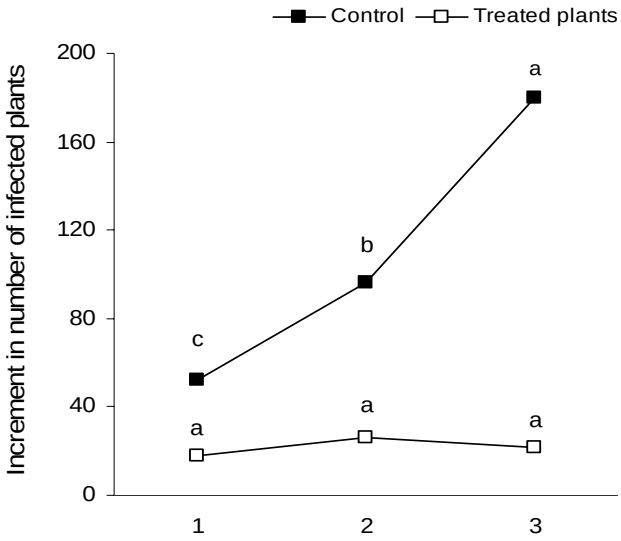
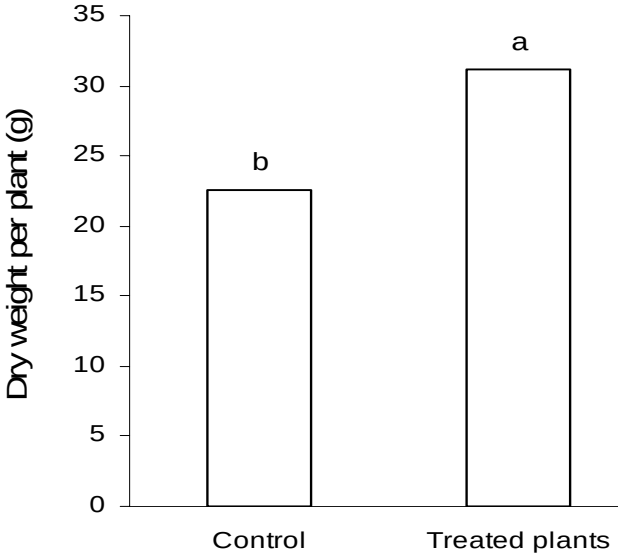


Fig. 5. Dry weight per plant at harvest in the field experiment. Different letters indicate significant differences between control and treatment



CONCLUSION

Results suggest that *N. muscorum* could be used as a bio-control agent to obtain higher yield and good health in horticulture. The bioactive substances could be advantageously used regardless of the treatment season. Results are useful for the biological control of the lettuce white mold caused by *S. sclerotiorum* and this treatment could be used for other crops with similar infection pattern. New assays are using these and other cyanobacterial metabolites for obtaining commercial products are now being sold in the market.

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