Ca-Lignosulphonate and sclerotial viability of *Sclerotinia sclerotiorum*

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Summary. Lignosulphonates, low cost by-products of the pulping process, have shown suppressive effects against some diseases caused by soil-borne pathogens. In this study, the effect of 1.5% v/v calcium lignosulphonate (Ca-Ls) amendment to two commercial potting mixes (peat + coconut fibres; PC; and municipal compost + peat + pumice; MCPP) on the viability of *Sclerotinia sclerotiorum* sclerotia was investigated. Sclerotia were buried in the Ca-Ls amended substrates for 30 days. Non-amended PC and MCPP, sterile sand and sterile PC with and without Ca-Ls were used as controls. The viability of sclerotia recovered from PC and MCPP amended with Ca-Ls was reduced by 50 and 42% respectively compared to control treatments. Ca-Ls amendment decreased sclerotial viability by enhancing the activity of the indigenous mycoparasitic fungi, *Fusarium oxysporum, Mucor* spp. and *Trichoderma* spp. The biocontrol ability of Ca-Ls against sclerotia was due to the stimulation of microbial activity and is, therefore, strictly dependent on the microbial composition of the substrate.

Key words: biocontrol, lignin product, sclerotia.

Introduction

Lignosulphonates (Ls) are low cost by-products of the acid sulphite wood pulping process. They have a complex lignin-like structure, containing mono- and polysaccharides. There are several types of Ls including ammonium, calcium, magnesium and sodium lignosulphonates. Some studies have shown the effectiveness of Ls soil application to control plant diseases caused by some soil-borne pathogens. Lazarovits *et al.* (2001) and Soltani *et al.* (2002) observed that ammonium lignosulphonate (A-Ls) amended to field soil in some Ontario potato crops significantly reduced the severity of common scab caused by *Streptomyces scabies* and

of verticillium wilt caused by *Verticillium dahliae*. In glasshouse experiments the enrichment of spent mushroom compost with both Ca-Ls and *T. atroviride* substantially increased the suppressiveness of the compost against disease caused by *Fusarium oxysporum* f. sp. *melonis* (Montanari *et al.*, 2004).

The mode of action of Ls against pathogens is not fully understood. In solution assays, Lazarovits (2001) observed that A-Ls treatment reduced *S. scabies* numbers, suggesting that a direct toxicity against the pathogen could be involved in the biocontrol activity of A-Ls. Abbasi *et al.* (2002) observed that the effectiveness of A-Ls foliar applications to control the bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* on tomato and pepper plants could be due to the increased resistance of the plant induced by A-Ls. Soltani and Lazarovits (1998) and Soltani *et al.* (2002) showed

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that the incorporation of A-Ls into soil increased fungal and bacterial numbers two- to eight-fold over control treatments, and that shifts in fungal and bacterial populations occurred. Among the soil fungi that increased in numbers after A-Ls application, the *Trichoderma* genus often became one of the dominant genera (Soltani *et al.*, 2002). Similar effects were observed by Van Beneden *et al.* (2010b) when Kraft pine lignin was amended to soil in a pot experiment conducted with two different soil types. These authors showed that shifts induced in the microbial community differed between soils. Both lignin amended soils showed a significant increase in Gram negative bacteria, and in one soil this was accompanied by a significantly higher increase in actinomycetes and fungi, compared with the other soil.

Sclerotinia sclerotiorum (Lib.) de Bary is a plant parasitic fungus causing severe disease to a wide range of plants. The pathogen survives as sclerotia in soil for several years. When appropriate environmental conditions exist, sclerotia can germinate either myceliogenically producing mycelium to infect plants, or carpogenically producing apothecia releasing ascospores to infect plants. In the past, sclerotia were killed by soil fumigation with methyl bromide. However, because of ozone depletion, methyl bromide is banned in almost all European countries and there are currently no effective chemical treatments available against sclerotia. The use of antagonist organisms, therefore, constitutes an alternative strategy to chemical control. Although sclerotia are highly resistant to adverse environmental conditions, they can be used as food source for soil animals, or be parasitized by soil micro-organisms such as bacteria and fungi (Coley-Smith and Cooke, 1971). In both field and glasshouse trials, the association of sclerotial decay with microbial activities is well documented (Coley-Smith and Cooke, 1971; McCredie and Sivasithamparam, 1985; Domsch *et al.*, 1993; Jones and Whipps, 2002; Jones *et al.*, 2003). For example, in a greenhouse trial the combination of a biological fungicide based on *Coniothyrium minitans,* a well known biocontrol agent of *S. sclerotiorum*, with a *Trichoderma* based product and Kraft pine lignin significantly reduced the viability of *S. sclerotiorum* sclerotia by increasing sclerotial parasitism by antagonists (Van Beneden *et al.*, 2010a). Van Toor *et al.* (2005) also observed

high mortality of sclerotia of *Ciborinia camelliae,* a fungus closely related to *S. sclerotiorum*, after burial in sterile pine sawdust colonised by a mixture of *Trichoderma* spp. isolates or by some white rot fungi. The combination of *Trichoderma* and white rot fungi increased the sclerotial mortality over that caused by each fungus alone. Several lignin-rich crop residues were tested for their ability to reduce *Verticillium dahliae* var. *longisporum* microsclerotia viability when incorporated into two different soils naturally infested by the pathogen; the effect of the lignin extracted from residues was also tested (Debode *et al.*, 2005). Results of this experiment showed that a reduction in sclerotial viability depended on soil, lignin and crop residue type. Crop residues with higher lignin content were more effective in reducing sclerotial viability than residues with lower lignin content; however the biocontrol ability of pure lignin was low. Therefore these authors concluded that the effect of crop residues incorporation on sclerotial viability cannot be explained solely by the lignin effect.

Since Ls seems to stimulate the density and activity of soil micro-organisms beneficial for plant health, the aim of this study was to investigate the effect of addition of Ca-Ls, a lignin rich by-product, to two commercial organic substrates used in glasshouse cultivation, on the sclerotial viability of *S. sclerotiorum*. These Ca-Ls amended substrates were also characterised for chemical properties and microbial composition.

Materials and methods

Fungal isolate and organic products

The isolate *S. sclerotiorum* MM165 from the collection of the Dipartimento di Protezione Valorizzazione Agroalimentare, University of Bologna, was used. It was stored as sclerotia and mycelium on Potato Dextrose broth and 15% glycerol at -80°C. To produce large numbers of mature sclerotia for bioassays, the method of Sansford and Coley-Smith (1992) was used.

Calcium-Lignosulphonate (Ca-Ls; Bretax C®) was supplied by Cartiere Burgo Srl (Tolmezzo, Udine, Italy) as 50% solid in water solution. It contains approximately 15–20% hexose and pentose sugars, normally mannose, glucose and xylose with small quantities of arabinose, fructose and galactose, and 5% polysaccharides (http://www.

Table 1. Populations of aerobic bacteria, total fungi, *Trichoderma* species in the organic substrates PC (88% peat + 12% coconut fibres) and MCPP (25% municipal compost + 15% peat + 60% pumice) 3 weeks after amendment with 1.5% (v/v) calcium lignosulphonate (Ca-Ls). Each microbial characterisation was determined in three samples of each treatment. Microbial enumeration was determined by plate count technique.

Treatment	Aerobic bacteria[a] log CFU g^{-1} dw	Total fungi[a] log CFU g^{-1} dw	*Trichoderma*[a] log CFU g^{-1} dw	Total microbial activity conc.[a] (μg) FDA hydrolysed $min^{-1}g^{-1}$ dw
PC	<4	7.9 a	4.9 a	3.8 a
PC + Ca-Ls	10.5 b	9.0 b	7.4 b	6.6 b
MCPP	10.5 a	7.8 a	<2	10.3 a
MCPP + Ca-Ls	10.8 b	8.1 b	6.2 b	10.3 a

[a] Means in the same column within each substrate followed by different letters are different at $P \leq 0.05$ according to the Student's *t*-test.

burgocom/burgo/Ligninsolfonati_it/index.htm). It is characterised by the following chemical parameters: pH = 4.19, C tot = 435 g kg^{-1}, N tot = 20 mg kg^{-1}. It was used at 1.5% v/v ratio, which in previous toxicity tests (data not shown) proved nontoxic for test plants (*Lepidium sativum*).

Two commercial organic substrates were used: a potting mix consisting of 88% peat plus 12% coconut fibres (PC; A400M Cocopor, Stender Italia Srl., Fauglia, Pisa, Italy), and a compost based potting mix consisting of 25% municipal solid waste compost, 60% pumice plus 15% peat (MCPP, Geobloom, Nuova Geovis Srl, S. Agata, Bologna, Italy). Prior to use, samples of each substrate amended or not with 1.5% v/v Ca-Ls, were characterised in terms of microbial (Table 1) and chemical properties (Table 2). Chemical analyses were performed at the Chemical Soil Laboratory of the Bologna Agriculture Faculty. Total microbial

activity was measured by the rate of hydrolysis of fluorescein diacetate (FDA), as outlined by Inbar *et al.* (1991). Fluorescein release was measured by the absorption at 490 nm with a spectrophotometer (Beckman Coultar DU530). FDA activity was expressed as μg FDA hydrolyzed min^{-1} g^{-1} dry weight. The numbers of aerobic bacteria, total fungi and *Trichoderma* spp. were determined by plate count technique on semi-selective media (Postma *et al.*, 2003), and expressed as log CFU g^{-1} dry product, as follows: aerobic bacteria were counted on 1/10 tryptic soy agar (4 g TSA, 13.5 g agar, 100 mg cycloheximide L^{-1} deionised water) after 7 d at 20°C; total fungi were counted on 1/4 PDA agar (10 g PDA, 16 g agar, 100 mg streptomycin sulphate, 10 mg tetracycline hydrochlorate and 2 mL Triton 1^{-1} deionised water) after 7 d at 20°C; *Trichoderma* species were distinguished on 1/4 PDA by colour, growth and morphological characteristics.

Table 2. Chemical properties of the organic substrates PC (88% peat + 12% coconut fibres) and MCPP (25% municipal compost + 15% peat + 60% pumice) 3 weeks after amendment with 1.5% (v/v) calcium lignosulfonate (Ca-Ls).

Treatment	pH (in H_2O)	C Total g kg^{-1}	N Total g kg^{-1}	C/N ratio
PC	5.8	520.0	16.2	32.0
PC + Ca-Ls	5.4	583.1	16.2	36.0
MCPP	8.0	295.2	18.3	16.1
MCPP + Ca-Ls	7.3	313.2	18.3	17.1

Each microbial characterisation (FDA test or population density) was conducted for three replicate samples collected from each organic product with and without Ca-Ls.

Pot bioassay for sclerotial viability determination

Organic substrates were distributed separately in plastic containers (50×50×70 mm) covered with plastic lids that allowed air exchange. Each container was filled with 70 mL of each substrate and a sterile distilled water solution of Ca-Ls was added to obtain 1.5% v/v Ca-Ls. Control treatments consisted of non amended PC and MCPP and sterile sand and sterile PC with and without Ca-Ls. In the absence of Ca-Ls, the same amount of sterile distilled water was added to the substrate. Ten replicates (containers) for each treatment were made, and placed in a growth chamber for 3 weeks at 20±2°C, 60% relative humidity in the dark and substrates were maintained at 60% water content. Ten fresh sclerotia 2–4 mm diam. were then randomly buried in each container and maintained in the growth chamber at the same conditions indicated earlier. Thirty days after burial, all sclerotia were recovered from each substrate; individually washed under running water, surface-sterilised for 2 min in 1% NaClO, rinsed three times in sterile, distilled water and blotted dry. Each sclerotium was then cut into two halves, one half was plated onto a Petri dish containing Neon, a semi-selective medium for *S. sclerotiorum* (Steadman *et al.*, 1994). The production of oxalic acid by the pathogen caused a change in the colour of the medium, from blue to light yellow. Sclerotia causing change of medium colour as well as formation of typical mycelium were considered viable. The other half of the sclerotium was plated onto a Petri dish containing modified PDA (Postma *et al.*, 2003). Dishes were incubated at 20°C for 1 week in the dark and examined daily. The fungal colonies appearing on each half of the decayed sclerotia and on surrounding PDA agar were counted, fungi isolated in pure culture by standard techniques, and identified by morphological characteristics. The experiment was repeated twice and the same batch of PC and MCPP stored at 4°C in the dark was used. To verify the potential suppressive effect of the fungi most frequently associated with sclerotial decay, plastic containers (50×50×70 mm) were filled with sterile sand (70

mL) and amended with 1.5% Ca-Ls. Immediately after addition of Ca-Ls or sterile distilled water, the containers were separately inoculated by a spore suspension obtained by standard technique from a pure culture of each fungus, to obtain a final concentration of 10^6 conidia mL^{-1}. Ten fresh sclerotia per container were randomly buried in the sand 3 weeks after the Ca-Ls amendment. In the control containers sclerotia were buried in sand not inoculated with the test fungi and amended or not with Ca-Ls. Six replicates were used for each treatment. Containers were placed in a growth chamber at 20±2°C, 60% relative humidity in the dark and substrates were maintained at 60% water content. Thirty days after burial, all sclerotia were recovered from each substrate; individually washed under running water, surface-sterilised for 2 min in 1% NaClO, rinsed three times in sterile, distilled water and blotted dry. Each sclerotium was then plated onto a Petri dish containing Neon (Steadman *et al.*, 1994), and the viability was determined as reported earlier.

Statistical analysis

Data were analysed using Statgraphics Plus 2.1 (1996). Percentage data were tested for normality and arcsine transformed prior to analysis. Back transformed means are presented. Data were subjected to two factor ANOVA and their interactions were significant ($P \leq 0.05$) when the Student's *t*-test was performed to assess differences between data.

Results

Ca-Ls amendment increased the populations of microbial groups in both PC and MCPP substrates, and the total microbial activity in PC (Table 1). Moreover, Ca-Ls decreased pH values, in particular when added to MCPP, and slightly increased C total content, whereas N total content was not modified by Ca-Ls amendment (Table 2).

Since the two repetitions in the sclerotial viability and fungal incidence experiments showed comparable results, data were combined and means presented in the results are the average of 20 values per treatment for a total of 200 sclerotia per treatment. Non viable sclerotia were discoloured, soft and sometimes watery when subjected to needle pressure.

Table 3. Percent of viable sclerotia of *S. sclerotiorum* and incidence (%) of potential parasite, *F. oxysporum, Mucor* spp., and *Trichoderma* spp. recovered from dead sclerotia, 30 days after their burial in the organic substrates PC (88% peat + 12% coconut fibres) and MCPP (25% municipal compost + 15% peat + 60% pumice) or sterile sand and PC amended or not with 1.5 % (v/v) calcium lignosulphonate (Ca-Ls). All sclerotia were viable after burial in sterile substrates amended or not with Ca-Ls, and no fungi were isolated from these sclerotia (data not reported in the table).

Treatment[a]	Viable sclerotia (%)	*F. oxysporum* (%)[b]	*Mucor* spp. (%)[b]	*Trichoderma* spp. (%)[b]
PC	95.0 b	0	0 a	0 a
PC + Ca-Ls	46.8 a	0	2.5 a	70.5 b
MCPP	66.8 b	35 a	0 a	2.5 a
MCPP + Ca-Ls	38.7 a	66.1 b	30.0 b	5.5 a

[a] Each treatment had ten replicates randomly distributed in the growing chamber, the experiment was performed twice with comparable results. Data were combined and those presented in the table are the average of 20 values per treatment for a total of 200 sclerotia per treatment.
[b] See [a] in Table 1.

MCPP alone showed the major impact on reducing sclerotia viability. The addition of Ca-Ls reduced the incidence of viable sclerotia by 50.7% in PC and by 42% in MCPP (Table 3). *Trichoderma* spp. were the most frequently isolated fungi from decayed sclerotia after burial in PC + Ca-Ls. They were not isolated from non amended PC. *Fusarium oxysporum* and *Mucor* spp., were the fungi most frequently isolated from decayed sclerotia after burial in MCPP. The numbers of *Mucor* spp. were increased two-fold after burial of sclerotia in Ca-Ls amended MCPP. No fungi were isolated from sclerotia buried in sterile substrates with or without Ca-Ls. More isolates of *F. oxysporum, Mucor* and *Trichoderma* spp. were obtained from decayed sclerotia after burial in Ca-Ls amended substrates. Three isolates of *F. oxysporum, Mucor* and *Trichoderma* were tested for their ability to reduce sclerotial viability, and data from the more active isolate is presented (Table 4). In the *Trichoderma* treatment without Ca-Ls, all sclerotia were viable, whereas Ca-Ls addition reduced the number of

Table 4. Percent of viable sclerotia of *S. sclerotiorum* 30 days after their burial in sterile sand inoculated separately with fungal spores (10^6 conidia mL^{-1}) of *Trichoderma* spp., *Mucor* spp. and *F. oxysporum* and amended or not with 1.5% (v/v) calcium lignosulphonate Ca-Ls.

Treatment	Viable sclerotia[a] (%)
Untreated[b]	100
Ca-Ls	100
Fusarium oxysporum MM12	33.1 b
Fusarium oxysporum MM12 + Ca-Ls	0 a
Mucor spp. MM3	91.7 b
Mucor spp. MM3 + Ca-Ls	72.2 a
Trichoderma spp. MM5	100 b
Trichoderma spp. MM5 + Ca-Ls	42.1 a

[a] Means followed by different letters within each fungus with or without Ca-Ls are different at $P \leq 0.05$ according to the Student's *t*-test.
[b] Control treatments consisted of sclerotia buried in sterile sand amended or not with Ca-Ls.

viable sclerotia by 58%. In the *Mucor* treatment without Ca-Ls, sclerotial viability was high, and was reduced by 21% by Ca-Ls addition. The lowest number of viable sclerotia was recovered from the *F. oxysporum* treatment; in the absence of Ca-Ls, 67% of buried sclerotia were not viable, whereas in the presence of Ca-Ls, all sclerotia were not viable. All sclerotia buried in sterile sand with/without Ca-Ls were viable.

Discussion

Our results showed that, under the experimental conditions used, the amendment of commercial peat and compost based substrates with Ca-Ls decreased the viability of *S. sclerotiorum* sclerotia. The suppressiveness of Ca-Ls against sclerotia cannot be attributed to a direct toxicity since did not show any effect on sclerotial viability when it was amended to sterile sand. These findings agree with previous Petri dish plate assays (data not shown), in which no effect on sclerotial viability was observed when sclerotia were plated on a growth medium amended with 1.5% Ca-Ls. Results of this study indicate that the suppressive effect of Ca-Ls is a microbial-mediated phenomenon. Ca-Ls amendment decreased sclerotial viability by enhancing the activity of the indigenous sclerotial parasites, presumably in response to increased nutrient availability. On the basis of our data, we could not determine which mechanisms of parasitism were stimulated by Ca-Ls addition. It is well known that several mechanisms are involved in the biocontrol ability of microbial antagonists towards plant pathogens: competition for space and nutrients, production of toxic metabolites and extra-cellular lytic enzymes and induction of plant resistance. In a greenhouse experiment, Van Beneden *et al*. (2010a, b) observed that Kraft pine lignin addition to a soil significantly reduced the viability of *R. solani* sclerotia together with an increased mycoparasitism by antagonists (Van Beneden *et al*., 2010b). Furthermore, these authors showed that lignin addition to a soil increased the activity of manganese peroxidase (Van Beneden *et al*. 2010b), an extra-cellular enzyme produced by lignin degrading fungi that can break down melanin (White and Traquair, 2006). They, therefore, suggested that increased sclerotial parasitism in lignin amended soil resulted from production of

the enzyme thereby damaging the sclerotia walls, and making sclerotia more susceptible to antagonists (Van Beneden *et al*., 2010b). Melanin degradation could be one of the mechanisms involved in the suppressive effect of lignin addition to soil against sclerotia. Van Toor *et al*. (2005) also observed high mortality of *C. camelliae* sclerotia 11 weeks after their burial in sterile pine sawdust colonised by a mixture of *Trichoderma* spp. isolates or by some white rot fungi. In addition, these authors demonstrated the secretion of melanin-degrading enzymes by cultures of selected white rot fungi when grown on the DOPA- melanin medium. However since there was not clear evidence of rind bleaching and melanin degradation after burial in substrates inoculated with these white rot fungi, Van Toor *et al*. (2005) supposed that sclerotia degradation was caused by denaturation of other rind components such as chitin and ß-glucans by *Trichoderma* spp. and rot white fungi. In the present study, there is also no evidence of melanin degradation. Sclerotia were parasitized by mycoparasitic fungi after burial in sterile Ca-Ls amended substrates in absence of ligninolityc fungi. Melaninases production, therefore, does not seem to be a fundamental condition for sclerotial parasitism as observed by Van Toor *et al*. (2005). *Trichoderma*, *Mucor* and *F. oxysporum* were the fungi most frequently isolated from degraded sclerotia. Tests carried out in sterile sand confirmed the mycoparasitic competence of these fungi and agreed with earlier published results in which these fungi were sclerotial colonisers and were able to reduce sclerotia survival (Merriman, 1976; Van Beneden *et al*., 2007). Soltani *et al*. (2002) also observed that colonies of *V. dahliae* derived from microsclerotia recovered from A-Ls amended substrate, were more heavily infested with the competing fungi *Trichoderma* spp. and *Talaromyces flavus* than those from untreated substrate. However, the reduction of sclerotial viability observed in this study, could be due to other biotic factors stimulated by Ca-Ls and not only specifically to the parasitic activity of *F. oxysporum*, *Mucor* spp. and *Trichoderma* spp. We did not, for example, isolate *Coniothyrium minitans*, a very efficient sclerotial parasite able to infect sclerotia in a relatively short time (Van Beneden *et al*., 2010a). This fungus could be present in the organic substrate, but displaced or masked by other colonisers such as

Trichoderma spp. (McCredie and Sivasithamparam, 1985; Jones *et al.*, 2003). Organic substrates vary considerably in their natural microbial population. Microbial diversity and density can change in relation to origin and age of the substrate and this could modulate the control effect of Ca-Ls on sclerotial viability. Van Beneden *et al.* (2010b) observed that the effect of lignin amendment on sclerotial viability of *R. solani* was dependent on the initial soil microbial composition. These authors showed that lignin addition to two different soils significantly reduced the sclerotial viability in one soil, whereas only a slight, not significant reduction of sclerotial viability was observed in the other soil. In this study, PC and MCPP were characterised by differences in microbial composition. These became more pronounced when enriched with Ca-Ls as indicated by the reduction in sclerotia viablity. The biocontrol ability of PC was related to the stimulation of indigenous *Trichoderma* spp. by Ca-Ls amendment. The biocontrol ability of MCPP against sclerotia was related to mycoparasitim by *Mucor* spp. and mainly *F. oxysporum*, stimulated by Ca-Ls.

In conclusion, our findings confirm the concept that amendment of suitable organic substrates by Ca-Ls, a low cost by-product of the pulping industry that is available in great quantity, could enhance the suppressive ability of indigenous parasites against sclerotia, thus reducing the potential primary inoculum of *S. sclerotiorum*. However, the use of Ca-Ls in a biocontrol strategy against *S. sclerotiorum* needs to be further validated, in particular, how the reduction in viability of sclerotia can reduce disease incidence.

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