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Effect of Sclerotial Distribution Pattern of *Sclerotinia sclerotiorum* on Colonizing Ability of *Trichoderma harzianum*

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Field studies were conducted over two seasons during the summers of 1997 and 1998 to investigate the effects of different spatial arrangements (random or highly aggregated) of sclerotia of *Sclerotinia sclerotiorum* and alginate pellet types (bran or polyethylene glycol) on colonization of sclerotia by *Trichoderma* spp. Treatment with alginate pellets increased the mean percentages of sclerotia colonized by *Trichoderma* spp. in both years. Distribution patterns of sclerotia affected the mean percentage of sclerotia colonized by *Trichoderma* spp. in both years, indicating that a highly aggregated distribution of sclerotia was more favorable to colonization by *Trichoderma* spp. The effects of the different pellet types (bran or PEG) were not siginificant in both years ($P > 0.05$). The application of higher densities (200 pellets per 1 m^2) of alginate pellets resulted in higher mean percentages of sclerotia colonized by *Trichoderma* spp. in 1998 ($P < 0.05$), but did not in 1997.

KEYWORDS: Biological control, Sclerotial distribution pattern, *Sclerotinia sclerotiorum*, *Trichoderma* spp.

Sclerotinia sclerotiorum is a widely distributed and destructive soilborne plant pathogen, causing severe economic losses in a range of many crops including potato, bean, carrot, rapeseed, and canola (Purdy, 1979). Sclerotinia white mold of potato caused by *S. sclerotiorum* (Lib.) de Bary is a serious problem in the Pacific Northwest of USA. Sclerotia of *S. sclerotiorum* serve as survival structures and the main source of primary inoculum in field soil (Coley-Smith and Cooke, 1971). Control of the pathogen is limited to the use of long rotations, soil fumigation, and fungicide sprays due to the wide host range of this pathogen and its ability to survive for more than three years as sclerotia (Steadman, 1979). One approach to developing alternative methods is biological control. Several fungi have been reported as mycoparasites of sclerotia or hyphae of *S. sclerotiorum*, thus reducing inoculum potential of the pathogen in soil (Budge and Whipps, 1991; Inbar *et al.*, 1996; Gerlagh *et al.*, 1991, 1999; McLaren *et al.*, 1996). *Trichoderma harzianum* isolate ThzID1 has been reported to be mycoparasitic on sclerotia of *S. sclerotiorum* (Bin *et al.*, 1991; Knudsen *et al.*, 1991a). Several formulation methods have been developed for biocontrol fungi which may improve attributes of storage, establishment, or growth in soil (Fravel *et al.*, 1985; Lewis and Papavizas, 1987; Knudsen *et al.*, 1991b). Knudsen *et al.* (1991b) reported that incorporation of polyethylene glycol 8,000 (PEG) into alginate pellets containing *T. harzianum* enhanced hyphal extension of the fungus in soil, but did not enhance production of conidia at pellet surfaces. They also suggested that ability of the biocontrol agent to effectively explore the three-dimensional soil space via hyphal growth may be more important than proliferation via spatially localized sporulation, for control of sclerotia-forming pathogens. Thus, the extent of mycoparasitism may depend on the density and spatial arrangement of sclerotia and of hyphae of the biocontrol agent in soil (Bin *et al.*, 1991; Knudsen *et al.*, 1991a). In this study, we determined the effect of random vs. highly aggregated spatial distributions of sclerotia on the control efficacy of *T. harzianum* in potato field soil, and whether the incorporation of additives (wheat bran or PEG) into alginate pellets makes a difference in the ability of *T. harzianum* to colonize sclerotia of *S. sclerotiorum*.

Alginate pellets of *T. harzianum* were made as described previously (Bin *et al.*, 1991). Three 1-cm^2 agar plugs from a culture plate (PDA, 7 days) were placed into 500 m*l* of potato dextrose broth in 1-liter flasks, and incubated at room temperature on a rotary shaker at 120 rpm with a 12-hr light cycle for a week. The mycelial biomass (37 g, wet weight) was then strained, rinsed with sterile distilled water, and added to 100 m*l* of 1% aqueous sodium alginate solution. The mycelium biomass-alginate mixture was mixed with two grams of wheat bran or twenty grams of PEG. Then, the mixture of ThzID1-alginate solution was added dropwise to 0.25 M aqueous $CaCl_2$. Pellets formed in the $CaCl_2$ solution were removed within 10 min, rinsed with sterile distilled water, and allowed to air-dry on waxed paper for two days. The pellets were stored in glass beakers at 4°C before use. The mean dried hyphal weight per pellet was 1.8 mg for ThzID1-bran pellets and 1.5 mg for ThzID1-PEG pellets. The mean weight of bran per pellet was 2.7 mg and the mean weight of PEG per pellet was 2.1 mg. Pellets used

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in all experiments were two-four weeks old.

To produce sclerotia of *S. sclerotiorum*, an isolate of *S. sclerotiorum* was grown on PDA for seven days. Mycelial disks were transferred to autoclaved sliced carrots contained in 2-liter Erlenmeyer flasks. After six-eight weeks of incubation at room temperature, sclerotia were harvested, rinsed with sterile distilled water, and air-dried. To stimulate carpogenic germination, sclerotia were surface-sterilized with sterile solution (1 min, 10% ethanol, 10% bleach in sterile distilled water). Sclerotia then were placed on top of 1,200 g sterile sand in foil plates (38-cm width×26-cm length). Sterile water was added (280 m*l* per plate), and plates were covered with aluminum foil, and incubated at 10°C for four weeks in the dark. Sclerotia germinated myceliogenically were removed at four-day intervals. After four weeks, the remaining sclerotia were harvested, attached to plastic toothpicks with cyanoacrylate glue, and allowed to dry overnight. Sclerotia were stored at 4°C, and surface-sterilized as above before use.

Field experiments were conducted in 1997 and 1998. Treatments were applied in wooden plots at the Plant Science farm of the University of Idaho, near Moscow. Microplots were one square meter and were buried to a depth of ten centimeter. Each microplot was planted with sixteen seed pieces of either certified Russet Burbank potatoes on July 26, 1997 or certified Norkota Russet potatoes on May 20, 1998. Seed potatoes were spaced at 0.33 m. Non-germinated or virus infected potato plants were replaced during the growth period. Microplots were separated from each other by border rows (approx. 0.5 m) of winter wheat in 1997 and spring wheat in 1998. On August 4, 1997 and July 20, 1998, pellets of *T. harzianum* isolate ThzID1 were treated as follows. A bulk of the soil was sieved through approximately five mesh, and ten kilograms of the soil were placed in a 20-*l* plastic bag. Pellets were added in and vigorously mixed with the soil. Then the mixture was scattered on the surface of a microplot and covered with another ten kilograms of sieved field soil. Treatments were: 1) no ThzID1, 2) forty pellets of ThzID1 per square meter incorporated with wheat bran, 3) two hundred pellets of ThzID1 per square meter incorporated with wheat bran, 4) forty pellets of ThzID1 per square meter incorporated with PEG, 5) two hundred pellets of ThzID1 per square meter incorporated with PEG. Sclerotia of *S. sclerotiorum* on plastic toothpicks were placed in each microplot at a depth of two centimeter according to randomized X-Y coordinate numbers at a density of eighty sclerotia per plot on August 7, 1997 and of a hundred sclerotia per plot on July 21, 1998. Sclerotia were arranged in two different patterns: 1) a random distribution and 2) a highly aggregated distribution of five sclerotia per spot in 1997 and of ten sclerotia in 1998. Treatments were arranged in a completely randomized design with four replicates. Therefore, there were forty microplots each year. To maintain high soil moisture, microplots were watered at one or two-day intervals, during the experimental period except on rainy days. Soil moisture content was measured every two or three days during the experimental period by sampling the soil of each microplot randomly. Soil temperature data were obtained from the weather station of Parker Farm at the University of Idaho near the experimental field. After four weeks, sclerotia were harvested from each microplot and labeled as to X-Y coordinate numbers. Sclerotia were then surface-disinfested with sterile solution for 5 min and placed on PDA containing 50 mg/m*l* of streptomycin. The proportion of sclerotia colonized by *Trichoderma* spp. was recorded after a two-weeks incubation period at 25°C. *Trichoderma* species were identified by colony color, morphology of conidia and conidiophores. Although apothecial production was observed in both years, weather conditions were not conducive to the disease development. Thus, the disease was not rated in either year.

Data in percentages were transformed before statistical analysis according to arsin [sqrt (%/100)]. Analysis of variance for a completely randomized design was carried out on the data of proportions of sclerotia colonized by *Trichoderma* spp. Orthogonal contrasts were conducted to compare effects of distribution patterns of sclerotia, and pellet types and densities of *T. harzianum*.

Soil moisture levels showed relatively uniform moisture contents (approximately between -300 and -1000 kPa) during the first two weeks in both years (data not shown). Soil temperatures were between 17 and 26°C in 1997 and between 18 and 28°C in 1998. Averages of both maximum and minimum temperature were higher in 1998 than in 1997 (data not shown). Mean percentages of sclerotia colonized by *Trichoderma* spp. in 1997 and 1998 are shown on Tables 1 and 2. Application of alginate pellets of *T. harzianum* to a potato field resulted in a higher mean percentage of sclerotia colonized by *Trichoderma* spp.

Table 1. Mean percentages of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. in 1997 field experiment[a]

Treatment[b]	Number of pellet[c]	Distribution pattern of sclerotia[d]	
		Random	Highly aggregated
Control	0	15.34 (±2.04)[e]	18.40 (±3.75)
Bran-alginate pellet	40	20.22 (±3.35)	28.79 (±3.57)
	200	25.06 (±2.30)	37.87 (±6.23)
PEG-alginate pellet	40	26.02 (±1.49)	29.98 (±6.16)
	200	28.10 (±3.17)	28.90 (±2.35)

[a]Values represent means of sclerotia colonized by *Trichoderma* spp.
[b]Microplots were nontreated or treated with alginate pellets formulated with wheat bran or PEG8000.
[c]Number of each pellet type applied to a microplot (1 m^2).
[d]Eighty sclerotia were randomly distributed for random distribution, with five sclerotia per spot being randomly distributed for aggregated distribution.
[e]The standard error of the mean.

Table 2. Mean percentages of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. in 1998 field experiment[a]

Treatment[b]	Number of pellet[c]	Distribution pattern of sclerotia[d]	
		Random	Highly aggregated
Control	0	18.62 (±0.93)[e]	20.82 (±1.16)
Bran-alginate pellet	40	24.77 (±0.83)	31.51 (±3.78)
	200	30.38 (±0.27)	29.64 (±0.27)
PEG-alginate pellet	40	24.45 (±0.97)	28.85 (±1.74)
	200	28.53 (±1.96)	31.59 (±1.14)

[a]Values represent means of sclerotia colonized by *Trichoderma* spp.
[b]Microplots were nontreated or treated with alginate pellet formulated with wheat bran or PEG8000.
[c]Number of each pellet type applied to a microplot (1 m^2).
[d]One hundred sclerotia were randomly distributed for random distribution, with ten sclerotia per spot being randomly distributed for aggregated distribution.
[e]The standard error of the mean.

than non-treated controls in both years ($P < 0.05$). Knudsen *et al.* (1991a) previously pointed out that colonization of sclerotia in the field resulted mainly from initial hyphal growth from pellets, based on no increased population of *Trichoderma* spp. recovered in field samples over time. This colonization accelerates loss of viability of the sclerotia and the production of apothecia from the sclerotia (Dos Santos and Dhingra, 1982; Gerlagh and Vos, 1991). Because sclerotia are the only source of inoculum for primary infection in fields (Coley-Smith and Cooke, 1971), mycoparasitism may result in the reduction of inoculum potential in soil (Knudsen *et al.*, 1991a). For example, Huang (1980) reported that *Coniothyrium minitans* reduced the incidence of *Sclerotinia* wilt of sunflower but did not reduce the secondary spread of the disease caused by direct contact with diseased roots. He speculated that the reduction in disease mainly resulted from the control of sclerotia. Indeed, many indigenous microorganisms have been known to be involved in the degradation of sclerotia in natural soil (Steadman, 1979; Willetts and Wong, 1980). In this study, we could not differentiate the introduced *T. harzianum* strain ThzID1 from indigenous *Trichoderma* spp. However, the background levels of colonization by indigenous *Trichoderma* spp. was always lower than those of added ThzID1, showing higher level of colonization than those of previous studies (Bin *et al.*, 1991; Knudsen *et al.*, 1991a). Soil environmental conditions (i.e., soil temperature and moisture) in this study likely were more conducive to activation of dormant propagules of indigenous *Trichoderma*. However, there is no available information on the role of indigenous *Trichoderma* spp. in the control of *Sclerotinia* disease in field conditions.

Colonization of sclerotia by *Trichoderma* spp. may depend on the density and spatial arrangement of the target propagules of *S. sclerotiorum* (Knudsen *et al.*, 1991a). Highly aggregated distribution of sclerotia resulted in significantly higher percentages of sclerotia colonized by *Trichoderma* spp. in comparison with random distribution of sclerotia in both years ($P < 0.05$). This result implies that sclerotia colonized by *Trichoderma* may serve as a nutrient source for further hyphal growth of *Trichoderma*, which may extend to and colonize nearby sclerotia. However, very little is known about the distribution of sclerotia of *S. sclerotiorum* in soil. Hoes and Huang (1975) reported approximately two to three sclerotia of *S. sclerotiorum* per kilogram of nonrhizosphere soil, but twenty-four sclerotia per kilogram of soil in the rhizosphere of diseased sunflowers.

For mycoparasitism to occur in soil, an introduced fungal agent must explore via hyphal growth through three-dimensional soil space to the target sclerotia. The addition of PEG to alginate pellets of *T. harzianum* stimulates radial growth of hyphae, while the addition of wheat bran stimulates more hyphal density or more sporulation of *Trichoderma* (Knudsen *et al.*, 1990, 1991b). We speculated that with more radial growth of hyphae of *T. harzianum*, more colonization of sclerotia occurs in soil. However, our results showed no difference in pellet types ($P > 0.05$). The effect of pellet density of ThzID1 on colonization of sclerotia by *Trichoderma* spp. was not significant in 1997 ($P > 0.05$), but significant in 1998 ($P < 0.05$). During experimental periods, soil moistures and temperatures were favorable for hyphal growth of *T. harzianum* (Knudsen and Bin, 1990). Possibly, biotic factors such as indigenous microorganisms and microfauna may diminish the effect of PEG addition on radial growth of hyphae (Dandurand *et al.*, 1993; Bae and Knudsen, 2001).

A single sclerotium may produce approximately 2.3×10^8 ascospores (Schwartz and Steadman, 1978). Our results indicate that the application of *Trichoderma* alginate pellets to soil can reduce inoculum potential of *S. sclerotiorum* and its colonization is more effective when sclerotia are highly aggregated in fields. However, because a single application of alginate pellets to soil results in a limited colonization of sclerotia by *Trichoderma* spp., alternative strategies such as soil application plus an area spray of alginate pellet powders on diseased plants will be necessary to increase the biocontrol efficacy in fields.

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