Effective Microorganisms for Controlling the Phytopathogenic Fungus Sclerotinia Sclerotiorum in Lettuce

H. Tokeshi[1], M. C. Alves, A. B. Sanches[2] and D. Y. Harada[2]
[1]Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, SP, Brazil and
[2]Mokichi Okada Foundation, Ipeúna, SP, Brazil

Abstract

Soils treated with Effective Microorganisms (EM) were found to be suppressive to the soil-borne plant pathogenic fungus, *Sclerotinia sclerotiorum*, EM also improved certain soil physical properties including a decrease in hardpan density, increased soil aggregation, and improved drainage. All of these benefits were obtained in half the time compared with organic biodynamic agriculture or minimum tillage.

The mechanisms responsible for the control of *Sclerotinia sclerotiorum* were studied by burying sclerotia in a) suppressive soil, b) a + water at a 5 cm depth, c) a + water at a 10 cm depth, and d) a + water at a 15 cm depth. The treated soil was placed in pots equipped with a water reservoir to maintain the soil columns at different heights above the water table and to ensure uniform soil moisture and humidity. Drainage was provided for removal of excess water from irrigation or rainfall. A field study was conducted according to a completely randomized design with 4 treatments and 10 replications. Each pot was placed in a 50 x 50 cm plot at a predetermined depth and 50 sclerotia per pot were sown at a 0.5 cm depth. Parameters assessed were: number of apothecia in 5 growth periods; number of fertile and aborted sclerotia; surface soil moisture; number of apothecia per sclerotium; volume of residual water in pots; number of natural apothecia in the field; and incidence of disease in commercial plantings. The results showed that surface soil moisture is one of the main factors which controls apothecia production. The number of apothecia in natural soil was lower than for the other treatments at the 1% level of probability, and sporulation did not coincide with the plant's maximum susceptibility period at the 5% level of probability.

The following control mechanisms were noted: a) evasion; b) reduction in the number of apothecia; c) abortion of germinated sclerotia; d) reduction in the number of soil sclerotia; and e) competition for nutrients at the plant surface. Leveling of the soil in the experimental area affected the results and caused the loss of some plots. Consequently, the data were analyzed as a 2 x 2 factorial, transformed into log (x + 0.5), and utilized a uni- and multi-variate, non-parametric analysis with 8 replications. Results for the natural soil differed statistically at the 10% level of probability and at the 5% level of probability for the other treatments.

Introduction

Agencies of the U.S. Department of Agriculture have emphasized the proper management of crop residues for controlling soil erosion by wind and water, preventing environmental pollution by sediment and agrichemicals, and improving soil and water quality. Crop rotations and management of crop residues at the soil surface through versions of conservation tillage and reduced tillage have increased soil productivity and resulted in higher economic returns compared with conventional agriculture, including monoculture systems (Faeth et al., 1991; Karlen et al., 1994).

Studies have shown that soils treated with Effective Microorganisms (EM) become suppressive to the white mold disease caused by the fungal pathogen *Sclerotinia sclerotiorum* (Tokeshi and Lima, 1994; Tokeshi et al., 1995a,b,c; Tokeshi et al., 1996). Results have also shown that EM significantly improved certain soil chemical and physical properties such as increased particle aggregation, rate of water infiltration, macropore percentage, rate of latex paint penetration, organic matter content, cation exchange capacity, water retention capacity, microbiological activity, and decreased bulk density and compaction. These are the same benefits that have been reported to occur in nature farming and organic farming systems (Reganold et al., 1993). However, with the use of EM, these beneficial effects are evident in a much shorter time. Farmers have also reported that their use of EM in conjunction with nature farming and organic farming methods has suppressed or controlled

certain soil-borne fungal pathogens including *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. All of these pathogens require moist soil conditions for maximum growth and activity.

In view of this and our earlier studies with S. *sclerotiorum*, we selected this organism to investigate the effect of EM on it's growth, activity and epidemiology of disease in lettuce. Accordingly, epidemics due to *S. sclerotiorum* will only occur after the development of fructification bodies called apothecia (Saito, 1973; Lumsden, 1979; Abawi and Grogan, 1979). The conditions necessary for apothecia formation are:

a) The sclerotia must remain buried in moist soil to undergo "conditioning" prior to apothecia formation.
b) The "conditioned" sclerotia will produce apothecia if buried at a soil depth not to exceed 3 cm.
c) The soil must be shaded by sufficient vegetative cover to minimize exposure to sunlight and rapid drying of the soil surface.
d) The soil moisture must be maintained at or near saturation by irrigation or rain-fall for at least 10 days; if soil drying occurs, apothecial formation declines abruptly (Steadman and Nickerson, 1975; Steadman, 1979; Abawi and Grogan, 1979).

Thus, if these conditions are not met, the formation of apothecia can be markedly suppressed in the soil and the incidence of the disease caused by *S. sclerotiorum* will be reduced accordingly.

The purpose of this paper was to test the hypothesis that EM can modify soil chemical and physical properties, and especially soil moisture relationships, that will create a soil environment that is suppressive to the growth and development of *S. sclerotiorum*.

Materials and Methods

The sclerotia were produced in the laboratory on cleaned and sterilized wheat grain that was buried for 45 days in moist soil of the experimental area and "conditioned" to enhance the formation of apothecia (i.e., carpogenesis) accordingly (Saito, 1973; Steadman and Nickerson, 1975; Abawi and Grogan, 1979). Experiments were initiated from July 15 to September 14, 1994 in area M-3 of a farm owned by Mr. Mário S. Mine of Suzano County, State of São Paulo. The M-3 area had been treated with EM during an earlier study (Tokeshi and Lima, 1994; Tokeshi et al., 1995a,b,c; Tokeshi et al., 1996).

After the sclerotia were conditioned for carpogenesis they were washed, dried, classified by size, and divided into uniform groups of 50 sclerotia each and stored in a refrigerator until needed. The treatments were:

a) Natural suppressive soil with no modifications;
b) Natural suppressive soil with water at a 15 cm depth;
c) Natural suppressive soil with water at a 10 cm depth;
d) Natural suppressive soil with water at a 5 cm depth.

The soils were placed in false bottom pots which were perforated, and set on a plastic sponge kept moist by strips which conducted water to the soil from a 6-liter capacity reservoir. Soil moisture was controlled by capillarity and drainage of excess irrigation water or rainfall was provided by lateral holes in the pots. The plastic pots (9-cm diameter) were prepared with 5-cm, 10-cm, and 15-cm soil columns and, in the natural soil treatment, a plastic ring similar to those of the pots was used.

The soil was sieved to ensure homogeneity before being placed in the pots with reservoirs full of water. The pots were buried at different depths according to their height in such a manner as to keep their surface level with the adjacent soil. The experimental area had an undesirable 15 percent slope; consequently to eliminate this variable in preparing the 1 x 10 m plot area, the soil was leveled by cutting and filling to a height of 75 cm.

Twenty of the 50 x 50 cm plots were located in the cut area and 20 in the soil-filled area. The experiment was a completely randomized design with 4 treatments and 10 replications. Each plot

contained a single buried pot, with 50 sclerotia sown in the center of each pot at a 0.5-cm depth. The treatments were randomly distributed in the cut and the soil-filled areas. However, since the leveling interfered with some results, the data were analyzed as a 2 x 2 factorial with the data transformed into log (x + 0.5) and a uni- and multi-variate, non-parametric analysis was used, according to Negrillo (1992). The averages were compared by Tukey's test at probability levels of 10, 5 and 1 percent. Upon completion of the experiment it was noted that the sclerotia in three adjoining plots in the cut area were partially destroyed by unidentified microorganisms and had produced few apothecia; thus, this reduced the size of the experiment to 8 replications.

Approximately 29 days after the experiment began apothecia and surface soil was collected at intervals of 7-8 days for a total of five evaluation periods over 60 test days. The total number of apothecia was obtained by counting during the evaluation periods. The moisture content of the 4-cm layer of superficial soil was also determined. At the end of the experiment all of the remaining sclerotia were collected and the number of sclerotia with fertile and aborted apothecia was counted. An apothecium was considered aborted when only it's pedicels were found. This was attributed to desiccation of the apex which prevented development of the hymenium of the ascus and ascospores. The residual water volume in the pots was measured to determine whether there was adequate water and if its position had influenced the volume of water evaporated or consumed by the pot.

The experiment was conducted in a commercial lettuce production field in which the incidence of *S. sclerotiorum* was determined in approximately 15 percent of the area of weekly harvest. To quantify the number of apothecia produced from natural sclerotia, ten areas of 1 m^2 each were established in which natural apothecia were counted weekly. The natural soil sclerotia potential was evaluated in 9 soil samples of 1kg each collected at random throughout the farm. The samples were analyzed according to the method described by Hoes and Huang (1975) which indicated an average of 0.56 sclerotia per kg of soil, with a bulk density of 1.1g cm^{-3}. This method estimated that 1.68 sclerotia were capable of germinating per square meter of soil to a 3-cm depth.

Results

During the experiment the lettuce fields were irrigated normally and the production of apothecia was initiated 29 days after the experiment began. The number of apothecia produced in the soils at different depths above the water table during five periods of evaluation, and the number of sclerotia germinated with fertile and aborted apothecia were counted. A sclerotium was considered germinated if it produced only one apothecium; however each sclerotium produced one or several apothecia. Therefore, the total number of apothecia per plot counted in the laboratory differed from the number of sclerotia germinated in the field.

The number of apothecia produced in the five evaluation periods were significantly different at the 1% level of probability for treatment, period and interaction (treatment x period) with a coefficient of variation of 82.0%. In comparing treatments by Tukey's test the natural soil differed from the others at the 1% level of probability and there were also significant differences between periods at the 1% level. Since interaction occurred between treatments x periods, the average number of apothecia per treatment grouped by period and treatment was compared (Tables 1 and 2). Tukey's test showed that the natural soil treatment differed from the others at the 1% level of probability in the second, third and fourth evaluation periods.

The non-parametric statistical analyses of the total fertile apothecia per plot showed that the natural soil differed from the other treatments at the 5% level of probability (Table 3). The non-parametric statistical comparisons of the total aborted apothecia (Table 4) showed that the natural soil differed from the 15-cm depth treatment at the 5% level of probability, but did not differ from the other treatments. During the apothecia collection periods it was noted that the plots placed in areas where the soil had been cut tended to have a higher moisture content and produced more apothecia than plots located in the soil-filled areas. In order to test this hypothesis, within each treatment, the moisture of the surface soil of plots in the cut or soil-filled areas was compared. Since the number of replications was different, the non-parametric analysis (2 x 2 factorial) was used.

Table 1. Average Number of Germinated Sclerotia in Soil at Different Depths above the Water Table during Five Periods of Evaluation.

Evaluation period	Soil depth (cm)	Average	Significance	
			5%	1%
First	Natural	0.0	a	A
	15	3.1	a	A
	10	0.9	a	A
	5	0.0	a	A
Second	Natural	0.0	b	B
	15	12.6	a	A
	10	9.4	a	A
	5	7.4	a	A
Third	Natural	0.6	b	B
	15	13.6	a	A
	10	11.5	a	A
	5	8.1	a	A
Fourth	Natural	2.1	b	B
	15	10.6	a	A
	10	13.2	a	A
	5	11.7	a	A
Fifth	Natural	15.7	a	A
	15	8.5	a	A
	10	11.2	a	A
	5	13.2	a	A

Averages with common letters are not significantly different at the indicated levels of probability.
"Natural" denotes a naturally-occurring, sclerotia-suppressive soil with no modifications.

Table 2. Average Number of Germinated Sclerotia in Soil during Five Periods of Evaluation at Different Depths above the Water Table.

Soil depth (cm)	Evaluation period	Average	Significance	
			5%	1%
Natural	First	0.0	b	B
	Second	0.0	b	B
	Third	0.6	b	B
	Fourth	2.1	b	B
	Fifth	15.7	a	A
15	First	3.1	b	B
	Second	12.6	a	A
	Third	13.6	a	A
	Fourth	10.6	a	A
	Fifth	8.5	a	AB
10	First	0.9	b	B
	Second	9.4	a	AB
	Third	11.5	a	A
	Fourth	13.2	a	A
	Fifth	11.2	a	A
5	First	0.0	b	B
	Second	7.4	a	B
	Third	8.1	a	B
	Fourth	11.9	a	B
	Fifth	13.2	a	B

Averages with common letters are not significantly different at the indicated levels of probability.
"Natural" denotes a naturally-occurring, sclerotia-suppressive soil with no modifications.

Table 3. Number of Sclerotinia sclerotiorum Apothecia Produced in Soils at Different Depths above the Water Table.

Replications	Natural	Soil depth to water table (cm)		
		15	10	5
A	133	128	101	154
B	36	178	147	31
C	6	200	110	54
D	21	152	71	134
E	11	102	90	100
F	34	110	79	114
G	36	123	121	77
H	83	112	91	81
Average	45.0a	138.1b	101.0b	93.1b
% Change	32.6	100.0	73.1	67.4

Averages with common letters are not significantly different at the 5% level of probability.
Percent change in numbers of apothecia for treatments are based on the 15-cm soil depth as 100.0%.
"Natural" denotes a naturally-occurring, sclerotia-suppressive soil with no modifications.

Table 4. Germinated and Aborted Sclerotia in Soils at Different Depths above the Water Table.

Replications	Natural	Soil depth to water table (cm)		
		15	10	5
A	3	0	12	2
B	37	0	1	37
C	46	1	0	23
D	38	1	3	5
E	45	1	1	1
F	33	1	2	0
G	32	0	1	0
H	16	0	0	0
Average	31.2a	0.5b	2.5a	8.5a
% Change	62.5	1.0	5.0	17.0

Averages with common letters are not significantly different at the 1% level of probability.
Percent change is based on the reduction of 50 sclerotia initially added to each pot.
"Natural" denotes a naturally-occurring, sclerotia-suppressive soil with no modifications.

Results indicated that the average moisture content of the natural soil treatment was significantly higher for plots in the cut (C) area (22.0%) compared with plots in the soil-filled (SF) area (20.4%) at the 10% level of probability. There were significant differences between the five evaluation periods. Multiple comparisons showed that the first evaluation period differed from the second and third periods at the 1% level of probability, but did not differ from the others. Statistical analyses of the results of soils maintained at 5-, 10-, and 15-cm depths above the water table showed that the soils in the cut area had a significantly higher moisture content than those in the soil-filled area at the 5% level of probability. The non-parametric statistical analysis of residual water in pots revealed a significantly higher volume of water for pots placed in the cut areas compared with those in the soil-filled area at the 1% level of probability. There were no statistically significant differences among treatment soils at the 5-, 10-, and 15-cm depths.

The number of sclerotia germinated per period was transformed into a percentage of the total (50) implanted and the result was compared with the lettuce growth stages. This showed that the highest peak of lettuce susceptibility did not coincide with the fungus sporulation peak in a soil that was naturally suppressive to *S. sclerotiorum*. The germination percentage curve of the remaining treatments showed that the sporulation peak coincided with maximum lettuce susceptibility. The average percentage incidence of *S, sclerotiorum* in commercial lettuce production in the experimental area was 1.05 percent and involved approximately 15 percent of the commercial area

harvested per week. Weekly counting of apothecia produced from natural sclerotia in the 10 m^2 areas was negative during the entire evaluation period.

Discussion

The results of this experiment indicated that surface soil moisture is one of the main factors that controls apothecia production. This was demonstrated when the natural soil markedly reduced the number of apothecia compared with the other treatments, and the differences were significant at the 1% level of probability. These results are in agreement with others (Saito, 1973; Adams and Ayers, 1979; Abawi and Grogan, 1979; Lumsden, 1979; Steadman, 1979).

The interactions showed that the natural soil suppressed the production of apothecia so that the plant's maximum susceptibility period did not coincide with the production peak of *S. sclerotiorum* apothecia and ascospores. These conclusions were corroborated by statistical analyses which showed that natural soil is suppressive to *S. sclerotiorum* because of changes in the soil and physical properties reported by Tokeshi and Lima (1994); Tokeshi et al. (1995c); and Tokeshi et al. (1996).

The high water infiltration capacity and the absence of soil compaction caused the irrigation water and natural rainfall to infiltrate rapidly for storage in the deeper horizons. Rapid drying of the soil surface which often follows is beneficial because it provides a means for controlling Sclerotinia (Hayes et al., 1986; Atwell, 1990; Reganold et al., 1993; Tokeshi and Lima, 1994; Tokeshi et al., 1995a; Tokeshi et al., 1996).

The interaction between treatment and evaluation period showed that in addition to producing a lower number of apothecia and germinated sclerotia, the natural soil caused a delay in sclerotia germination. This delay promotes disease control by the evasion mechanism because the plant's maximum susceptibility doesn't coincide with the ascospore emission peak. This was confirmed by the statistical analysis presented in Tables 1 and 2. Results indicate that all treatments were equivalent in the first and fifth periods; however in the second, third and fourth periods the natural soil differed from the others at the 1% level of probability. Since these periods coincide with the plant's maximum susceptibility, it is evident that the suppression of the disease is, to a large extent, due to the delay in production of apothecia.

In the 10- and 15-cm soil depth treatments, the production of apothecia in the second and fifth periods is similar at the 1% level of probability. This indicates that the production of ascospores continues to be high during the period when the plant is most susceptible to the fungus. In the 5-cm soil depth treatment, the results are intermediate to the other treatments, and showed a tendency for a higher sporulation in the fifth period of evaluation. Since the same soil was used in all four treatments, the initial microflora and microfauna were equivalent; therefore the most likely variable among treatments was the soil surface moisture which confirms the hypothesis that control of this pathogen depends on the soil moisture content.

The exact mechanism or mode of action whereby antagonistic microorganisms destroy or suppress sclerotia, directly or indirectly, with Effective Microorganisms (EM), is still unclear. There is some indication in this study that EM microorganisms act more on changing soil properties that, in turn, can indirectly suppress the disease. Table 3 shows that the average number of apothecia of *S. sclerotiorum* produced in the natural soil treatment was significantly lower than the other treatments at the 5% level of probability. The percent reduction in apothecia for the natural soil, 10-cm soil depth, and 5-cm soil depth was 32.6, 73.1 and 67.4 percent, respectively, compared with the 15-cm soil depth treatment as 100 percent.

Table 4 shows that the frequency of germinated and aborted sclerotia was higher in naturally-suppressive soil compared with the other treatments. The average number of sclerotia that germinated and aborted in the natural soil was 31.2 out of 50 sclerotia applied and represented a significant reduction of 62.5 percent. The other treatments showed a very low abortion frequency and that almost all of the viable sclerotia produced fertile apothecia.

The fact that suppressive soils do not prevent sclerotia germination but abort them after germination is desirable from an epidemiological viewpoint, since this reduces the sclerotia potential and, in a

few years, may actually deplete the soil's sclerotia bank. The occurrence of control by evasion helps to reduce the soil's sclerotia bank due to the lack of a susceptible host. During periods of continued rainfall, disease-suppressive soils have been reported to lose their capacity for controlling the disease. The most likely explanation is that the soil surface remains wet for extended periods which favors the growth and development of *S. sclerotiorum* (Saito, 1973; Abawi and Grogan, 1979).

Among the various ways in which a disease-suppressive soil can control the incidence of *S. sclerotiorum* are the following:

a) Controlling by evasion,
b) Reducing the number of apothecia,
c) Causing the abortion of germinated sclerotia,
d) Reducing the soil's sclerotia bank,
e) Preventing infection by ascospores,
f) Causing antibiosis on the surface of healthy plants.

The results showed that the pot system adopted was very efficient, since all the water reservoirs were kept well-supplied until the end of the experiment. The cut area, with a general average of 3.71 liters of residual water per pot, consumed less water than the soil-filled area, with a general average of 2.05 liters per pot. The difference in consumption was about 80 percent and showed that drainage and evaporation were greater in the soil-filled areas, and caused a lower production of apothecia. These results emphasize that agricultural practices which facilitate more efficient drainage and drying of the superficial soil layer (e.g., subsoiling, reduced tillage, crop rotations and raised beds) are vital for controlling soil moisture and, thereby, controlling soil-borne diseases (Faeth et al., 1991; Reganold et al., 1993; Karlen et al., 1994).

Since the commercial lettuce production area acted as a spore trap, it detected ascospores in the experimental area and showed that the climatic conditions were favorable to the occurrence of *S. sclerotiorum* epidemics. The absence of epidemics in the field was probably due to the control mechanisms operating in the disease-suppressive soil. The statistically significant differences, at the 0.1% level, between the treatments by the uni- and multi-variate, non-parametric analysis (Negrillo,1992) suggest that changes in the soil environment are responsible for biological control of the disease.

The loss of three plots in the cut area due to the destruction of sclerotia showed that other biological control systems were acting in the experimental area. The efficiency of these other systems, however, is probably more restrictive since it caused the partial loss of the sclerotia and affected less than 7.5 percent of the experimental plots.

Conclusions

Use of Effective Microorganisms (EM) to achieve a more sustainable agriculture showed that these microorganisms act in a holistic manner, changing the soil's chemical and physical properties, and mainly the aggregation of particles that cause rapid drying of the soil surface layer. Drying of the superficial soil layer delayed fungus sporulation, increased abortion of apothecia, suppressed *S. sclerotiorum* in lettuce by evasion mechanisms, increased competition for nutrients, enhanced antibiosis, and reduced the soil's sclerotia bank.

Control of other pathogens that cause stem and root rot, such as *Sclerotium rolfsii*, *Fusarium* spp., *Pythium* spp., *Phytophthora* spp. and *Rhizoctonia solani* in disease-suppressive soils is likely the result of changes in soil properties and the soil environment that enhance soil aeration and drying. This, in turn, probably increases the activity of competitive saprophytic microorganisms which are better adapted to the drier superficial soil layer, despite wide variations in the soil water content. This hypothesis, however, requires further study to confirm it's validity.

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