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Biological destruction of conidia of *Verticillium biguttatum*

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Abstract

Because biological control of *Rhizoctonia solani* in potato with conidial suspensions of the mycoparasite *Verticillium biguttatum* was often less successful in sandy soils than in loamy soils, we examined soils of potato fields for the presence of organisms destructive to conidia of *V. biguttatum*.

Representatives of conidiophagous testate amoebae were frequently present on sclerotium disks of *R. solani* infected with *V. biguttatum* in all soils studied and were most active under moist conditions. Conidiophagous naked amoebae were also numerous, except for two loam soils, and were not sensitive to moist conditions. Conidiophagous ciliates were found in rather low numbers and were most frequently isolated from coarsely structured soils under moist conditions. Conidiophagous flagellates were very infrequently observed.

A bacterial type, parasitizing and killing conidia and hyphae of *V. biguttatum*, was observed in all soils studied. It produced clusters of cocci fixed to the outside of conidia and hyphae and was most active under moist soil conditions.

The possible role of protozoan predators and bacterial parasites in the biological control of *R. solani* in potato with *V. biguttatum* applied at planting is discussed.

Introduction

Fungal conidia are a valuable source of food for predatory species of the meso- and microfauna and parasitic bacteria. Success of biological control using fungal conidia may therefore be affected by organisms destructing conidia in the soil.

Until now, most studies on conidiophagous organisms were aimed at amoebae as predators of fungal conidia. A review on this research is given by Old and Chakraborty (1986). Ciliates (Petz et al., 1985), flagellates (Hekman et al., 1992) and bacteria (Old and Robertson, 1970; Old and Wong, 1972; Clough and Patrick, 1976a, b) however, may also affect fungal conidia.

In experiments aimed at controlling *Rhizoctonia solani* Kühn in potato using *Verticillium biguttatum* Gams we observed that the control in pleistocene sandy soils was often considerably less effective than in marine loam soils (Jager and Velvis, 1985, 1986; Jager et al., 1991). From these studies it could be calculated that a reduction of the sclerotium index, a measure for damage due to formation of sclerotia on new tubers of harvested progeny tubers of 40% or more was attained on only 4 out of 19 fields (21%) on slightly acid pleistocene sandy soils and on 22 out of 34 fields (65%) on neutral holocene marine loam soils.

Biological factors inhibitory or destructive towards conidia of *V. biguttatum* were supposed to be more active in the sandy soils and thus causing the reduced effect of biological control there. The presence of organisms lethal towards conidia of *V. biguttatum* in potato fields was studied in soils of both types and compared to gain some insight whether these organisms could affect *V. biguttatum* by destructing its conidia and hyphae and so the effect of control of *R. solani* in potato.

Materials and methods

Mass production of conidia

V. biguttatum was grown on agar plates containing: mannitol 16 g; yeast extract (Oxoid, Basingstoke, England) 5 g; Ca-glycerophosphate 1 g; Mg-glycerophosphate 0.5 g; KH_2PO_4 2 g; agar (Oxoid no. 3) 12 g, micronutrient solution 5 ml (Bunt and Rovira, 1956) and water to 1 l. After about ten days' growth on agar plates conidia were suspended in 1/4 Ringer solution, prepared from Ringer solution tablets (Oxoid). The suspensions were filtered through cotton wool to remove hyphal fragments and washed by centrifugation (at 100 g for 15 min) and resuspension. A stock suspension in 1/4 Ringer solution containing about $3–5.10^8$ conidia ml^{-1} was stored at 1 °C. Conidial numbers were assessed with a haemacytometer.

Predators (protozoa)

Isolation of conidiophagous protozoa. Individual protozoa containing conidia of *V. biguttatum* were transferred from cultures in which the number of protozoa increased and the number of conidia disappeared under a dissection microscope (Wild M3) with an extended pipette to a drop of suspension with *V. biguttatum* conidia in 1/4 Ringer solution ('nutrient droplet') in small (3 cm diam.) plastic Petri dishes. After multiplication, some individuals (2–5) were again transferred to a fresh nutrient droplet. Cultures were freed from contaminating bacteria by growing them in successive nutrient droplets with 50 mg l^{-1} kanamycin-sulphate and nalidixid acid. Cultures also contaminated by fungi were, when free from bacteria, treated as follows: 2–5 cysts were selected from a fungus-free area before transferring to a fresh nutrient droplet. The isolation and multiplication procedure was repeated until the culture proved to be free of fungi.

Wanted protozoa were mass-cultured when pure, using conidia of *V. biguttatum* as food source.

Qualitative changes. Glass slides coated with a thin smear of *V. biguttatum* conidia were gently air-dried and placed between two layers of moist soil, each 4–5 mm thick, in Petri dishes. Details of the soils are given in Table 1.

To prevent drying of the soil, the dishes were placed in plastic bags and kept at room temperature.

Slides were examined microscopically, under phase contrast illumination (magnification 400 ×), over a period of 1–5 weeks.

Table 1. Some properties of the soils used in the experiments

Location	Org. matter (%)	pH (KCl)	Soil type
Haren	3.7	5.0	Pleistocene sand
Zeijerveld	7.2	4.5	Pleistocene sand
Rolde	4.5	4.9	Pleistocene sand
Borgercompagnie	20.0	5.5	Pleistocene, reclaimed peat
Kloosterburen	1.7	7.0	Holocene sandy loam
Marknesse	2.4	7.3	Holocene loam
Bellingwolde	3.7	7.4	Holocene silty clay

Observations in soil suspensions with conidia. Fresh, homogenized soil (200–300 mg) or 1 ml of a suspension of 50 g soil in 100 ml 1/4 Ringer solution was added to 20 ml 1/4 Ringer solution in 100-ml Erlenmeyer flasks to which 2–4 drops of the stock suspension of conidia were added. The soil suspension was shaken by hand and settled for 5 min before use. Incubation was at 20 °C. Observations were made every 4–7 days from the start during four to six weeks. A small amount of suspension was taken from the bottom with a Pasteur pipette for observation under phase contrast illumination.

Incubation in soil. In previous experiments, the presence of conidiophagous protozoa was examined in a conidial suspension with a soil inoculum under conditions which may differ from those in soil. To observe the activity of conidiophagous protozoa under more natural conditions, seven different soil types were investigated (Table 1).

Conidial masses of *V. biguttatum* grown on sclerotium disks of its host *R. solani* were used as a baiting system for conidiophagous protozoa. Sclerotium disks (3 mm diam.) were cut from sclerotia grown on malt extract (15 g/l), neutralized bacteriological peptone (5 g/l) agar (12 g/l)(MPA), (Oxoid preparations). Sclerotium disks infected with *V. biguttatum* by dipping them in a conidia suspension (Jager and Velvis, 1988) were placed between two 4–5 mm thick layers of soil in Petri dishes separated by nylon gauze. The water content of the soil was kept at 40 or 80% of the water holding capacity (WHC) of the soils. The WHC was assessed according to a modification of a method described by Piper (1944). Eight sclerotium disks were placed in each Petri dish. The dishes were incubated at 20 °C. After 35 days, three sets of eight disks from each soil and moisture level were examined for proto-

zoan numbers and types. The disks were squashed in 5 ml of a mineral P&J solution (Prescott and James, 1955). The obtained suspension was passed over a 0.5 mm screen, further diluted 1:4 in a conidial suspension of *V. biguttatum* ($10^6 \cdot ml^{-1}$ P&J) in 96-well microtiter-plates (MTP; flat bottom type, Greiner, Frickenhausen, Germany) and examined after two weeks using an inverted microscope. Numbers of protozoa were estimated using a most probable number (MPN) method (Darbyshire et al., 1974; Rowe et al., 1977). Only species that had ingested conidia were counted in the wells of the MTP's. As we were interested in the increase of protozoa on local accumulations of fungal conidia in the different soils, the amount of protozoa was expressed in numbers per sclerotium disk, rather than per unit of soil.

Effect of two conidiophagous protozoa on survival of V. biguttatum *on sclerotium disks of* R. solani *in soil.* The effect of two protozoa, commonly occurring in the soils of the previous experiment, on colonization and survival of *V. biguttatum* was studied in a γ sterilized sandy soil (Rolde). A naked amoeba resembling the genus *Hyalodiscus* and a testate amoeba resembling the genus *Cryptodidugia*, but sometimes with more or less filiform pseudopodia, up to 25 μm, protruding from a basal lobe (following the description of Lee et al., 1985) were used. Petri dishes (9 cm diam.) were filled with 40 g soil, WHC adjusted to 40 or 80%, which corresponded for this soil with the water contents at pF 2.6 and 1.8 respectively. Twenty MPA-cultured sclerotium disks were placed on the soil surface in each dish. Aliquots of 10 μl of a *V. biguttatum* conidia suspension or of a mixed suspension of conidia plus one of the protozoa were carefully dripped on the sclerotial surface. The conidial density was 10^6 ml^{-1}; the protozoan density 10^4 ml^{-1}. Protozoa had been precultured in conidial suspensions of *V. biguttatum* in P&J solution. The disks were covered with nylon gauze plus another 40 g soil. Three Petri dishes were used for each treatment and these were incubated at 20 °C.

At intervals three disks were removed from each dish and assessed for the number of conidia and protozoa. The disks were squashed and suspended in 75 ml sterilized tap water. A dilution series (1:10) was prepared. From each dilution 1 ml was mixed to a counting plate with malt extract agar (7.5 g malt extract (Oxoid), 12 g agar, water 1 l, plus 50 mg l^{-1}, each of neomycin-sulphate, oxy-tetracycline and streptomycin-sulphate to suppress bacterial growth). After about 10 days the numbers of colony forming units (cfu) of *V. biguttatum* were calculated from suitable dilution plates. For the assessment of the numbers of protozoa the first suspension was further serially diluted 1:4 in MTP as described in the preceding section.

Parasites (bacteria)

Observations on parasites. Bacteria producing black conidia of *V. biguttatum* were observed on slides, in experiments with diluted soil suspensions and with soil incubation as described under 'predators'. To make an inoculum of these parasites free from soil debris soil was suspended in 1/4 Ringer solution (1:5, w:v) and shaken, settled for 5 min after which the supernatant was decanted and centrifuged for 15 min at 200 g. The supernatant was filtered through a 1.2 μm pore membrane filter. A few drops of a suspension of *V. biguttatum* conidia were added to 5 ml filtrate, made up to about 20 ml in an Erlenmeyer flask with 1/4 Ringer solution and incubated at 20 °C. Samples were taken from the bottom of the flask with micropipettes for microscopical observations.

Microscopical observations of parasitized conidia. Part of the observations were made with a confocal UV laser scan microscope. Preparations for these observations were stained according to Mayfield (1975) with the Mg salt of 1-anilino-8-naphtalene sulfonic acid, MgANS (B.D.H.). In an attempt to separate conidia and adhering parasitic bacteria an ultrasonic treatment with a Sonyprep 150 (MSE) was used for up to one minute at an amplitude of 25 μm.

Effect of moisture content on destruction of conidia. The effect of the moisture content (40 and 80% WHC) of the soil on the development of parasitic bacteria on conidia of *V. biguttatum* was studied in sandy soil from Haren, obtained from the soil incubation experiment (see 'Predators'). Equal amounts of soil (dry weight basis) of both moisture levels (day 35) were treated as described under 'Observations on parasites'. After ten days of incubation the effects on the destruction of conidia were assessed. Conidia were classified as: 1. intact 2. recently parasitized (white or light grey under phase contrast), and 3. parasitized, empty (black).

Destruction rate of conidia. A volume of about 10 ml was taken from the suspension with the highest level of parasitization in the experiment on the effect of mois-

ture content mentioned above and treated as described under 'Observations on parasites'. Conidia were sampled after 0, 1, 5, 7, 9 and 12 days of incubation at 20 °C and observed microscopically.

Statistical methods

Statistical evaluation of differences between average values of comparable figures was tested using Student's t-test.

Results

Predators

Smears on object glasses. The observation of smears of conidia on glass slides after one to five weeks in the soil showed that healthy, white conidia were disappearing. Grey conidia became more numerous, but most striking was the appearance of conidia that were black under phase contrast illumination. The total number of white and grey conidia strongly decreased from 50–100 per field of view to 0–10 after 4–5 weeks of incubation.

Testate amoebae were the only predators observed occasionally with this method; the method did not meet our requirements and was discarded.

Observations in soil suspensions with conidia. Conidiophagous ciliates were usually present in suspensions of sandy and reclaimed peat soils and also in crumbly silty clay after at least one week of incubation (Figure 1A). Small numbers were infrequently observed in suspensions of the marine loam soils. Conidiophagous testate amoebae were present in all soils (Figure 1B). Conidiophagous naked amoebae were present in all soils; large conidiophagous types were observed in the sandy soils and in the reclaimed peat (Figure 1C). A representive of *Vampirellidae* was infrequently observed in the latter soil only. Conidiophagous flagellates were not observed in the soils studied.

Incubation in soil. The results of predation and parasitation of conidia of *V. biguttatum* on sclerotium disks of *R. solani* infected with this fungus in various soils at 40 and 80% WHC are presented in Table 2.

Although different members of the main groups of protozoa were present on *Verticillium* infected sclerotium disks as predators of conidia, testate and naked amoebae were most numerous and widely distributed

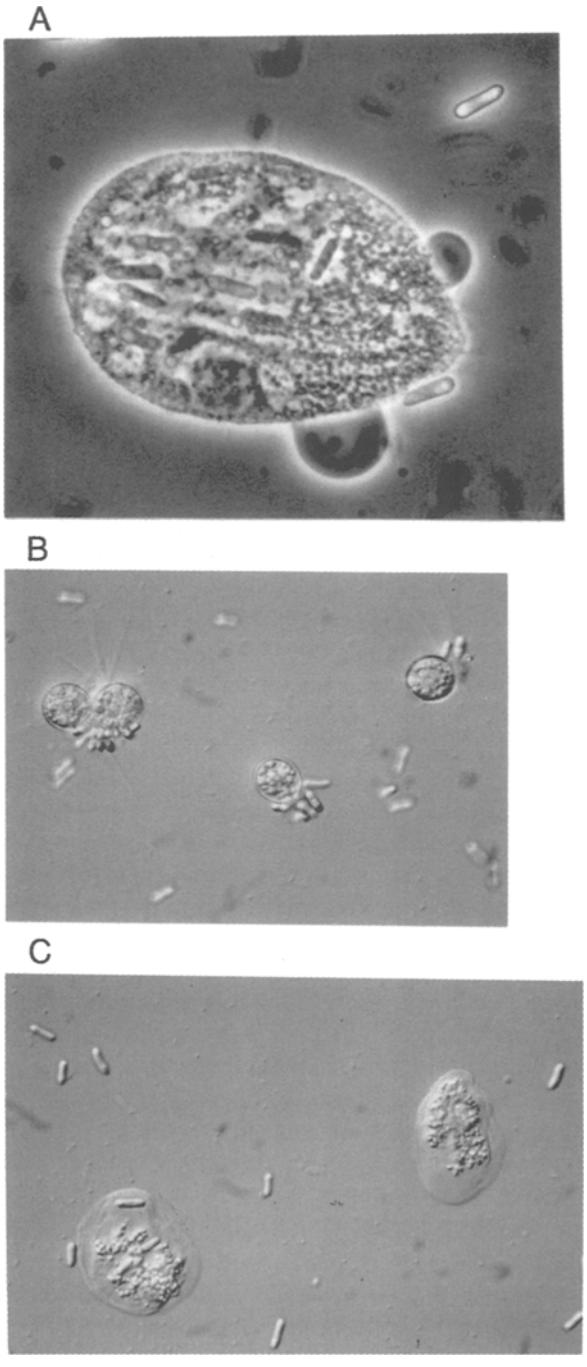

Figure 1. Frequently occurring conidiophagous protozoa consuming conidia of *Verticillium biguttatum.* (A) Ciliate, collapsing under the coverslip (phase contrast); (B) Testate amoeba (interference contrast); (C) Naked amoeba (interference contrast). Length of conidia: 8–10 μm.

Table 2. Occurrence of conidiophagous protozoa and parasitic bacteria in some soils

Location	WHC[1]	Protozoa[2]				Bacteria[2]
	(%)	Testac.	Amoeba	Ciliates	Flagell.	(black con.)
Haren	40	–	++++	–	–	–
(sandy soil)	80	+++++	+	++	+	+++++
Zeijerveld	40	–	++	–	–	+
(sandy soil)	80	++++	+++	+	+	–
Rolde	40	+	++++	–	–	+++
(Sandy soil)	80	+++	++	–	–	++++
Borgercompagnie	40	++	+	–	–	+
(recl. peat)	80	++++	++	+	+	+++++
Kloosterburen	40	++	+	–	–	+
(sandy loam)	80	+++++	+++	+	+	++++
Marknesse	40	+	+	–	–	–
(loam)	80	++++	+	+	–	+
Bellingwolde	40	+	–	–	–	–
(silty clay)	80	+++++	+	+	–	+

[1] WHC=water holding capacity

[2] converted to numbers per sclerotium disk. Exact calculation was not possible because of interference between different types of protozoa and bacteria. Therefore density is broadly classified into six categories: – = not detected; + = < 10; ++ = 10–50; +++ = 50–100; ++++ = > 100; +++++ = > 1000.

over all soil types. About four types of conidia predating testate amoebae were observed, but one type (Figure 1B) resembling *Cryptodifflugia* was most abundant. It had a clear preference for moist soil conditions and could easily be cultured on conidia of *V. biguttatum*.

Naked amoebae increased in numbers on sclerotium disks in most soils as far as could be checked, they mostly belonged to the same type (Figure 1C) resembling the genus *Hyalodiscus*. They proliferated on sclerotium disks in dry as well as in moist soil; in case of two sandy soils they even were more numerous in relatively dry soil (40% WHC).

Conidiophagous ciliates were detected only in small quantities on sclerotia in moist soil: in this experiment regardless of soil type. Flagellates consuming conidia were only found incidentally and only under moist soil conditions. They did not multiply after transfer to a conidial suspension of *V. biguttatum*.

Effect of two conidiophagous protozoa on survival of V. biguttatum *on infected sclerotium disks of* R. solani *in soil and increase in protozoan numbers.* The amount of conidia of *V. biguttatum* on sclerotia increased rapidly at both moisture levels (Figure 2A). Within 10 days the density increased by a factor > 1000 to a maximum of more than one million per sclerotium disk. At this early stage there was no effect of protozoa and the protozoan populations did not yet reach detectable numbers (Figure 2C). The response to moisture conditions became obvious in the course of time.

At 40% WHC only naked amoebae had significantly reduced conidia numbers after 37 and 59 days of incubation. Testate amoebae were not observed as active predators at this moisture level. After 108 days of incubation the numbers in all treatments were reduced to values between 2 and 6 $\times$ 10^4 per sclerotium. The cause of the strong reduction in the control is not clear (contaminating organisms lethal to conidia have not been observed).

At 80% WHC both testate and naked amoebae caused a substantial decline in the conidial density. The testate amoebae seemed to be more effective initially than the naked amoebae and their density was at least 120 times higher after 37 and 59 days incubation (Figure 2D). Compared with the control, testate and naked amoebae had substantially reduced the density of V. biguttatum conidia (Figure 2B). At this moisture level no decrease was observed in the conidial density of the control after 108 days.

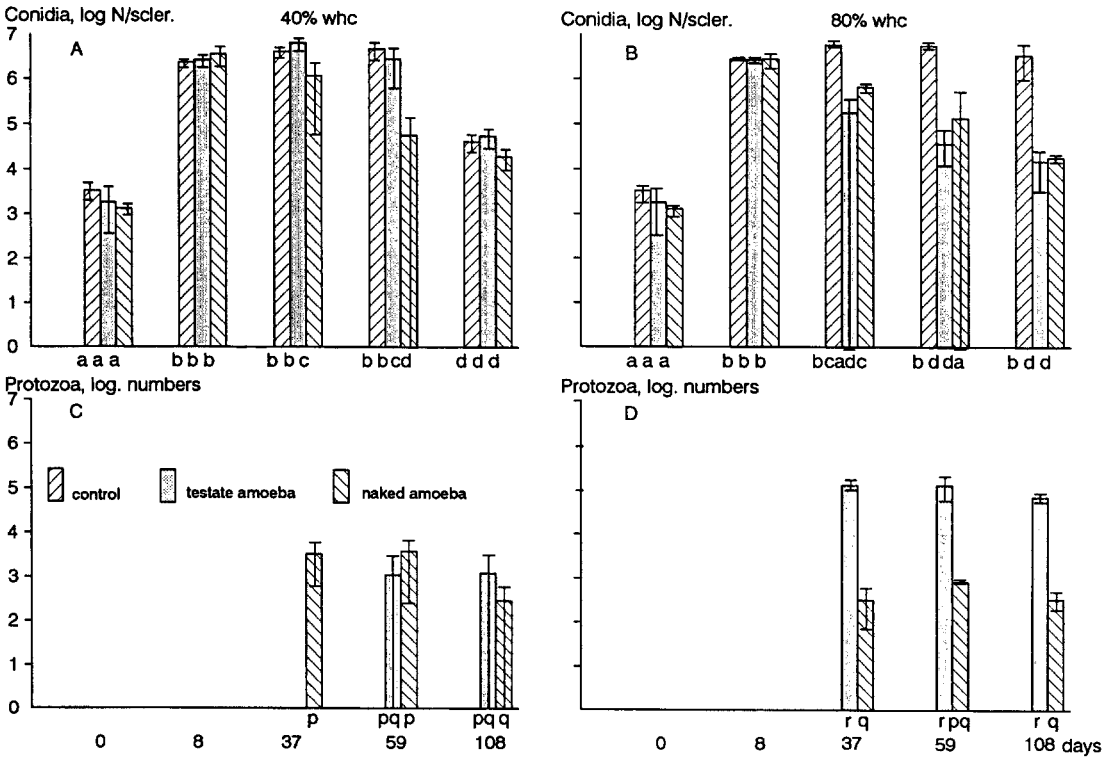

Figure 2. Effect of a testate and a naked amoeba on conidial density of *V. biguttatum* grown on sclerotial disks of *R. solani* in soil at 40% (A) and at 80% (B) of water holding capacity (WHC) in course of time. Density of the conidiophagous protozoa at 40% (C) and at 80% (D) of WHC. The sandy soil used was sterilized by γ-radiation. Remark: log. numbers are given per sclerotium disk. Values given in the columns with different letters differ statistically significantly at $p < 2.5\%$. The standard error of the mean is given as a vertical bar.

Parasites

Observations on parasitized conidia. A variable proportion of the conidia of *V. biguttatum* in smears on object glasses, in suspensions with a soil inoculum, and of those on parasitized sclerotia of *R. solani* in soil appeared black when observed under phase contrast illumination (Table 2). In bright field these conidia were poorly visible and in dark field only the conidial cell wall proved to be present, indicating that they were empty. Most of these black conidia wore bunch-like appendices (Figure 3A, 4A) but dead conidia without these appendices were also present (Figure 3B). The latter were already present in small quantities (2–6%) among conidia produced by the fungus in pure culture. Their number could increase further due to the feeding activity of some testate and naked amoebae (Figure 3B) and for other yet unknown reasons. The organism causing the bunch-like appendices (Figure 4A) seems to be a parasitic bacterium. Parasitic organisms were observed in an earlier stage of parasitizing as one single cell on apparently intact conidia. Figure 3C shows parasitized (black) hyphae bearing the 'bunches' and healthy (white) hyphea. The parasite turned out to be Gram positive.

Studies with a confocal UV Laser Scan Microscope showed that a cluster of coccoid cells with a diameter of 0.8–1.0 μm was formed at the outside of the conidia. A connection between the cluster of cocci and the conidium was not observed. The cluster and the conidium, however, could not easily be separated. The connection between the cluster of cocci and the conidium broke only after ultrasonic treatment for one minute at an amplitude of 25 μm; the cocci were also separated then. Conidia were observed, with a ruptured wall, probably where the cluster had been positioned (Figure 4B), pointing to a strong connection. The nature of this connection is not yet known. Confocal laser scanning with steps of 0.2 μm in depth showed no connections.

Effect of soil moisture content. Parasitic organisms occurred more frequently in moist (80% WHC) than in dry (40% WHC) soil (Table 2). The parasitic activity

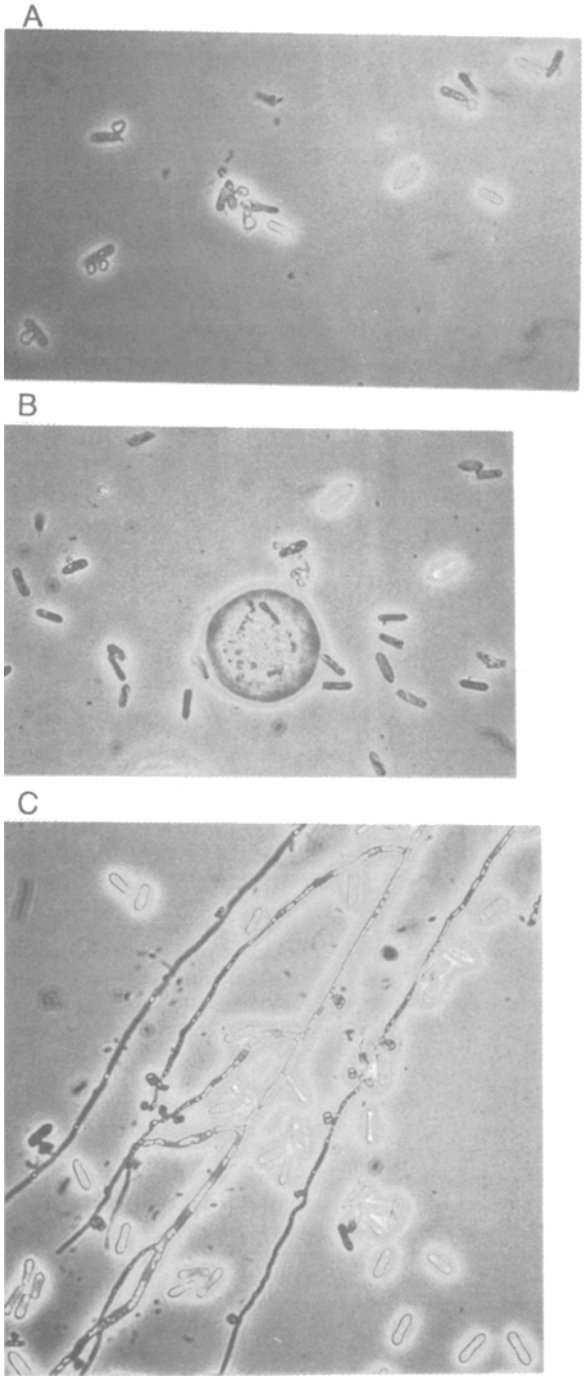

Figure 3. 'Black conidia' (phase contrast). (A.) Intact (white) and parasitized (black) conidia with small bunches; (B.) Black conidia (empty hulls) as a result of digestion of contents by naked amoebae. Empty hulls are excreted; (C.) Hyphae of *V. biguttatum* infected by parasites. (Black hyphae and conidia with small bunches next to healthy, white hyphea).

in a filtrate of moist soil also proved to be higher than in a filtrate of dry soil (Figure 5). In the conidial suspension with filtrate of dry soil 85–90% of the conidia was still intact after 10 days of incubation, while in the suspension with filtrate of the moist soil only 45% was in a good condition.

Rate of destruction. Parasitism proceeded quite rapidly (Figure 6) and was very destructive (an active inoculum from the preceding experiment was used). Within 12 days almost all conidia were killed by the parasite.

Discussion

Approximation of numbers

The amount of conidiophagous organisms grown on *V. biguttatum* infected sclerotium disks was estimated using the MPN method. Although the MPN method is less suitable for the estimation of slowly multiplying organisms like testate amoebae in a mixture of protozoa, the method seems sufficient for the experimental conditions in our soil incubations. A direct observation of protozoa in the suspension of squashed sclerotium disks was obscured by the many particles in the suspension, and did not allow a proper discrimination between conidiophagous and bacteriophagous species. Besides, the method was applied in a situation of enrichment where only a restricted number of protozoan types, able to grow on fungal conidia as specific source of food, were expected to multiply. As conidia of *V. biguttatum* were also the only source of food in the wells of the micro-titre-plates, conidiophagous organisms could easily be detected, including testate amoebae. There may, however, have been some interference between testate and naked amoebae in the soil incubations in non-sterilized soils (Table 2). The method of dilution in MTP supplemented with fungal spores also offered the opportunity for a good observation of parasitized conidia.

Predators

Our data did not univocally point to a general difference in activity of conidiophagous organisms on sclerotium disks of *R. solani* infected with *V. biguttatum* buried in coarse and fine-textured soils. Theoretically, protozoa have a better chance to operate in sandy soils than in loams and clays (most soil pores in diameter classes 6–30 μm and 0.2–1.2 μm, respectively (Hassink et al., 1993)), because of their relatively large

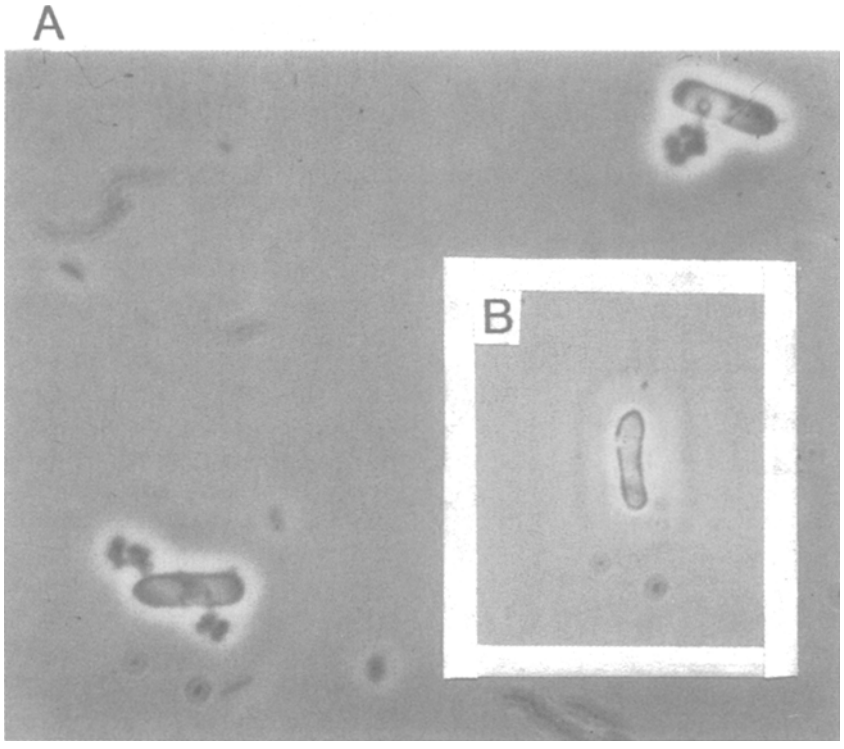

Figure 4. (A) Conidia of *V biguttatum* with bunches of cocci: parasitic bacteria (phase contrast); (B) Ruptured empty conidium after sonification (phase contrast).

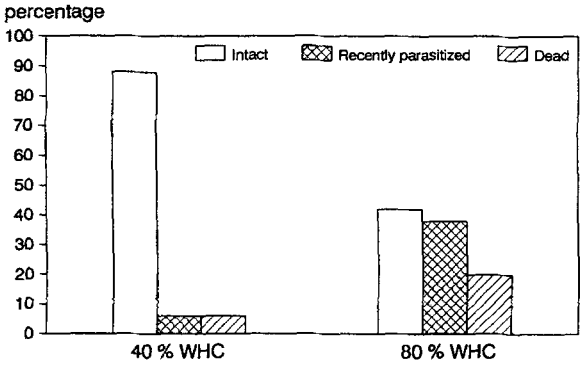

Figure 5. Quality of *V. biguttatum* conidia, infected by parasites from sandy soil at 40 and 80% WHC during ten days incubation in 1/4 Ringer solution at 20 °C.

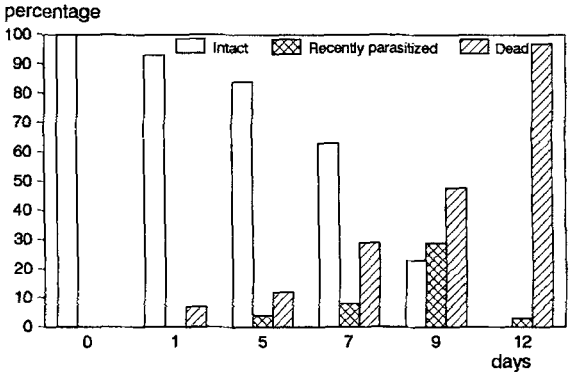

Figure 6. Rate of destruction of conidia of *V. biguttatum* by parasites in 1/4 Ringer solution during 12 days incubation at 20 °C. (The inoculum was from a suspension with very active parasitism).

size (5–80 μm). Hassink et al., 1993), however, did not find a relationship of the biomass of amoebae and flagellates with a special pore size class. Biomass probably depends more on available food than on sufficient space. In soil, circumstances on the surface of sclerotial disks or of subterranean plant parts may locally overcome spatial limitations. The number of conidiophagous organisms developing on conidia of *V. biguttatum* on sclerotium disks of *R. solani* in soil, as in our experiments, depends on the amount of organisms in the soil layer in direct contact with the sclerotium disk and also on the possibility to move through the sieved soil to the sclerotium disks. The water content of the

soil is probably more important for swimming than for creeping organisms.

In all soils tested, protozoa conidiophagous to *V. biguttatum* were present in varying numbers. Their development on *V. biguttatum* infected sclerotium disks of *R. solani* was strongly influenced by soil moisture conditions. Of the different types of protozoa, testate amoebae and naked amoebae responded most to conidia of *V. biguttatum* on sclerotium disks incubated in soil (Table 2).

Testate amoebae increased markedly in numbers on sclerotium disks in all soils used when the soil was rather moist (80% WHC). On disks in dry soil (40% WHC) they were not observed, or only in very low numbers. Also in the soil incubation in sterilized soil at 40% WHC they multiplied very slowly (Figure 2C).

Naked amoebae, growing on sclerotium disks, appeared to be not sensitive for dry soil conditions (Table 2, Figure 2). In two soils there seemed to be a preference of naked amoebae for dry conditions, but the difference with moist soil may have been caused by lack of competition by testate amoebae. The numbers of naked amoebae were very low on the sclerotium disks in the two heavier soils (loam and clay).

Swimming organisms such as ciliates and flagellates were not observed as inhabitants of sclerotium disks under dry soil conditions (40% WHC), but were found in very low numbers when the sclerotium disks were incubated in moist soil, The ciliates we isolated could easily be cultured using conidia of *V. biguttatum*. Conidiophagous flagellates were also observed in very low numbers. Unlike the isolate described by Hekman et al. (1992) we were unable to culture the flagellates on conidia of *V. biguttatum*. The conidia we observed within the these flagellates probably were ingested facultatively.

Parasites

Besides by predatory protozoa, conidia were attacked by a parasitic organism. Its activity, which appeared to be highly increased by moist soil conditions, was low on sclerotium disks in the two fine-textured soils but also in one of the sandy soils (Table 2).

Parasitization of conidia also occurs when a filtrated soil infusate is added to conidia. Pores of 1.2 μm allow the infective agent to pass. Formation of appendices to conidia and their destruction thus is caused by an external factor < 1.2 μm. The appendices are small bunches of uniform cocci with a diameter of 0.8–1.0 μm, of which a number is situated at some distance from the conidium. Form and size are in the range of those of bacteria. We assume that the observed object indeed is a bacterium and not an artifact like spheroplasts.

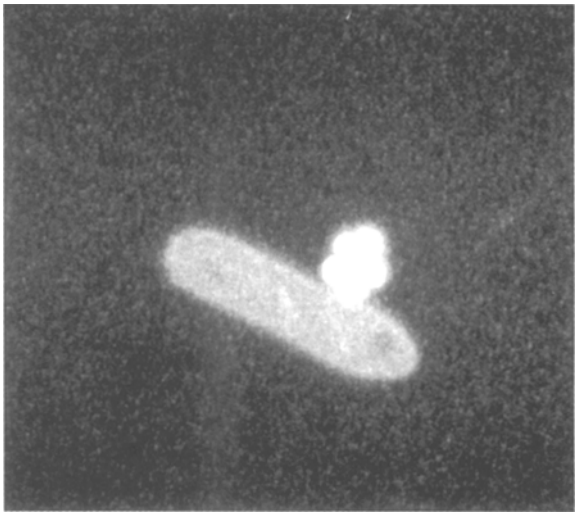

Figure 7. Conidium of *V. biguttatum* with a cluster of bacterial parasites (confocal laser scan microscope image).

This bacterium differs considerably from those already described. The latter include helically lobed bacteria (Old and Bobertson, 1970; Old and Wong, 1972) and motile coccoid form (Clough and Patrick, 1976b. These, however, did not form clusters of cocci on parasitized conidia and hyphae. We only observed the non-motile parasitic stage of a parasite which forms the bunches of cocci. Although in figure 4A stalks seem to be present between the bunch and the sclerotium, connections could not be shown between cocci and conidium and between the cocci mutually when using confocal laser microscopy (Figure 7). However, connections proved to be very firm.

The nature of the connection between the cluster of cocci and a conidium and the way of penetration of a conidium by a parasite need further study. The infective stage of the parasite probably is motile. Its identity is not known and needs to be elucidated in a further study.

Influence of predators and parasites on effect of biological control of R. solani

Conidia of *V. biguttatum* can survive in non-rhizosphere soil for more than four years (Van den Boogert and Velvis, 1992) so rapid loss of viability in the subterranean phytosphere of the potato plant may be caused by detrimental factors as predators and parasites. Applied to seed tubers in spring, for biological

control of *R. solani*, conidia have to be viable during the growth of the potato plant to protect it against infection by *R. solani* and to prevent formation of sclerotia on the harvest. Biological control of *R. solani* with *V. biguttatum* applied at planting was often less effective in slightly acid sandy soils than in neutral marine loams (Jager and Velvis, 1985, 1986; Jager et al., 1991). The density of *V. biguttatum* on subterranean plant parts, in the soil and on tuber-borne sclerotia was also very low in two sandy soils, even after inoculation in spring (Jager and Velvis, 1995). This may be caused by differences in activity of predators, parasites and/or other detrimental factors like fungicides applied to soil.

In this study we presented the most prevalent types of conidiophagous protozoa and a bacterial parasite which occur in the soils studied. They may contribute to the reduced effect of biological control. Although we observed some tendency to a greater activity of certain conidiophagous groups in coarse-structured soils than in fine structured soils, data were too divergent to draw conclusions for effects on biological control under field conditions. All of the soils contained organisms that could effectively predate or parasitize conidia of *V. biguttatum*. Moist soil conditions appeared to be favorable for the activity of a number of predators and the parasite. In moist years they thus could negatively affect biological control of *R. solani* with *V. biguttatum* in potato. Nothing is yet known about interfering or compensating effects between the different groups in the complex soil environment. Besides, there are other possible mechanisms which may contribute to the observed differences in the effect of biological control: differences in inoculum density of the pathogen, differences in antagonistic effects, differences in 'false germination' of conidia of *V. biguttatum* by non-host fungi (Van den Boogert et al., 1989) between soils etc. A more detailed study is needed to enravel these interactions on the mechanisms acting on the soil-potato interface, where biological control is taking place.

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