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EXPERIMENTAL ARTICLES

Development and Relations of *Fusarium culmorum* and *Pseudomonas fluorescens* in Soil

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Abstract—The development of *Fusarium culmorum* and *Pseudomonas fluorescens* in soil, and the relations between them, were studied using membrane filters containing the fungus, the bacterium, or both microorganisms; the filters were incubated in soil. *F. culmorum* was identified by indirect immunofluorescence; the GUS-labeled strain was used to visualize *P. fluorescens.* It was found that *F. culmorum* introduced in soil can develop as a saprotroph, with the formation of mycelium, macroconidia, and a small amount of chlamydospores. Introduction of glucose and cellulose resulted in increased density of the *F. culmorum* mycelium and macroconidia. *P. fluorescens* suppressed the development of the *F. culmorum* mycelium in soil, but stimulated chlamydospore formation. Decreased mycelial density in the presence of *P. fluorescens* was more pronounced in soil without additions and less pronounced in the case of introduction of glucose or cellulose. *F. culmorum* had no effect on *P. fluorescens* growth in soil.

Key words: interaction of microbial populations in soil, *Fusarium culmorum*, *Pseudomonas fluorescens.*

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F. culmorum is found in humid, semihumid, and semiarid zones of cereal cultivation throughout the world. The fungus causes diseases of cereals, which result both in decreased yields with low grain quality and in contamination with mycotoxins.

Bacteria of the genus *Pseudomonas* are well-known biocontrol agents for a number of phytopathogenic fungi, including *Fusarium* species. The effect of fluorescent pseudomonads is, however, not constant [1], but depends on a number of factors. The type of soil is known to influence the survival of pseudomonads introduced into soil [2]; the degree of plant disease suppression depends on the relative density of the phytopathogenic fungus and the antagonistic bacterium [3, 4]. Compared to environmental conditions, the *P. fluorescens* : *F. culmorum* ratio was demonstrated to have less effect on the capability of bacteria to suppress fungal growth and to promote plant growth [5].

When introduced into soil, antagonistic bacteria can decrease the numbers of phytopathogenic fungi; the fungi, however, also affect bacterial antagonists. For example, *F. graminearum* suppressed *Pseudomonas* sp. growth under conditions of the simulated corn rhizosphere [6]. Fusaric acid produced by *F. oxysporum* strains was found to inhibit antibiotic synthesis by several *P. fluorescens* strains [1]. Thus, understanding of the behavior of each microorganism and their interactions under natural conditions (in soil, rhizosphere, and rhizoplane) is required in order to obtain the maximal protective effect of antagonistic bacteria.

The goal of the present work was to monitor the development of the phytopathogenic fungus *F. culmorum*, its bacterial antagonist *P. fluorescens*, and their interactions in soil.

MATERIALS AND METHODS

We have previously used membrane filters for the study of *F. culmorum* development; fungal macroconidia were applied to the filters and introduced into soil or rhizosphere. The indirect fluorescent antibody method was used to identify the fungal structures on the filters removed from soil. It was found that fungal development was similar in soil and on the membrane filter introduced into the soil [8]. The same methodical approach was used in the present work.

Since competition for nutrients with soil microorganisms is one of the mechanisms of *Fusarium* suppression in soil [9, 10], the development of *F. culmorum* and *P. fluorescens* and their interactions were monitored in soil with carbon sources of different availability for the fungus and the bacterium (glucose and cellulose).

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Fusarium culmorum 30 was isolated from a diseased barley plant in the Leningrad oblast. Infection with this strain caused root and stem rot of barley in greenhouse experiments. *F. culmorum* was grown on Czapek agar for seven days at 25°C and the macroconidia were washed and separated from the medium by filtration through nylon fabric.

Pseudomonas fluorescens 2137 was kindly provided by L.V. Kravchenko and N.M. Makarova (Research Institute of Agricultural Microbiology). When tested on a nutrient medium, the strain inhibited the growth of some phytopathogenic fungal strains, including *F. culmorum* 30. The reporter GUS was introduced into the *P. fluorescens* genome by transposon mutagenesis with Tn5. The GUS-labeled strain retained the antifungal activity of the parent strain. Strain 2137 *gus* was cultivated for one day on agarized potato medium (pH 7.0).

The development of *F. culmorum* and *P. fluorescens* and their interrelations were studied by the membrane filter method in a nonsterile sod-podzol loam soil. To determine the effect of organic matter on soil microorganisms, glucose or crystalline cellulose were introduced (0.1% and 1 % Wt/wt of dry soil, respectively). The soil was thoroughly mixed and distributed unto 1.5-l vessels (1800 g each). The soil was moistened two weeks prior to the experiment. Throughout the experiment, the soil humidity was maintained at 60% of its moisture capacity. The soil respiratory activity was determined by CO_2 production using a TSVET-100 gas chromatograph.

To assess *F. culmorum* development, 20 μl of the suspension of fungal macroconidia (6×10^4 /ml) was applied within a 0.45-cm circle on the surface of a Synpor 0.23 μm membrane filter. To assess *P. fluorescens* development, 20 μl of bacterial cell suspension (2×10^7/ml) was applied to the same area of another filter. For the study of interrelations between the fungus and the bacterium, the above-mentioned amounts of fungal and bacterial suspensions were applied to a single filter; each organism was inoculated in two areas, and the distance between the sites of fungal and bacterial application was 0.7 cm. Immediately after the application, the filters were air-dried at room temperature and introduced into sterile nylon bags (pore diameter, 200 μm). The filters were submerged 5–6 cm deep into soil and incubated for 12 h (only the filters with *P. fluorescens*), 1, 3, 5, 8, 13, 22, and 55 days. At the end of each incubation period, four filters were removed. The filters were dried at room temperature. *F. culmorum* was identified by the indirect fluorescent antibodies method. The procedures for the isolation of *F. culmorum* water-soluble proteins, immunoglobulin isolation, determination of their specificity, and staining of the fungi on membrane filters have been described previously [8].

The stained filters containing *F. culmorum* were examined under an Axiolab fluorescence microscope (Carl Zeiss, Germany) under ×200 and ×400 magnification. At least 30 microscope fields of random location were examined on each filter. The presence of bacteria, including lytic ones, was determined. The *F. culmorum* conidia and chlamydospores were enumerated separately; the mycelium density was evaluated according to the five-grade scale: 1, <1 m/cm^2; 2, 1–2.3 m/cm^2; 3, 2.3–3.6 m/cm^2; 4, 3.6–5.5 m/cm^2; and 5, >5.5 m of mycelium per 1 cm^2 of the filter.

The filters with *P. fluorescens* were treated with the solution containing 5 ml 0.1 M phosphate buffer (pH 7.0), 50 μl 10% SDS solution, and 25 μl 2% X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt); the solution was prepared immediately before use. The filters were placed on filter paper saturated with this solution and incubated for 24 h at 30°C. After staining, colonization sites were visible as blue spots; the coloration intensity depended on the number of bacteria. The complete area of the filter was examined under the microscope (×70), and the *P. fluorescens* concentration was assessed according to the eight-grade scale: 1, the blue spot occupied <25% of the microscope field; 2, 25–50%; 3, 50–75%, and 4, >75%. In the case of bright blue coloration, indicating high densities of bacteria, the value for the spot area was doubled: 2, 4, 6, and 8, respectively.

To study the interrelations between the fungus and the bacterium, one half of the filter (two application spots) was stained for immunofluorescent determination of the fungus, while another was treated with the X-Gluc-containing buffer to reveal bacterial development.

For statistical treatment of the results, the Stat Soft Statistica v. 6.0, 1995 software package was used.

RESULTS

***F. culmorum* development in control soil and in soil with glucose or cellulose.** When introduced in soil on membrane filters, *F. culmorum* macroconidia germinated; a branched mycelium was formed with new macroconidia and chlamydospores.

By the first day of observation, the introduced macroconidia germinated practically in all the variants; chlamydospores were formed in some germinated macroconidia. The macroconidia usually germinated in two growth tubes and formed hyphae of the length and branching depending on growth conditions (Fig. 1). Thus, a mycelial grid was formed on membrane filters and persisted for at least 22 days.

In all the variants, formation of new macroconidia on the mycelium was already visible on the first day (Fig. 1). In soil, macroconidia usually germinated in one growth tube; the germinated conidia were detectable throughout the experiment, even on the 55th day in soil with cellulose. Chlamydospores of both conidial and mycelial type were formed.

In the unsupplemented soil, *F. culmorum* numbers remained relatively stable for 22 days (Fig. 2a). The number of macroconidia on the mycelium increased

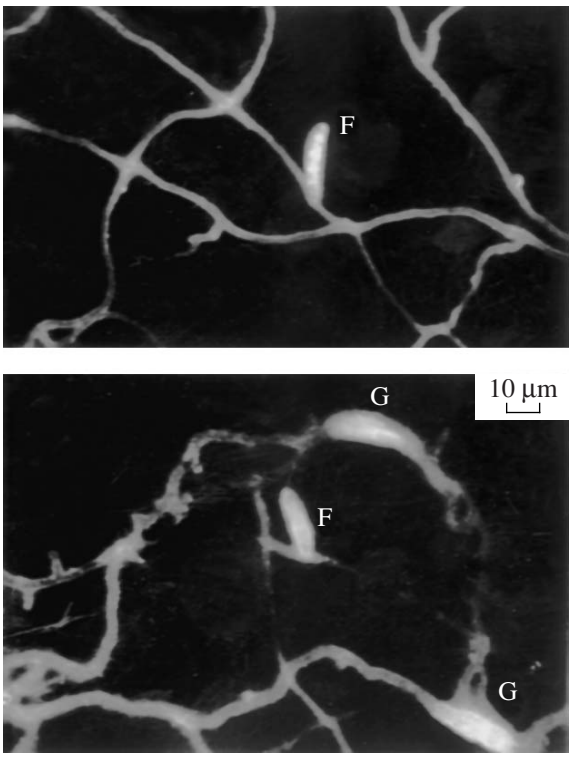

Fig. 1. Development of *F. culmorum* on a membrane filter in soil: germinated macroconidium (G), branched mycelium, newly formed macroconidium (F). The immunofluorescent method was used to identify *F. culmorum.*

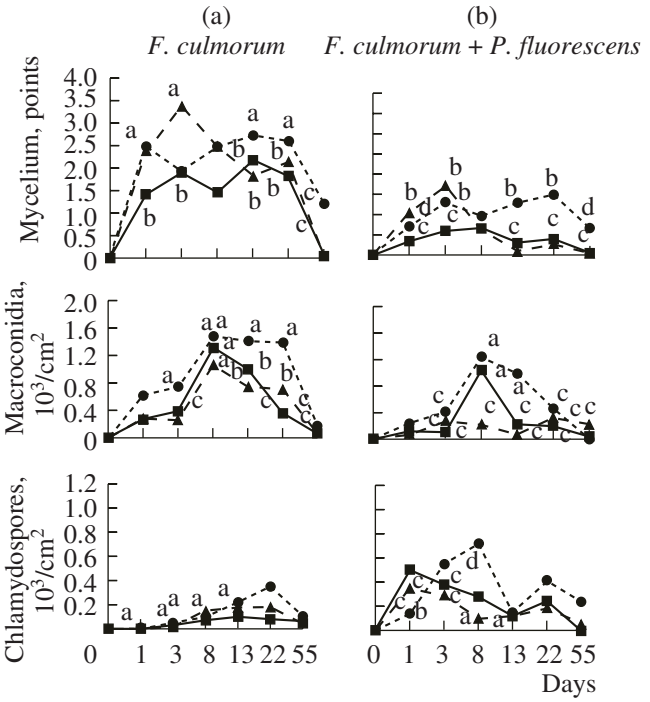

Fig. 2. Dynamics of mycelium development, formation of macroconidia and chlamydospores by *F. culmorum* (a) on a filter in soil without supplements (■), supplemented with glucose (▲), and supplemented with cellulose (●). The same in the presence of *P. fluorescens* (b). Growth of *F. culmorum* mycelium was assessed using a five-point scale (1–5). The differences between the points marked by different letters are significant ($p < 0.05$).

sharply after the third day; by the 13th day, however, its decrease had commenced, although the density of the mycelium was still stable. Few chlamydospores were formed; their number was stable throughout the experiment.

Introduction of glucose or cellulose resulted in increased *F. culmorum* density on the filters (when grown on nutrient medium, *F. culmorum* is equally capable of utilizing both carbohydrates as carbon sources). Glucose caused an intense, albeit brief, increase of mycelial density; only at one point, however, was the number of macroconidia higher than in the control. In soil with cellulose, the elevated level of both mycelium and macroconidia was maintained for a long time; a tendency was observed towards an increase in chlamydospore number.

By the end of the experiment, on the 55th day, the number of fungal structures on the filters was low in all the variants; the relatively high density of the *F. culmorum* mycelium was retained only in soil supplemented with cellulose.

In the course of incubation, membrane filters with *F. culmorum* were colonized by other fungi and bacteria. When stained, the colonizing fungi obtained black or brown coloration and thus were easily discernable from *F. culmorum*. No effect of the colonizing soil fungi on *F. culmorum* was detected; however, the effect of soil bacteria was persistent. On the fifth day, bacterial colonies were already visible in all experimental variants as light spots against a dark red background. Bacterial aggregations were often observed around *F. culmorum* lysing structures (Fig. 3); after immunofluorescent stain, these microcolonies emitted the same fluorescence as *F. culmorum* structures, probably due to the presence of fungal cell residues among bacterial cells. The amount of bacteria colonizing the filter increased with time. Bacterial density and the degree of the lysis of fungal structures were similar in all the variants.

The addition of glucose or cellulose to the soil resulted in increased CO_2 emission (Fig. 4). In soil with glucose, a significant, short-time increase of the respiration rate was observed; in soil with cellulose, the respiration rate was high throughout the experiment.

Development of *P. fluorescens* in control soil, in soil supplemented with glucose or cellulose, and in the presence of *F. culmorum*. After 12 h of incubation in soil, *P. fluorescens* was already practically absent from the inoculation site; it colonized the adjoining filter areas. Bacterial numbers in unsupplemented soil were low and did not change significantly in the course of 22 days (Fig. 5a). Introduction of cellulose had no effect on *P. fluorescens* density. In the variant with glu-

cose, bacterial numbers started to increase on the fifth day and remained stable until the 22nd day.

On the filters with both *P. fluorescens* and *F. culmorum*, the same dynamics of bacterial density was recorded as in the case of *P. fluorescens* development without the fungus (Fig. 5b). In soil with glucose in the presence of *F. culmorum*, bacterial density increased on the third day; without the fungus, an increase occurred on the fifth day. Compared to growth without the fungus, in the presence of *F. culmorum*, bacterial numbers on the 13th day were higher both in unsupplemented soil and in soil with cellulose. No significant effect of the fungus on *P. fluorescens* growth in soil was detected.

In soil with glucose, *P. fluorescens* developed with a prolonged lag phase; bacterial density started to increase on the third or even on the fifth day. On the 55th day, *P. fluorescens* density was low in all the variants, as was evident from the very few "blue spots" on the filters.

No pronounced taxis of the bacterium towards the fungus was revealed; the "blue spots" indicating the presence of *P. fluorescens* were located chaotically; however, in some cases bacteria were distributed along the fungal hyphae.

Effect of *P. fluorescens* on *F. culmorum* development. No effect of *P. fluorescens* on *F. culmorum* germination was revealed; in all the variants, macroconidia germinated by the first day almost completely. However, *P. fluorescens* inhibited the subsequent mycelial growth; this effect was most pronounced in unsupplemented soil (Fig. 2b). Even on the third day, microscopy of the filters incubated in unsupplemented soil revealed only short growth tubes of the germinating macroconidia. The dynamics of *F. culmorum* mycelial growth with and without *P. fluorescens* was similar in all the variants.

Introduction of glucose or cellulose resulted in a decrease in the inhibitory effect of *P. fluorescens* on *F. culmorum* mycelial growth; this effect, however, still persisted. During the first three days, the density of mycelium in soil with carbohydrates and bacteria was the same as in the case of *F. culmorum* in soil without supplements and bacteria (Fig. 2a). In the presence of bacteria, mycelial density rapidly decreased in soil with glucose, while in the presence of cellulose it remained high until the end of the experiment. Since cellulose was introduced in a higher amount, its persistence in soil may explain the persistent density of mycelium in the presence of cellulose. Cellulose can persist in soil for a long time because it is not available to some of the soil microorganisms.

In unsupplemented soil and in soil with cellulose, *P. fluorescens* had no strong inhibitory effect on macroconidia formation by *F. culmorum*. The most pronounced inhibition of macroconidia formation by *P. fluorescens* occurred in soil with glucose.

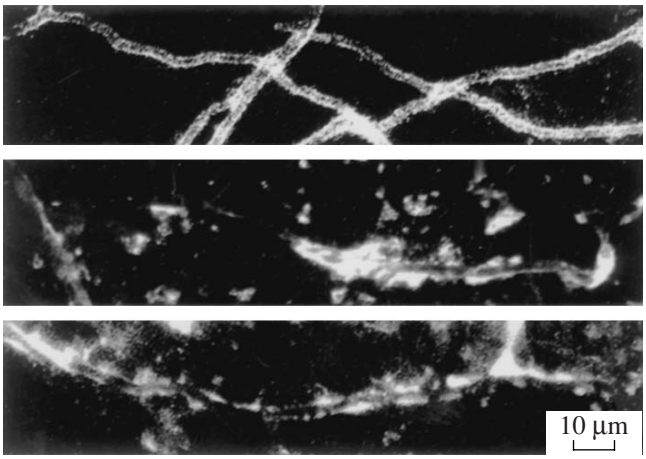

Fig. 3. Lysis of *F. culmorum* mycelium by soil bacteria. The immunofluorescent method was used to identify *F. culmorum*.

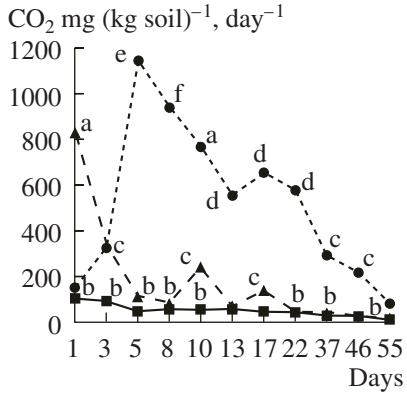

Fig. 4. Dynamics of CO_2 production in soil without supplements (■), supplemented with glucose (▲), and supplemented with cellulose (●).

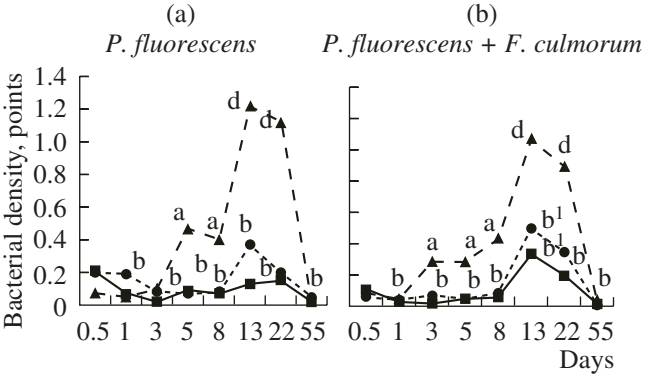

Fig. 5. Dynamics of *P. fluorescens* growth (a) on membrane filters in soil without supplements (■), supplemented with glucose (▲), and supplemented with cellulose (●). The same in the presence of *F. culmorum* (b). Growth of *P. fluorescens* was assessed using an eight-grade scale (1–8). The differences between the points marked by different letters are significant ($p < 0.05$).

In all cases, *P. fluorescens* stimulated chlamydospore formation; this effect was most pronounced in soil with cellulose and less pronounced in soil with glucose. Microscopy revealed that some of the macroconidia introduced to the filters had changed into chlamydospores. In all the variants, the stimulatory effect of *P. fluorescens* on chlamydospore formation by *F. culmorum* lasted only until the 13th day of the experiment.

Microscopy of the filters revealed that while the picture of characteristic bacterial lysis of the fungal mycelium was common for the filters containing the fungus alone, it occurred only rarely in the in the presence of *P. fluorescens*; in this case, lysis occurred only in soil with glucose or cellulose during the first three days (Fig. 3). *P. fluorescens* inhibited the development of mycelium but did not lyse it.

DISCUSSION

The application of membrane filters, to incubate fungi in soil, and of immunofluorescent identification revealed that *F. culmorum* introduced in soil can develop as a saprotroph, with the formation of mycelium, macroconidia, and a small amount of chlamydospores. In the presence of plant debris (cellulose under the conditions of our experiment), *F. culmorum* can maintain high levels of mycelium and macroconidia for a long time.

De Boer and coworkers [11] compared growth of *F. culmorum* in sterile sand and in dune soils and found that the latter suppressed mycelial growth. The authors therefore expressed doubt in the capability of potentially pathogenic *F. culmorum* to grow in the rhizosphere and to reach the roots. Our results demonstrated that *F. culmorum* can form a branched mycelial net in soil and maintains high density for a long time, although all natural soils are to some extent suppressive; *F. culmorum* develops in sterile soil more actively than in the native one [8]. In our experiment, significant amounts of *F. culmorum* mycelium and macroconidia were present for 22 days even in soil without organic supplements. Although lytic bacteria surround the mycelium from the first day and their number increases with time, the processes of lysis and biomass growth are balanced. We have previously demonstrated that *F. culmorum* on a membrane filter in barley rhizosphere can maintain a high density of mycelium and macroconidia for four months [12].

Introduction of glucose and cellulose resulted in higher densities of mycelium and macroconidia, but not of chlamydospores. This work, together with previous ones, revealed that chlamydospores are not the dominant *F. culmorum* structure in soil.

The effect of carbohydrates primarily on chlamydospore germination in various *Fusarium* species has been previously studied. Glucose was found to stimulate chlamidospore germination [13, 14], while cellulose suppressed it [15, 16]. In the experiment, stimulation of the development of the fungus by both glucose and cellulose after one day was observed. Macroconidia of *F. culmorum* are known to be subject to soil fungistasis but to overcome it quickly [8]. It was therefore interesting to determine the density of mycelium, macroconidia, and chlamydospores formed in soil and the effect of carbohydrates on these processes, rather than the germination of introduced propaguls.

P. fluorescens survived on membrane filters in soil for 55 days, although in all the variants its density decreased significantly during this time. The capability of introduced pseudomonad strains to survive in soil for prolonged periods is well known [17].

Introduction of glucose into the soil resulted in a significant increase of *P. fluorescens* on the membrane filters; high bacterial density persisted after 22 days, when the microbiological activity of soil and the *F. culmorum* density decreased significantly. Elevated numbers of *P. fluorescens* in soil with glucose were the possible reason for the decrease in *F. culmorum* mycelial density, as well as the more pronounced decrease in the numbers of macroconidia and chlamydospores, compared to unsupplemented soil and to soil with cellulose.

No visible effect of *P. fluorescens* on the germination of *F. culmorum* macroconidia was found; in all the variants, practically all macroconidia germinated by the first day. Elad and Baker [18] have demonstrated that the introduction of pseudomonads into soil suppressed significantly chlamydospore germination in *F. oxysporum, F. solani*, and *F. graminearum*. The germination of *F. culmorum* macroconidia is probably less dependent on environmental conditions.

In all cases, the effect of *P. fluorescens* was expressed as the inhibition of development of *F. culmorum* vegetating structures (mycelium, conidia) and stimulation of the formation of the resting ones (chlamydospores). In soil with cellulose, the inhibitory effect was observed even on the 55th day of the experiment, when *P. fluorescens* on the filters was insignificant; the density of the *F. culmorum* mycelium was significantly lower than in the variant when the fungus developed under the same conditions without the bacterium. The stimulation of chlamydospore formation by *P. fluorescens* did not last long in any variant. Formation of chlamydospore-like structures by *F. oxysporum* f. sp. *radicis-lycopersici* in the presence of strain *P. chlororaphis* PCL 1391 has been reported by other researchers [19].

Several mechanisms of the action of pseudomonads on *Fusarium* phytopathogenic fungi are known. Antagonism, including competition for carbon and iron, is among the direct effects [20, 21], as well as the production of antifungal compounds [22, 19]. Development of *F. culmorum* together with *P. fluorescens* on filters in soil resulted only rarely in the lysis of fungal structures. Competition for carbon was probably the mechanism of the antagonistic effect of the bacterium under exper-

imental conditions; moreover, introduction of carbohydrates partially removed the inhibitory action of *P. fluorescens*. However, since the inhibitory action of the bacterium did not disappear completely upon the addition of carbohydrates (even of cellulose, which is not utilized by *P. fluorescens*), competition for carbon was not the only mechanism of suppression of the fungal growth in this experiment. Similarly, the introduction of carbohydrates did not eliminate the factor (signal) stimulating chlamydospore production.

Thus, it was shown that *F. culmorum* can compete both for cellulose and glucose introduced into the soil. The fungal growth is limited by soil bacteria and the introduced *P. fluorescens*; however, in the presence of an available nutrient source, the phytopathogen can maintain high densities of mycelium and macroconidia, i.e., of the structures that provide for its expansion. In the case of unfavorable conditions (in our experiment, coinoculation with *P. fluorescens*), the fungus responds by production of high numbers of chlamydospores, the structures that provide for its survival under such conditions. The flexible survival strategy of the phytopathogenic fungus probably explains the possible failures in the application of biocontrol strains, especially in field experiments when a number of factors can affect every microorganism and their interrelations.

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