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Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*

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Abstract

In wet soils, low oxygen conditions often develop that favour disease development by many soil-borne plant pathogens. The introduction of a biocontrol agent, to suppress disease development, would require that the agent remains metabolically active under such conditions. Denitrifying bacteria can maintain this metabolic activity by switching to nitrate respiration. In the rhizosphere, plant roots not only supply carbon as an electron donor, but also cause a localised lowering of oxygen concentrations, conditions favourable for nitrate respiration. Two strains of *Bacillus subtilis*, showing strong inhibition of a number of pathogenic fungi on agar plates, and the capacity to grow under anoxic and anaerobic conditions when provided with nitrate, were used to study the possible involvement of nitrate respiration in fungal disease control. The effect of the addition of nitrate on the activity of these antagonistic strains was studied under anoxic conditions using the sealed plate method of Fiddaman and Rossal [Fiddaman, P.J., Rossal, S., 1995. Plant Pathol. 44, 695–703]. The assay tests the activity, measured as a reduction in fungal growth, of antifungal volatiles (AFV) produced by the bacteria. The in vitro experiments showed that antagonism by the *B. subtilis* strains towards *Fusarium oxysporum* varied under anoxic conditions, depending on the nitrate availability and agar used as a growth medium. AFV activity was increased by the presence of nitrate in the medium at concentrations of 10 mM or more. Nitrate respiration may therefore have an important role in the control of fungal root diseases by allowing denitrifying soil-borne bacteria to remain metabolically active in wet soils with low oxygen concentrations.

Keywords: Biocontrol; Nitrate reductase; Antifungal volatile; Antagonism

1. Introduction

In the rhizosphere, denitrification is stimulated by the presence of plant roots due to the enriched carbon supply in exudates and a localised lowering of the oxygen concentration (Woldnedorp, 1963). This limited oxygen causes most rhizobacteria to maintain metabolic activity by switching to nitrate (NO_3^-) as an alternative respiratory electron acceptor (Stewart, 1988; Berks et al., 1995). In soils of high matric potential (i.e. high moisture availability) and low oxygen tension, crop losses due to seedling and root diseases are most severe. Under these conditions, nitrate respiration is likely to be the major form of respiration, being carried out by the soil microbial population and of great importance for any antagonistic control of root disease. The availability of nitrate would therefore be of significance in maintaining the metabolic activity

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of a bacterial biocontrol agent in wet soils with poor oxygenation.

Bacillus subtilis is a Gram-positive, spore forming saprophyte capable of growing anaerobically when nitrate is available as a terminal electron acceptor (Priest, 1993). If *B. subtilis* were able to utilise nitrate under soil conditions where oxygen was limited, then this may indicate a niche where the role of *B. subtilis* in the soil has not previously been considered or exploited. Many strains of *B. subtilis* have shown the potential to be used as biocontrol agents against fungal pathogens (Fravel, 1988). Work presented to date suggests that the principal mechanism of this antifungal action involves the production of antibiotics (Loeffler et al., 1986; McKeen et al., 1986; Fravel, 1988), especially within soil microsites (Wright, 1956). However, it is likely that several mechanisms are acting in concert to achieve control including the production of volatiles, which have a significant effect on soil microbiology (Linderman and Gilbert, 1975). *B. subtilis* strains also produce volatiles which have been shown to antagonise a range of organisms including the soil-borne plant pathogens *Rhizoctonia solani* and *Pythium ultimum* (Wright and Tjompson, 1985; Fiddaman and Rossal, 1993).

Fiddaman and Rossal (1994) have shown, for selected strains of *B. subtilis*, that active antifungal volatiles (AFV) are produced on a range of growth media and soils and the activity of these volatiles can be enhanced by adding complex carbohydrates (e.g. D-glucose and peptones) to the media.

The aim of the present study was to assess whether nitrate respiration by soil-borne bacteria increases biological control activity under conditions of oxygen limitation. This involved in vitro interaction experiments on media containing different nitrate concentrations. The biocontrol activity of two *B. subtilis* strains against plant pathogenic fungi and the effects of the addition of nitrate to the growth medium were investigated to establish if the addition of nitrate enhanced the activity of any produced AFV.

2. Material and methods

Two strains of *B. subtilis*, MBI600 and MBI205 (Fiddaman and Rossal, 1995) were obtained from Microbio (Hemel Hempsted, UK). An isolate of *Fusarium oxysporum* was obtained from Dr. Duncan White (Department of Agriculture, Aberdeen University, UK). Isolates of *Phytophthora cryptogea*, *Phytophthora infestans*, *Phytophthora frageriae*, *Phytophthora cactorum*, *Phytophthora cambivora*, *Phytophthora sojea* and *Pythium ultimum* were obtained from Dr. David Cook (SCRI, Dundee, UK). Isolates of *Sclerotinia sclerotiorum*, *Macrophomina phascolina* and *Rhizoctonia solani* were obtained from Dr. Siripun Chidpuree (Department of Plant and Soil Science, Aberdeen, UK).

Antagonistic contact plate studies comprised a 5 mm fungal disc placed on one side of a nutrient agar or potato dextrose agar (PDA) plate and a wire loop inoculum of either the *B. subtilis* MBI600 or MBI205 streaked down the other. The plates were then incubated at room temperature, 25 or 30°C and visually inspected for the development of inhibition zones and the ability of the fungus to grow over the bacterial streak.

For all in vitro antifungal assays, methods based on the unsealed and sealed plate methods of Fiddaman and Rossal (1993) were used with the following alterations. The bacterial cells were prepared from 10 ml Luria–Bertani broth overnight cultures. The 5 mm fungal disc was removed with a number 2 borer and placed on PDA plates. Measurements of the resultant fungal colony were taken every 24 h for a 10-day duration.

The PDA and nutrient agar media were amended with different concentrations of potassium nitrate for culturing the *B. subtilis* MBI600 and MBI205 strains. Each plate set-up was repeated in triplicate. The bacterial inoculum added to each plate was 1×10^6 CFU in 1 ml. The anoxic conditions for the incubation were obtained using 3.5 l anaerobic gas jars with *Campylobacter* system gas packs (Oxoid).

Bacterial biomass was measured at the end of each trial. Two millilitres of methanol was added to each plate and a spreader used to liberate the cells from the agar surface. One millilitre of this suspension was then removed into a pre-weighed microtube and the cells pelleted at 10 000 g for 10 min. The supernatant was discarded and the pellet air-dried and weighed.

Statistical analysis of the data was carried out using an unpaired *T*-test. Probabilities of less than 5% ($p \leq 0.05$) were used to indicate significant differences between treatments. Association between biomass and

fungal diameter was tested using correlation analysis (Minitab v.12).

3. Results

The contact plate experiment to test for antagonism against the fungus by the bacteria showed that the *B. subtilis* strains antagonised *S. sclerotiorum*, *P. infestans*, *P. cactorum*, *P. cambivora*, *P. sojae*, *P. infestans*, *M. phascolina*, *R. solani* and *F. oxysporum*. The MBI205 strain showed some antagonism toward *Pythium ultimum* when exposed to it using this method, although no antagonism towards *P. ultimum* was exhibited by MBI600.

F. oxysporum was selected as the test fungus for subsequent trials due to its speed of growth over the PDA test plates and the fact that it showed strong inhibition when the bacteria were present. *B. megaterium* and *B. subtilis* 346 were also tested using the contact plate method but neither showed any antagonism toward the test fungi.

An unsealed, aerated plate study showed that, under anoxic conditions (4% O_2), there was a significant ($p \leq 0.05$) reduction in the rate of fungal growth across the PDA plates. Fungal growth was, however, not significantly affected by the addition of the MBI strains. Statistical analysis of the results also showed that the addition of nitrate to the bacterial medium caused no significant difference in the radial growth of the fungi between treatments (data not shown).

Sealed plate studies results showed that a significant reduction in fungal disc diameter was obtained under anoxic conditions only when nitrate was added to the medium, supporting *Bacillus* cultures, at concentrations of 10 and 100 mM (Fig. 1). Bacterial biomass did not correlate with fungal colony diameter at the end of each sealed plate trial (Fig. 2).

4. Discussion and conclusion

The ability of many soil-borne bacteria to control plant diseases and to carry out dissimilatory nitrate reduction is well established. Here, we have evaluated the potential of one strategy for increasing biocontrol activity (i.e. to utilise nitrate as an alternative electron acceptor in order for soil-borne biocontrol bacteria to remain active under conditions when many pathogens are virulent).

Bacillus spp. which show antagonism are ideal for biocontrol experiments as they are often soil isolates

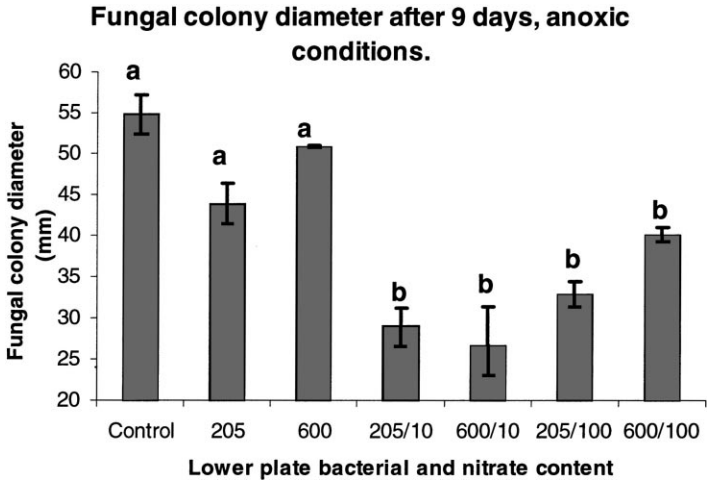

Fig. 1. Fungal growth in a sealed plate experiment carried out under anoxic conditions. The graph shows the mean diameter on day 9 of the *F. oxysporum* colony. '205' and '600' refer to the MBI *B. subtilis* strain on the lower plate and '205/10', '205/100', '600/10' and '600/100' refer to strains MBI205 and MBI600 with 10 or 100 mM nitrate additions to the medium. The fungal 'Control' is shown, as are the standard error bars. Different letters indicate significantly different means, based on an unpaired *T*-test, $p < 0.05$.

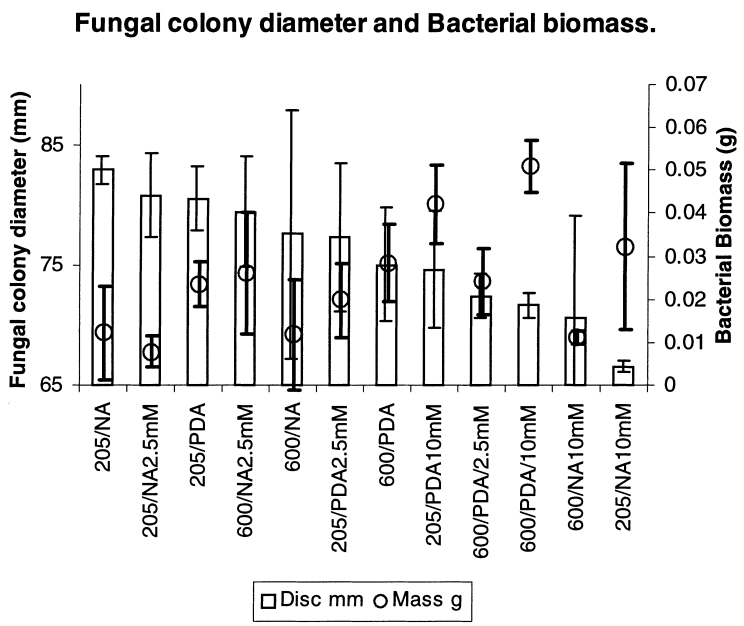

Fig. 2. Fungal diameter (bars) and bacterial biomass (circles) from an anoxic sealed plate experiment after 10 days. The bacteria, medium and nitrate concentrations shown on the *x*-axis have been ranked according to descending mean fungal diameter. Standard deviations are indicated as error bars for both biomass (light) and diameter (bold).

capable of sporulation, a characteristic which facilitates long term storage and formulation (Powell et al., 1990). Both the MBI *Bacillus* strains showed good inhibition of a number of fungal antagonists in plate studies, but in order to monitor the quantitative effects of nitrate additions under conditions bearing some similarity to the rhizosphere, the sealed plate method was used.

The results showed that under anoxic conditions, *B. subtilis* MBI600 and MBI205 exhibited increased antagonism toward *F. oxysporum* when nitrate was present in the growth medium. Whether this was due to nitrate being utilised as a terminal electron acceptor or for the production of nitrogen-based antagonistic molecules (e.g. NO) is unclear and requires further work. Addition of potassium nitrate at 100 mM did not have a more pronounced effect on reduction of the fungal colony diameter than 10 mM additions, suggesting that the nitrate concentrations could be reduced. Indeed, subsequent experiments using 1, 2.5 and 5 mM have shown no significant increase in antagonism, suggesting that 10 mM potassium nitrate may be close to optimum for AFV production by the *Bacillus*.

The final radial diameter of the fungal colony did not correlate with the bacterial biomass present at the end of the trial. Reasons for this are unclear, although Fiddaman and Rossal (1994) found that some media generate effective AFV activity from high bacterial biomass, whilst others with negligible biomass produced similar antagonism.

The results of these plate trials suggest that nitrate respiration may play an important role in the antagonistic control of root diseases under soil conditions where crop plants are most susceptible to attack. Trials such as this are designed to aid the development of biocontrol strategies for use in sustainable agriculture. Experiments are now required to investigate whether the observed role of nitrate in control of plant pathogenic fungi translates to disease control in the plant-soil system.

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