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The role of bacteria-emmited cyanogenic volatile metabolites in biocontrol

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Abstract In a study on biocontrol of tomato *Fusarium* wilt, the rhizobacterium *Pseudomonas fluorescens* T58 was selected after evaluation under greenhouse conditions. This isolate was found to produce hydrogen cyanide and the role of this volatile metabolite in biocontrol was subsequently investigated. Effect of the volatiles on microbial growth and their role in inducing pathogenesis-related proteins in plants were examined. *Fusarium* spore germination was slowed but not inhibited. *Fusarium* mycelial growth was also not inhibited but mycelia lost their characteristic pink pigmentation when exposed to the volatile metabolites. Activity of the plant defence related protein guaijakol peroxidase was increased significantly, but chitinase and ß-1,3-glucanase activities were notaffected. The populations of bacteria and other fungal organisms in a plant growth substrate were reduced upon exposure to the volatile bacterial metabolites. The study concluded that bacterial volatiles could contribute to biocontrol in various ways.

Key words: Biocontrol, cyanide, *Fusarium*, hydrogen, *Pseudomonas fluorescens*, tomato

Résumé Dans une étude de contrôle biologique de *Fusarium* wilt, le rhizobacterium *Pseudomonas fluorescens* T58 était sélectionné après évaluation dans les conditions de serre. Il a été trouvé que cet isolé peut produire du cyanure d'hydrogène et le rôle de ce métabolite volatile dans le contrôle biologique a été étudié. Les effets de volatiles sur la croissance de microbes et leur rôle dans l'induction des protéines associés aux pathogènes dans les plantes étaient étudiés. La germination des spores *Fusarium* était ralentie et pas inhibée. La croissance des micelles *Fusarium* n'était pas aussi inhibée mais ces derniers ont perdu la coloration de leur pigmentation quand ils étaient exposés aux métabolites volatiles. L'activité de protéines de défense des plantes, guaijakol peroxydase, était augmenté de manière significative, mais l'activité de chinitase et b-glucanase n'était pas affecté. Les populations de bactéries et autres fungi dans la croissance des plantes étaient réduites à l'exposition aux bactéries métabolites volatiles. En conclusion, les bactéries volatiles peuvent contribuer au contrôle biologique de plusieurs façons.

Mots clés: Contrôle biologique, *Fusarium*, hydrogène, *Pseudomonas fluorescens*, tomate

Introduction

Microorganisms produce many secondary metabolites, some of which are volatile. Production of hydrogen cyanide is predominantly associated with the genus *Pseudomonas* (Bakker and Schippers, 1987; Munif *et al.*, 2000) although not all *Pseudomonads* are cyanogenic. The occurrence of HCN-producing isolates varies significantly with habitat and with species. Approximately 50 % of potato rhizosphere *Pseudomonads* (Bakker and Schippers, 1987) and 18 % of all tomato endophytes (Munif *et al.*, 2000) were reported to produce HCN *in vitro*. Detection of HCN of microbial origin has been mainly done *in vitro*, and only rarely in soil. Failure to detect cyanide in the rhizosphere is thought to be due to assimilation and detoxification of the compound by soil microorganisms, thereby reducing its concentration and hence the chances of detection (Bakker and Schippers, 1987).

Hydrogen cyanide has been implicated in biological control of some pathogens, for example, suppression of black root rot of tobacco caused by *Thielaviopsis basicola* (Voisard *et al.*, 1989). However, in many cases a direct role of cyanide or other volatile metabolites in biocontrol has not been established. Theoretically, volatile metabolites could function by inhibiting the growth of pathogens in soil, thereby making the producer a better competitor (O'Sullivan and O'Gara, 1992) or alternatively, volatile metabolites could also act by inducing resistance (Voisard *et al.*, 1989). Cyanide-resistant respiration has been associated with increased resistance to *Meloidogyne incognita* in tomato (Zacheo and Bleve, 1987) and to *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana* (Simons *et al.*, 1999).

In this study the cyanide producing rhizobacterium *Pseudomonas fluorescens* T58 was selected for biocontrol of *Fusarium* wilt under greenhouse conditions and studied further. The objectives were to determine whether cyanogenic volatile metabolites produced by this bacteria (1) could reduce *Fusarium* spore germination and mycelial growth; (2) could induce changes in the activities of pathogenesis-related (PR) proteins in plants; (3) could alter microbial densities in soils.

Materials and methods

HCN production *in vitro*. *Pseudomonas fluorescens* T58 was cultured in 150 ml Tryptic Soy Broth (TSB) amended with 4.4 g glycine per litre (called TSG) while shaking at 100 rpm at 23 ± 2°C. To monitor production of HCN, the culture flasks were tightly sealed with parafilm and the volatile metabolites generated were delivered continuously through a 1 cm diameter plastic tube into 5 ml picric acid

solution contained in another sealed flask. The absorbance of the picric acid solution was checked at regular intervals at 480 nm (Alström and Burns, 1989).

Effect on *Fusarium* spore and mycelia growth. The bacteria were cultured in TSG as described above and allowed to grow for 24 h. In a separate flask *Fusarium* spore suspension was inoculated into potato dextrose broth (PDB). The volatiles generated by bacterial culture were delivered through a 1 cm diameter tube into the *Fusarium* culture so that the volatile metabolites produced by the bacteria would saturate the environment in which the *Fusarium* spores were germinating (Nejad and Johnson, 2000). The joined flaks were placed on a shaker at 100 rpm. Spore germination was checked under microscope at 12, 24 and 30 hr. After 5 days of growth the fungal cultures were filtered using cheesecloth and the dry weight of the mycelia determined.

The effect of volatile metabolites on *Fusarium* colony growth rate was determined on solid PDA medium. *P. fluorescens* T58 was streaked on TSG in one petriplate and allowed to grow for 24 h. In a separate plate a 5 mm diameter *Fusarium* mycelia plug was placed at the centre of PDA medium. The plate inoculated with bacteria was covered with the plate containing *Fusarium*, both plates were sealed tightly together with parafilm so that bacteria-emitted volatiles would saturate the environment in which the *Fusarium* mycelia was growing. Four replicate plates were used and mycelial radial growth was checked daily for up to seven days.

Effect on pathogenesis-related proteins. Tomato seeds cv Rheinlands Ruhm were germinated in organic substrate and transplanted to Murashige and Skoog nutrient solution after three weeks growth. Plants were placed in glass tubes of 2.5 cm diameter and 5.5 cm deep. Seven days after transferring, the plants were exposed to bacterial volatile metabolites by connecting a pipe from the flask with bacteria culture to the glass tube where plants were growing. The end of the tube was dipped in the MS nutrient solution near the plant roots. The plants being treated were kept in a climatic chamber at 23 ± 2 °C and a 12/12 h light / darkness cycle. After every 3 days, fresh 2 day-old bacterial cultures replaced the old ones. To ensure a constant level of metabolite in the root environment, the volume of MS nutrient solution was maintained at a constant 5 ml level. The effect of bacterial volatiles was compared to that of cyanide volatilised from 0.5 and 1 m*M* NaCN (Sigma-Aldrich). Control plants were grown in MS solution without any other treatment. Three plants were sampled after 1 and 7 days of exposure and tissues processed to extract and quantify the activity of the PR-proteins chitinase, ß-1,3-glucanase and peroxidase as described by Reitz *et al.* (2001).

Effect on populations of soil microorganisms. The initial population density of resident bacteria and fungi in an organic plant propagation substrate was determined. The substrate was then exposed to bacterial volatile metabolites, delivered to 10g of wetted substrate by connecting a pipe to bacteria cultures growing in broth as described above. The population density and growth rate of microorganisms in the substrates was determined after 1, 4 and 7 days of exposure.

Results

Spore germination. Without the volatiles most of the spores germinated within the first 12 h and proceeded to grow into dense mycelial mats. Exposure to cyanogenic volatiles significantly slowed down germination of conidia with almost 50% of the conidia failing to germinate in the initial 12 h (Fig. 1). Germination of spores that were exposed to cyanide volatilised from 1 mM NaCN was significantly reduced.

Mycelia growth. After 5 days growth in liquid medium the dry mycelial weight for cultures exposed to bacterial volatiles was reduced, but not significantly, when compared to cultures that had not been exposed to volatiles. There was almost no growth on cultures that were exposed to cyanide from 1 mM NaCN. On solid medium the radial growth of *Fusarium* mycelia was not reduced by volatile metabolites, but mycelia growing under exposure did not develop the pink pigment characteristic of the *Fusarium* isolate.

Activity of PR-proteins. Guaijakol peroxidase activity increased significantly in the stems of treated plants (Table 1). Chitinase activity was only nominally increased while ß-1,3-glucanase activity was not affected.

Microbial population densities in plant growth substrate. Exposing organic plant growth substrate to bacterial volatiles for 4 days caused a significant reduction in bacterial densities, but fungal densities were not significantly reduced after exposing substrate to volatiles for 1, 4 or 7 days (Fig. 2A, B).

Discussion

Results indicated that antibiosis was not a mechanism of biocontrol of the bacterial volatiles since they failed to inhibit *Fusarium* growth. However, the lack of

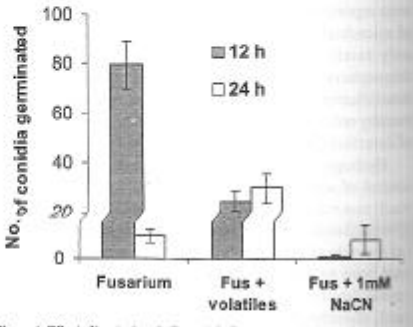

Figure 1. Effect of bacteria volatile metabolites on germination of *Fusarium* spores after 12 and 24h. Bars indicate standard deviation.

Table 1. Activities of guaijakol peroxidase, chitinase and ß-1,3-glucanase in stems seven days after exposing tomato plants continuously to volatile compounds from *Pseudomonas fluorescens* T58, 0.5 and 1 mM NaCN.

Treatment	Peroxidase (x10³)	Chitinase	ß-1,3-glucanase
Control	22.07[a] a	815 b	674.44 b
P.fluorescens T58	69.50 c	1065 b	642.22 b
0.5 mM NaCN1	46.38 b	1134 bc	646.33 b
mM NaCN	81.30 c	533 a	577.40 a

[a]Means are units min^{-1} g^{-1} fresh weight.
Means followed by different letters along the columns are significantly different according to Fisher's least significant difference test $P \leq 0.05$.

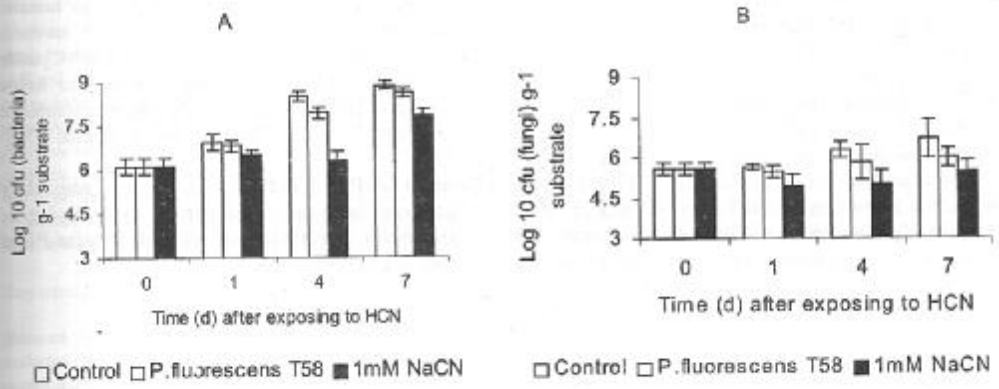

Figure 2. Effect of volatile compounds from *Pseudomonas fluorescens* T58 and 1 mM NaCN on bacterial densities (A), fungal densities (B) in greenhouse plant growth substrate. Control was untreated substrate, (n = 4). Bars indicate standard deviation.

pigmentation on mycelia when exposed to the volatiles could be an indicator of an effect on a biochemical process in the fungus. With the higher concentrations of cyanide from 1mM NaCN growth of spores and mycelia was almost totally inhibited. This could indicate that cyanide content in the bacterial volatiles in little amount and insufficient to reduce *Fusarium* growth. Alternatively, it could be that *Fusarium* has a way to overcome the effect of cyanide when at low concentration. Some fungi including some *Fusarium* can detoxify cyanide by producing the enzyme cyanide hydratase (Knowles, 1988).

The increase in peroxidase activity on treated plants could have implications for biocontrol because this enzyme is involved in synthesis of lignin and suberin, both of which are defense barriers often found in resistant cultivars (Bradley *et al.*, 1992). Peroxidases are also involved in regulation of phenol oxidation and in generation of free radical intermediates that are toxic to plant pathogens. The increase in peroxidase activity after exposure to bacterial volatiles was in the same magnitude to the increase triggered by exposing plants to cyanide volatilised from 1 mM NaCN, which confirms a direct role for cyanide in inducing this reaction.

Antoun *et al.* (1998) suggested the possibility that biocontrol agents cold affect the balance of microorganisms in the rhizosphere or on the phylloplane. In this study fungal populations were reduced more than bacteria when substrates were exposed to volatiles. Such a change in microbial populations could be enhanced and exploited for biocontrol if it favours increase of organisms that compete well against plant pathogens.

The results of this study showed that bacterial-emitted volatile metabolites have potential to contribute to biocontrol in various ways. However, under natural conditions production of volatiles by bacteria may not be sufficient to have an impact. It has been suggested that production of cyanogenic volatiles could be promoted by applying amendments to soil such as glycine rich substrates that favor synthesis of volatile cyanogenic metabolites by bacteria (Alström and Burns, 1989). More studies should be carried out to find other suitable amendments. Studies should also be done on volatiles from non-cyanogenic bacteria, which may also have antimicrobial potential, but have so far not been studied well.

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