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In vivo control of *Macrophomina phaseolina* by a chitinase and β-1,3-glucanase-producing pseudomonad NDN_1

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Abstract
The ability of rhizosphere-associated fluorescent pseudomonads to inhibit the growth of plant pathogenic fungi has generated interest in using them as an alternative to agrochemicals. In the present study, fluorescent pseudomonads were isolated from tomato rhizosphere and characterized. Isolates were screened for the ability to produce different antifungal compounds and plant-growth promoting factors. All the isolates were tested under *in vitro* conditions for the ability to inhibit *Macrophomina phaseolina* that causes charcoal rot in a number of plants. Isolate NDN_1 showed the maximum antagonistic activity against *M. phaseolina*, as well as maximum glucanase and chitinase activities. In pot studies, *Pseudomonas* NDN_1 not only controlled *M. phaseolina* but also enhanced seed germination and growth of the mustard test crop. The ability of this microorganism to form a symbiotic association with the roots of higher plants providing growth promoting capability as well as protection against soil-borne pathogenic fungi is briefly discussed.

Keywords: Fluorescent *Pseudomonas*, *Macrophomina phaseolina*, chitinase, β-1,3-glucanase, biocontrol

1. Introduction

In recent years, considerable attention has been paid to plant-growth promoting rhizobacteria (PGPR) to replace agrochemicals for the biocontrol of soil and seed borne plant pathogens (Gupta et al., 2001; Haas and Défago, 2005). Most of the bacteria exhibiting plant-growth promoting (PGP) activity belong to the Gram-negative group, including fluorescent pseudomonads (Duffy and Défago, 1999). The ability of rhizosphere-associated fluorescent pseudomonads to inhibit the growth of plant pathogenic fungi has generated interest in using them as crop protectants. Because of their catabolic versatility, their excellent root-colonizing abilities and their capacity to produce a wide range of antifungal metabolites, the soil-borne fluorescent pseudomonads have received particular attention (Compant et al., 2005). The fact that chitin and glucans are the main structural components of fungal cell walls (Bartnicki-Garcia, 1968) suggested that the hydrolases produced by many biocontrol pseudomonads were involved in antagonistic activity (Ajit et al., 2006).

However, their effect on growth of the pathogen and the resulting contribution of pathogen inhibition has received little attention. Chitinases which hydrolyze chitin (β-1,4-linked polymer of N-acetyl D-glucosamine), occur in a wide range of organisms. In plants, which lack chitin, the enzymes are thought to be a defense system against fungal pathogens as they can degrade isolated cell walls of the latter (Taira et al., 2002). Chitinases in fungi are thought to have autolytic, nutritional and morphogenetic roles. In bacteria, chitinases play a role in nutrition and parasitism (Dahiya et al., 2006). Several rhizobacterial antagonists belonging to the fluorescent pseudomonads produce chitinases which degrades the chitin present in fungal cell walls (Ajit et al., 2006). There are also reports of production of β-1,3-glucanase (β-1,3-glucan-hydrolase or laminarinase) by fluorescent pseudomonads (Nielson et al., 1998).

Macrophomina phaseolina is one of the most destructive fungal pathogens causing charcoal rot, dry root rot, wilt, leaf blight, stem blight and damping off disease in a wide range of plants (Singh et al., 1990). *M. phaseolina* has a very wide host range and therefore, it is not easy to assess host resistance/tolerance. Chemical management is

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often uneconomical and not feasible, because the pathogen is both seed and soil borne (Singh et al., 1990). Biocontrol thus offers a very good alternative for management of this pathogen. The present study was aimed at assessing the antagonistic potential of fluorescent pseudomonads against *M. phaseolina in vitro* as well as *in vivo* and determining the possible control mechanism.

2. Materials and Methods

Bacterial and fungal cultures

Fluorescent *Pseudomonas* strains were isolated from the rhizosphere region of tomato plants growing in the fields in Dehradun district of the state of Uttaranchal in Northern India. The region comes under the sub-Himalayan belt. The isolates were screened for production of fluorescence on King's B media (King et al., 1954) and pseudomonas fluorescein agar (HiMedia, Mumbai) (selective media for fluorescent *Pseudomonas*). Twelve such isolates were further characterized according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The isolates were grown and maintained on King's B medium at 28°C and 4°C, respectively. The plant pathogenic fungus, *M. phaseolina* was obtained from culture collection of Department of Microbiology, Institute of Biosciences & Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur, India. The fungus was grown and maintained on potato dextrose agar medium (Dawson et al., 1990) at 28°C and 4°C, respectively.

Production of antagonistic and growth promotory secondary metabolites

Siderophore production by the isolates was qualitatively detected by the universal chrome-azurol S (CAS) assay according to Schwyn and Neilands (1987). Production of hydrocyanic acid (HCN) was determined on tryptic soya agar as described by Kremer and Souissi (2001). Indole acetic acid (IAA) production was determined according to the method of Gupta et al. (1999). Ability to solubilise phosphate was detected according to Pandey et al. (2002).

Assay for antifungal extracellular enzymes

The bacterial isolates were grown in 100 ml of liquid LB medium (Braun et al., 1998) at 28°C for 1–6 days. The broth was centrifuged at 18,785 ×g for 20 minutes at 4°C, and the supernatant was treated with ammonium sulphate up to saturation. After standing overnight at 4°C, the precipitate was dissolved in deionised water and then dialyzed against phosphate buffer (pH 6.0) using Visking dialysis tubing (Medicell International, London, UK) (Lim and Kim, 1990). The dialyzed solutions were used as a source of enzyme. The chitinase activity was determined by measuring the release of N-acetyl-D-glucosamine (NAGA) from colloidal chitin, as per the method described by Miller (1959). Colloidal chitin was prepared from crab shell chitin (Sigma-Aldrich) by the method of Berger and Reynolds (1958). The assay mixture containing 0.5 ml of enzyme sample and 0.25 ml of 0.2% colloidal chitin in 1 M sodium acetate buffer (pH 5.5) was incubated at 37°C for 1 h. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of NAGA/min. Enzyme β-1,3-glucanase was assayed by the laminarin-dinitrosalicylic acid method (Miller, 1959). β-1,3-glucanase activity was determined with laminarin (Sigma-Aldrich) as a substrate and quantification of the reduced sugar colorimetrically. One unit of β-1,3-glucanase was defined as the amount of the enzyme releasing 1 μmol of glucose/min under these conditions (Miller, 1959).

In vitro *tests of fungal antagonism*

The antifungal potential of the isolates was checked using a dual culture method (Arora et al., 2001). Antibiosis of test strain against the fungal pathogen was recorded for up to 6 days of incubation at 28°C by measuring the diameter of the inhibition zone. Percentage inhibition was determined according to Arora et al. (2001). Five replicates of each set were used. The mycelia of *M. phaseolina* were taken after 6 days of incubation from the zone of inhibition and observed by electron microscopy (Gupta et al., 2001).

Screening for antifungal activity and chemical nature of antifungal compound(s) in culture supernatant

Culture supernatants of two isolates, NDN_1 and NDN_5, were tested for fungal antagonism towards *M. phaseolina*. Culture supernatant of the fluorescent *Pseudomonas* isolates were obtained by growing them on tryptic soya broth (100 ml) separately for 48 h at 28°C, and followed by centrifugation at 18,785 ×g for 20 min at 4°C. Cells were separated and culture supernatant was filtered using a 0.45 μm membrane filter. To 100 ml of supernatant, four fungal discs (6 mm) (cut from 5 days old culture of *M. phaseolina* grown on PDA) were mixed separately. Tryptic soya broth inoculated with fungal discs but without bacterial supernatant acted as control.

In the second set 100 ml of culture supernatants were incubated with *Bacillus subtilis* protease (Sigma-Aldrich, St. Louis, Missouri, USA) at 37°C for 24 h.

In a third experiment the heat treatment to supernatants was given at 100°C for 1 h in water bath. 6 mm discs from agar culture of *M. phaseolina* on PDA were put in protease treated and heat treated bacterial supernatants of both the isolates separately and incubated at 28°C for 5 days so as to determine the inhibition ratio (Lee and Kim, 2000). In total five replicates were taken for each set.

Table 1. Antagonistic and growth promontory characters of *Pseudomonas* isolates (NDN).

Characteristic activity	*Pseudomonas* isolates (NDN)											
	1	2	3	4	5	6	7	8	9	10	11	12
Siderophore	–	+	+	+	+	+	–	+	–	+	+	–
HCN	–	–	+	–	–	–	+	–	+	–	–	+
Chitinase	+	+	–	+	–	–	+	–	–	+	+	–
β-1,3-glucanase	+	–	–	+	–	–	+	–	–	–	+	–
IAA	+	+	+	–	+	+	–	+	+	+	–	+
Phosphate solubilisation	–	–	–	–	–	+	–	+	–	–	–	–

(–) = Negative result; (+) = Positive result.

Table 2. Quantitative chitinase and β-1,3-glucanase activities (mU/ml) by the pseudomonad isolates up to 6 days at 28°C.

Isolate	Time (h)											
	24h		48h		72h		96h		120h		144h	
	C	G	C	G	C	G	C	G	C	G	C	G
NDN_1	04±0.03	19±0.04	11±0.02	34±0.04	24±0.03	55±0.04	29±0.02	70±0.04	32±0.02	74±0.05	30±0.02	73±0.04
NDN_2	02±0.02	–	06±0.04	–	11±0.02	–	19±0.04	–	19±0.04	–	13±0.02	–
NDN_4	05±0.03	11±0.02	10±0.03	20±0.03	16±0.04	34±0.05	20±0.03	50±0.02	22±0.02	57±0.02	18±0.04	58±0.03
NDN_7	03±0.04	20±0.03	08±0.02	37±0.04	17±0.03	56±0.03	23±0.05	73±0.05	24±0.02	73±0.04	21±0.03	68±0.02
NDN_{10}	04±0.02	–	08±0.05	–	16±0.05	–	22±0.03	–	24±0.03	–	23±0.05	–
NDN_{11}	04±0.03	10±0.05	07±0.03	17±0.02	12±0.04	27±0.02	16±0.02	40±0.02	17±0.05	38±0.04	17±0.03	32±0.04

Results are mean of five replicates ± SD. C = Chitinase; G = β-1,3-glucanase.

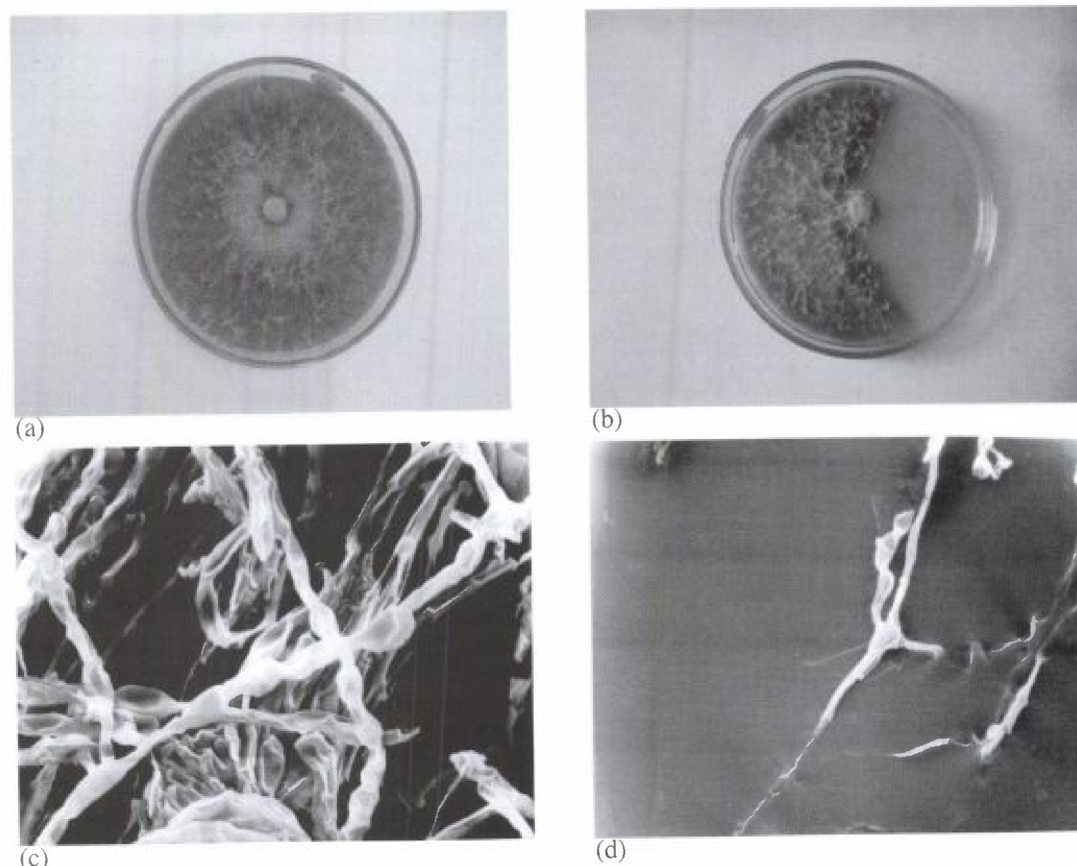

Figure 1. *In vitro* inhibition of *M. phaseolina* by *Pseudomonas* NDN_1. (a) Control (*M. phaseolina*); (b) Inhibition in presence of NDN_1; (c) Electron micrograph showing swelling of fungal hyphae; (d) Electron micrograph showing swelling and shriveling in hyphae.

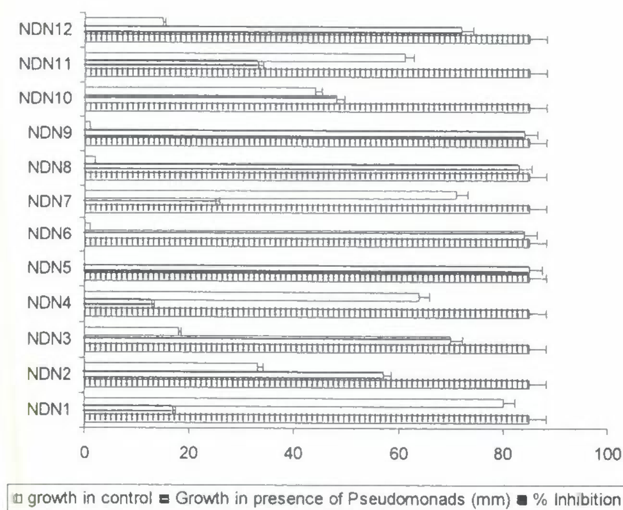

Figure 2. Inhibition of *M. phaseolina* by different isolates of fluorescent *Pseudomonas in vitro* conditions.

Table 3. Effect of seed bacterisation on percentage germination and seedling biomass in *M. phaseolina* infested soil.

Treatment	Seed germination (%)	Seedling biomass (g/plant) after 60 days
Control	74±0.03 b	12.10±0.02 b
Soil + MP	59±0.02 a	6.67±0.05 a
Soil + NDN_1	85±0.03 d	15.00±0.03 c
Soil + NDN_5	76±0.04 b	14.22±0.04 c
Soil + MP + NDN_1	80±0.02 c	11.54±0.03 b
Soil + MP + NDN_5	61±0.05 a	6.90±0.02 a

Control = soil + non-bacterised seeds; MP = *M. phaseolina*. Results are mean of five replicates ± SD. Means in the column followed by same letter are not significantly different (Duncan's multiple range test).

In vivo studies

Isolates *Pseudomonas* NDN_1 and NDN_5 were grown in King's B broth (500 ml) for 24 h at 28°C to get log phase culture (cfu 10^8 cells/ml). This culture was used for coating of the seeds of test crop, *Brassica campestris* (mustard) separately accordingly to Somasegran and Hoben (1994). For the control set, seeds were not coated and sown just as they were procured.

Earthen pots (24 × 12 × 12 cm) were filled with garden soil (loamy, pH 7.2). Fungal inoculum for the study was raised on oat grains according to Arora et al. (2001). The grain-based culture of *M. phaseolina* was mixed in soil so as to make the inoculum level of 10^5 cfu g^{-1} (Arora et al., 2001). Approximately 50 seeds were sown per pot. The experiment was designed with following treatments: (i) soil + non-bacterised seeds (Control); (ii) soil + *M. phaseolina* (and non-bacterised seeds); (iii) soil + bacterised seeds; (iv) soil + bacterised seeds + *M. phaseolina*. Five replicates of each set were taken. The plants were irrigated with tap water as and when required. Plants were harvested after 60 days to measure percentage seed germination, disease incidence and seedling biomass.

3. Results

Bacterial cultures, and production of antagonistic and growth-promoting compounds

All the twelve isolates were Gram-negative, non-spore forming rods, showing growth at 4°C and produced fluorescent and diffusible pigment on pseudomonas fluorescein agar. All the biochemical and physiological characters, production of water soluble yellow-green pigment confirmed that the twelve isolates belonged to the genus *Pseudomonas* (Holt et al., 1994). Out of the twelve isolates, eight produced siderophore as confirmed by the production of orange halo around there colonies on CAS agar. Only four of the isolates produced hydrogen cyanide and nine produced IAA. Only two of the isolates were capable of solubilizing phosphate (Table 1).

Enzyme assays

Six of the isolates were positive for chitinase and four for β-1,3-glucanase activity. Quantitatively, amongst all the isolates *Pseudomonas* NDN_1 showed maximum chitinase and β-1,3-glucanase activity. Both the enzyme activities increased with time and reached maximum after 5 days of incubation in King's B medium (Table 2).

In vitro *antagonism and chemical nature of antifungal compound*

Isolate NDN_1 showed the maximum antagonism against *M. phaseolina* as revealed by the presence of a clear zone of inhibition around the bacterial colony relative to control (Fig. 1a and b). Isolates NDN_5, NDN_6, NDN_8 and NDN_9 showed no inhibition (Fig. 2). An increase in incubation time (in dual culture of NDN and *Macrophomina*) corresponded with an increase in the inhibition zones for up to 5 days in case of NDN_1, NDN_2, NDN_4, NDN_7, NDN_{10}, NDN_{11} (data not provided). On further incubation, the fungal mycelia growing towards the interacting zone stopped and appeared to degrade. The isolate NDN_1 showed 80% and NDN_7 71% inhibition of fungal growth as compared to control. Scanning electron micrographs of the fungal hyphae, from the zone of inhibition, showed clear deformities. The hyphae became completely distorted, shriveling, swelling, and fragmentation were observable (Fig. 1c and d).

Cell free culture supernatant of isolate NDN_1 completely inhibited *M. phaseolina*. However, isolate NDN_5 did not show any inhibition as there was ample of fungal growth after 5 days of incubation, as in the control (without supernatant). Protease treated and heat treated supernatants of both the isolates NDN_1 and NDN_5 did not inhibit growth of pathogen.

In vivo *studies*

Plants growing in control soil did not show any sign of infection. The germination percentage in mustard was enhanced when the seeds were coated with NDN_1, even in the presence of *M. phaseolina* as compared to non-bacterised and NDN_5 coated seeds. After 60 days, plants raised from NDN_1 bacterised seeds showed no signs of disease in *M. phaseolina* infested soil. On the other hand, plants from non-bacterised seeds and those bacterised with isolate NDN_5 developed clear symptoms of charcoal rot, in presence of *M. phaseolina*. Seed bacterisation with *Pseudomonas* NDN_1 enhanced seedling biomass by 24% and 73% over uninfested and infested controls, respectively. Isolate NDN_5 was also able to enhance the seedling biomass by 17% in comparison to uninfested control. However, in presence of *M. phaseolina* there was no significant increment (Table 3).

4. Discussion

The fluorescent pseudomonad NDN_1 showed highest chitinase and β-1,3-glucanase activity as well as maximum inhibition of *M. phaseolina* in comparison to other isolates. The *in vivo* results were also in agreement to *in vitro* study clearly indicating the role of lytic enzymes in biocontrol of *M. phaseolina*. A variety of microorganisms exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases (Ryu et al., 2000; Nautiyal et al., 2002; Compant et al., 2005; Gupta et al., 2006). The cell wall of *M. phaseolina* is rich in chitins and glucans (Saikia et al., 2005), suggesting that the production of chitinases and glucanase by a co-cultured organism might be sufficient to inhibit the ability of this fungus to grow normally. Srivastava et al. (2001) reported control of charcoal rot of *Cicer arieitinum* by a chitinase producing *P. fluorescens*.

The study demonstrated that fluorescent pseudomonad strains have a characteristic mechanism of fungal antagonism. The protein free culture filtrate (protease treated) and heat treated culture filtrate of *Pseudomonas* sp. NDN_1 showed insignificant inhibition in comparison to cell free culture supernatant indicating that the inhibition of *M. phaseolina* was mainly due to hydrolytic enzymes. *Pseudomonas* NDN_1 showed maximum antifungal activity and enzyme activities simultaneously, again showing that the inhibition of *M. phaseolina* is related to enzyme activity. The role of chitinase and β-1,3-glucanase in inhibition of *Phytophthora capsici* and *Rhizoctonia solani* was confirmed by Arora et al. (2007). Although earlier reports indicated the ability of fluorescent pseudomonads to inhibit *M. phaseolina* (Shanmugam et al., 2001), a clear mechanism was not presented. The reduced antifungal activity of NDN_2 and NDN_{10} could be due to the absence of β-1,3-glucanase activity. This proposition is supported by studies revealing that some plant chitinases, with antifungal activity against filamentous fungi, can only inhibit the growth of some phytopathogenic fungi when combined with β-1,3-glucanase (Mauch et al., 1988). These results provide evidence that extracellular enzymes provide the mechanism for antagonism as only fluorescent *Pseudomonas* producing chitinase and β-1,3-glucanase caused significant inhibition of the pathogenic fungus *M. phaseolina*.

It was clear from *in vivo* (pot) studies that the *Pseudomonas* isolate NDN_1 not only inhibited the pathogen but also enhanced the growth of test crop. There are reports that support the role of pseudomonads in biocontrol (Validov et al., 2005) and plant growth promotion (Pandey et al., 2005). Thus the isolated strain NDN_1 has both biocontrol and plant growth promoting potential and may prove to be an alternative to the use of agrochemicals (fertilizers and pesticides) for a range of crops because this bacterium was isolated from the tomato rhizosphere but exhibited growth-promoting and fungal pathogen-control ability in mustard. *Pseudomonas* NDN_5 also showed growth enhancement of mustard in the absence of pathogen. Characteristic that mainly contribute to the ability of fluorescent *Pseudomonas* NDN_1 and NDN_5 to enhance plant growth may be the capacity to secrete IAA, resulting in rapid establishment of roots advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment (Patten and Glick, 2002).

Mustard is an important oil-seed crop and susceptible to the infection of *M. phaseolina*. This fungus is one of the most serious fungal pathogens causing significant economic losses (Dhingra and Sinclair, 1978; Pratt et al., 1998), particularly under dry, warm conditions in India. The pathogen is becoming resistant to more and more pesticides. Biological control by fluorescent pseudomonads may be the only alternative to hazardous chemicals which disturb the ecosystem and are toxic to soil biota as well as humans (De Weger et al., 1995). The present study clearly establishes the role of extracellular enzymes in the antagonism of fluorescent pseudomonads and also suggests that there is a symbiosis between this microorganism and the host plants. This can lead to enhanced seed germination and growth in the host which in turn supports a high population of the bacteria in the rhizosphere through exudates which are a cost to the plant. Clearly more research is needed to establish the closeness and nature of

this symbiosis and the range of hosts that can support the bacteria. Only when the symbiosis is well understood are we likely to be able to exploit it to provide optimum growth enhancement of the host and control of fungal pathogens.

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