Egyptian British Biological Society (EBB Soc)

Induction of resistance to tobacco necrosis virus in bean plants by certain microbial isolates

Sahar A. Shoman[1*], Nagwa A. Abd-Allah[1] and Ashraf F. El-Baz[2]

1. Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt
2. Department of Industrial Microbiology, Genetic Engineering and Biotechnology Research Institute, Sadat City, Univ. of Monofyia.

ABSTRACT

Four rhizosphere microorganisms (*Bacillus globisporus, Candida glabrata, Pseudomonas fluorescens,* and *Streptomyces gibsonii*) were isolated, identified, and tested for their capacity to protect *Phaseolus vulgaris* plants from tobacco necrosis virus (TNV) symptoms. Foliage treatment by the culture filtrate of each one of the four microbial isolates highly reduced the number of local lesions, while soil treatment by the same four microbial isolates showed lesser effects. Concerning foliage treatment, both *Pseudomonas fluorescens* and *Streptomyces gibsonii* culture filtrates showed the highest inhibitory effect to TNV infection (91.5 % and 97.2%, respectively). A number of *de-novo* synthesized proteins (induced proteins) was detected by Polyacrylamide-gel electrophoretic analysis in the culture-filtrate-treated bean leaves relative to the (water-treated) control. The determination of β-1,3-glucanase and peroxidase enzymatic activities in the extracted proteins showed high β-1,3-glucanase and peroxidase activities in the leaves treated with *Pseudomonas fluorescens* and *Bacillus globisporus* culture filtrates respectively, in contrast to the *Candida glabrata* culture-filtrate-treated leaves. However, *Streptomyces gibsonii* culture-filtrate-treated leaves showed the same enzymatic activities as that of the control. These results show that filtrates of the four rhizosphere microbial isolates reduced TNV incidence in bean plants through a process termed induced resistance (IR). However each microbial isolate could induce resistance by a distinct mechanism that was different from classic induced resistance against viral infection.

KEYWORDS: Induced resistance, Viral infection, TNV, Rhizosphere microbial isolates.

INTRODUCTION

Alternative strategies have been developed and implemented in recent years to reduce damage of crops by viral diseases. The developments of genetic engineering techniques have enabled the production of transgenic plants (Huang *et al*. 2002). Pathogen-mediated protection was derived from the concept of cross-protection (Gibbs & Harrison 1976), where plants infected with one viral strain become protected from subsequent infection by another related strain. The state of enhanced defensive capacity developed by a plant when appropriately stimulated is known as systemic acquired resistance (SAR) or induced resistance (IR) (Ryals *et al*. 1996). The involvement of IR in viral disease suppression has been studied for a wide range of biological control microorganisms (Weller *et al*. 2002) as well as chemicals and various plant-derived materials (Doubrava *et al*. 1988; Kessmann *et al*. 1994).

Maurhofer *et al*. (1994) evaluated the root-colonizing bacterium *Pseudomonas fluorescens* as an inducing agent against the lesion-inducing tobacco necrosis virus (TNV) in tobacco. They also observed a reduction in the number of TNV–induced lesions in *P. fluorescens*-treated plants. Raupach *et al*. (1996) were the first to show that treatment of cucumber or tomato plants with plant-growth-promoting rhizobacteria (PGPR) resulted in induced systemic resistance against systemic infection by cucumber mosaic virus (CMV).

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* Address for Correspondence

Zehnder *et al*. (1999) identified PGPR strains that protected tomato against systemic infection by CMV under greenhouse and field conditions.

The notion that the enhanced resistance apparent upon challenge inoculation depends on the same defensive mechanism as expressed after primary infection led to the identification of common metabolic alterations induced systemically in response to infection. When the microorganisms are applied to either host or non-host plants, they will induce symptoms of resistance reactions. Many of these reactions rely on rapid transcriptional activation of specific genes classified as plant defense-related genes, encoding what are called pathogenesis-related proteins (PR-Ps) (Van Loon *et al*. 1994). Typical examples include the genes encoding lytic enzymes such as chitinase and glucanases (Zhu *et al*. 1994), cell wall components like hydroxyproline rich glycoprotein and peroxidase (Mehdy 1994)

The present study was conducted to evaluate the potential of certain rhizosphere microbial isolates in inducing resistance and biocontrol of tobacco necrosis virus infection in beans. It also assesses the contribution of the inducible pathogenesis-related proteins in the observed resistance against TNV infection.

MATERIALS AND METHODS

Rhizosphere microorganisms were isolated from rhizosphere soil of *Phaseolus vulgaris* using the soil dilution plate method (Johnson & Curle 1972) on: 1/5 M_{32} medium for isolation of bacteria; King's medium B and 1/10 tryptic-soy agar (TSA) containing ampicillin, sodium salt (50 mgml^{-1}), cyclohexamide (75 mgml^{-1}) and chloramphenicol (12.5 mgml^{-1}) (TSA+ACC) for isolation of fluorescent pseudomonads; starch nitrate agar (SNA) (Kuster 1959), starch casein agar (SCA) and glucose yeast malt (GYM) for isolation of actinomycetes; and nutrient agar (NA) and peptone yeast malt (PYM) for isolation of yeast fungi.

The selected bacterial and fluorescent pseudomonad isolates were identified with Williams *et al*. (1989)**.** Identification techniques were carried out according to the recommended methods in Cappuccino & Sherman (1992). Morphological characteristics of individual colonies were recorded, and Gram staining, motility and some physiological and biochemical tests were carried out. The actinomycete isolate was identified and grouped to the genus level on the basis of standard morphological criteria and according to the presence/absence of the aerial mycelium, the distribution (aerial/substrate), the form of the spore and the fragmentation stability of the substrate mycelium (Cross 1989). Culture characteristics were carried out as described in Williams *et al*. (1989). The selected yeast fungi isolate was identified to species level (Barnett *et al*. 1990) using morphological criteria on corn-meal tween-80 agar medium, growth at different temperatures, cyclohexamide sensitivity test, starch-urease-gelatin hydrolysis tests and biochemical tests.

Microorganism isolates were inoculated in 250-ml conical flasks containing 50 ml of M_{32} medium for bacteria, TS medium for fluorescent bacteria, SN medium for actinomycetes and PYM for yeast fungi. The flasks were incubated for 3, 7 and 4 days respectively at 30-37°C for bacteria and 28°C for actinomycetes and yeast fungi. Then the culture filtrates were collected by centrifugation for 5 min at 10,000g to separate the microbial growth. The clear filtrate of each isolate was then collected and diluted twice (two-fold dilution) for spraying onto plant leaves.

French bean seeds (*Phaseolus vulgaris* cv. contender) were cultivated in 20-cm plastic pots and grown in a greenhouse under standard conditions for 12-14 days. The primary full-expanded leaves were obtained and plants were selected to be as uniform in size as possible.

Tobacco necrosis virus strain D (TNV-D) was supplied from the Virology Unit, Microbiology Dept. Faculty of Science, Ain Shams Univ. The viral inoculum was prepared from stored, frozen bean-leaf tissue, previously infected with TNV. Ten grams of leaf material was ground with 20 ml of distilled water in a mortar. The extract was strained through cheesecloth. The filtrate was diluted to give a suitable number of discrete local lesions on test plants.

For testing the ability of the four rhizosphere microbial isolates on reducing TNV symptoms on the host plant, two treatments were carried out.

(a) In the soil treatment, each of the four microorganisms was scraped from the plates with 20 ml of sterile distilled water; suspensions were centrifuged and the pellet resuspended in 20 ml of sterile distilled water for immediate soil inoculation. The treated soil was packaged in different pots (4 pots for each microbial treatment). Seeds of *Phaseolus vulgaris* were planted in each of them under greenhouse conditions with 32/25 ^{o}C day/night temperature. Two weeks later, when the primary leaves had developed, these were lightly dusted with carborundum and rub-inoculated with TNV inoculum. The local lesions were counted after 4 to 5 days on both treated and control plants (TNV- inoculated plants without soil treatment).

(b) In the foliage or exogenous treatment, about 100 ml of each microbial culture filtrate was collected and sprayed on the upper surface of the *Phaseolus vulgaris* primary leaves for two successive days (4 pots for each treatment). The leaves of both treated and control (plants sprayed with distilled water) were rinsed with tap water and mechanically inoculated with TNV suspension. After 4 or 5 days, the total number of discrete local lesions on the plants was counted. The viral inhibitory activity of the four microbial isolates was estimated using the following equation: Inhibition % was calculated as the difference in number of local lesions produced by treatment and control multiplied by 100 and divided by number of local lesions produced by the control.

Soluble proteins were extracted from both treated and control bean leaves by grinding the leaf tissues in a warning blender with cold phosphate-citrate buffer at pH 2.8 (30mM Na_2Hpo_4, 80mM Citrate, 5mM Ascorbate, 14mM mercaptoethanol), using 1 ml of buffer per gm of tissue. The homogenate was filtered through cheesecloth and centrifuged for 30 min at 20,000 g. The supernatant was dialyzed against Tris HCl buffer at pH 8.0 (50mM tris, 1mM EDTA, and 3mM mercaptoethanol). The protein content of the crude extract was analyzed by subjecting aliquots to polyacrylamide gel electrophoresis.

The extractable proteins were subjected to 15% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions according to Laemmli (1970). The relative amount of bean pathogenesis-related proteins was estimated by measuring the area under peaks obtained from the densiometric scans of PAGE. For molecular weight determination, polyacrylamide gel was run in the presence of 0.1 SDS and a mid-range marker kit of standard proteins (Promega).

Leaf samples were taken from both treated and control plants, frozen in liquid nitrogen, and then extracted for measuring the activity of the following enzymes:

a) β-1,3-glucanase. Leaf samples were extracted in two volumes of 0.2 M Tris HCl buffer (pH 7.8) containing 14mM β-mercaptoethanol. β-1,3-glucanase activity was assayed by incubating 62.5 μl of the leaf homogenate containing the enzyme for 2 hours at 40°C with 62.5 μl of 4% laminarin. The reaction was terminated by heating the sample in boiling water for 10 min. The amount of reducing sugar was measured spectrophotometrically at 492 nm after reaction with 372μl of 3, 5-

dinitrosalicylate. Final activity values are reported as *nktal*, defined as the enzyme activity catalyzing the formation of nmols glucose equivalents (Abeles *et al.* 1970).

b) Peroxidase. Enzyme activity was assayed according to Kim & Yoo (1996). 0.1 gm of leaf sample was homogenized in 1 ml 0.1M phosphate buffer (pH 6.0) for 5 min using a sonicator. Homogenates were then centrifuged at 12000 g for 5 min at 4 $^{\circ}$C and the supernatant subjected to the peroxidase activity assay. Peroxidase activity was determined at 30 $^{\circ}$C with a spectrophotometer (470 nm) following the formation of tetraguaiacol in a 3 ml reaction mixture containing: 1ml of 0.1 M phosphate buffer (pH 6.0); 1ml of 15 mM 2-methoxyphenol (guaiacol); 1ml of 3 mM H_2O_2; and 15μl of enzyme extract. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min.

The significance of differences between the mean values for treatment and control was estimated statistically using one-tailed student T-test.

RESULTS

The bacterial isolates were identified as *Bacillus globisporus* and *Pseudomonas fluorescens*. The actinomycete isolate was identified as *Streptomyces gibsonii*. The yeast fungi isolate was identified as *Candida glabrata*.

The effect of the four representative rhizosphere microorganisms (*Bacillus globisporus, Candida glabrata, Pseudomonas fluorescens,* and *Streptomyces gibsonii*) on the induction of resistance against TNV was investigated through both soil and foliage (exogenous) treatments. The results of the soil treatment showed that relative to control plants, treatment decreased the number of local lesions to about two-thirds with *Streptomyces gibsonii*, approximately one-half with *Candida glabrata* and *Pseudomonas fluorescens*, and even less with *Bacillus globisporus* (Table 1). With foliage treatment, the four tested microorganisms became even more effective in reducing the total lesion number, with reductions in excess of 90% with treatment by *Streptomyces gibsonii* and *Pseudomonas fluorescens* (Table 2).

Table 1: Induction of resistance or the inhibitory effect against tobacco necrosis virus (TNV) in *Phaseolus vulgaris* plants following soil treatment with one of four microorganisms (n = 4 replicates). SE= Standard Error. ** and * indicate "highly significant" and "significant" differences respectively between the mean values for the treatment and the control.

Microorganism	Mean number of local lesions ± SE	% of inhibition
Control	60.8 ± 9.9	
Bacillus globisporus	24.8 ± 4.5**	59.2
Candida glabrata	30.8 ± 6.5**	49.3
Streptomyces gibsonii	42.0 ± 4.9*	30.9
Pseudomonas fluorescens	31.8 ± 6.6**	47.7

Figure 1 shows some changes in the protein patterns separated on 15% SDS-PAGE of treated leaves relative to the control. Densiometric scans (Table 3) of the PAGE revealed the induction of *de-novo*-synthesized proteins in the intracellular fluid of treated leaves, undetectable in the fluid of control leaves. Treatment with *Streptomyces* and *Pseudomonas* resulted in the detection of two similar induced proteins (with molecular weights of 33.5 and 24.6 KDa), although in different amounts. Treatment with *Bacillus* and *Pseudomonas*

also gave similar proteins, with molecular weights of 41.2, 38.5, 31.4, and 22.8 KDa, in approximately similar amounts (Table 3). Treatment with _Candida_ gave the largest number of detectable protein bands, and these were very different from those of the other three microbial treatments.

Table 2: Induction of resistance or the inhibitory effect against tobacco necrosis virus (TNV) in _Phaseolus vulgaris_ plants using an exogenous treatment by spraying culture filtrates onto leaves (n = 4 replicates). Abbreviations as in Table 1.

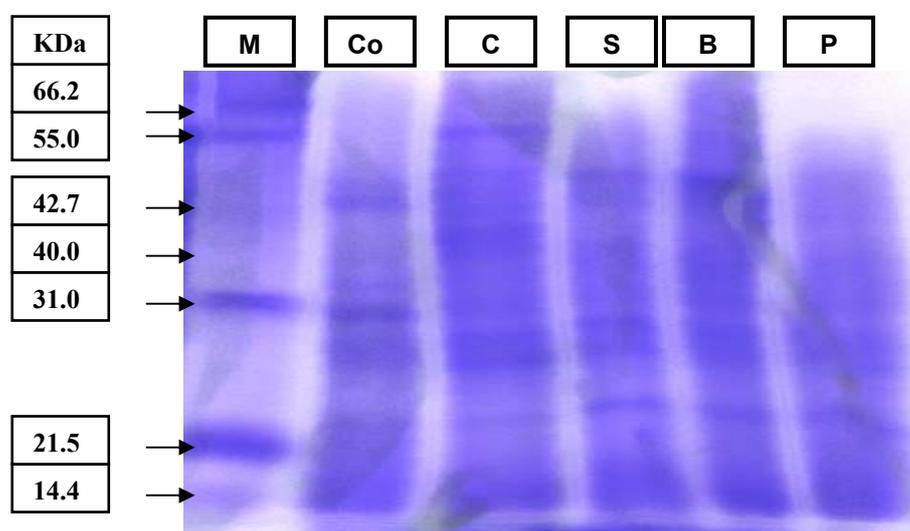| Microorganism	Mean number of local lesions ± SE	% of inhibition
Control	56.4 ± 6.3	
Bacillus globisporus	14.0 ± 2.0**	75.2
Candida glabrata	21.8 ± 5.0**	61.3
Streptomyces gibsonii	1.6 ± 0.4**	97.2
Pseudomonas fluorescens	4.8 ± 0.9**	91.5

Figure 1: Electrophoretic patterns in 15% polyacrylamide gel of soluble proteins extracted from leaves treated with culture filtrate of _Candida glabrata_ (C), _Streptomyces gibsonii_ (S), _Bacillus globisporus_ (B), or _Pseudomonas fluorescens_ (P) and then post-inoculated with TNV. The control (Co) represents soluble proteins extracted from water- treated control leaves. M = marker proteins with molecular masses in KDa.

It can be concluded from the analysis of protein patterns that: (1) the number and concentration of the _de-novo_ synthesized proteins varied among treatments; (2) there were similarities among certain low-molecular-weight proteins among some of the treatments. These proteins are probably PR-proteins.

Treated bean plants showed varied protection against TNV when compared with the control. The enzymatic activities of β-1,3-glucanase and peroxidase were monitored in the leaf extract of these plants, to investigate whether they participate in resistance of bean to TNV (Table 4). β-1,3-glucanase activity reached a maximum in the _Pseudomonas_-treated leaves; _Candida_-treated leaves were the same as control leaves; both _Streptomyces_-and _Bacillus_-treated leaves showed lower activity than the control. Peroxidase activity was much higher than the control in _Bacillus_-treated leaves, but lower in all other treatments.

Table 3: Densiometric computer analysis of protein patterns of *Phaseolus vulgaris* leaf homogenates extracted from leaves treated with culture filtrate of *Candida glabrata* (C), *Streptomyces gibsonii* (S), *Bacillus globisporus* (B), and *Pseudomonas fluorescens* (P) then inoculated with TNV. Column (Co) analysis of protein patterns extracted from water -treated control leaves.

Band number	Mol. Wt	Co Band%	C Band%	S Band%	B Band%	P Band %
1	66.2					
2	57.8	1.68	4.95	1.23	3.79	
3	53.6		3.11	0	3.28	0
4	47.1	5.70	2.70	7.08	11.1	8.83
5	44.4	7.80	4.71	6.58	5.66	7.12
6	44.1		4.82	0	0	0
7	42.3		3.02	0	0	0
8	41.8	1.06	4.54	3.04	2.35	5.33
9	41.3	2.14		3.55		
10	40.9		3.25	0	5.14	5.51
11	40.6	3.57		4.90	6.82	6.21
12	40.2		3.35	0	0	0
13	39.5	6.99		6.10		
14	38.1		4.66	0	6.43	6.30
15	37.6	6.54		5.03		4.86
16	36.2	4.91	5.76		4.14	
17	35.2		0	0	0.77	1.59
18	33.7	23.7	2.42			
19	33.5		0	1.90	0	0
20	32.6		0.71	0	2.09	4.51
21	32.3	1.98				
22	31.5		0	0	0.83	2.38
23	29.7	4.15			0.91	
24	27.6		0	2.60	0	0
25	25.5	4.63			3.17	
26	23.5		6.62	9.60	0	5.11
27	22.9	7.66				
28	22.5		0	0	8.92	10.8
Total number of bands		14	14	11	15	12

Table 4: β-1,3-glucanase and peroxidase activities in the protein extract of treated and then TNV-inoculated bean leaves.

Treatment	β-1,3-glucanase (nktal/g)	peroxidase activity (U/g)
Control (Co)	350	3
Bacillus globisporus (B)	250	7
Candida glabrata (C)	350	1
Streptomyces gibsonii (S)	250	2.5
Pseudomonas fluorescens (P)	500	2.5

DISCUSSION

In the present study we evaluated the efficacy of four rhizosphere microbial isolates to induce resistance and biologically suppress viral infection by TNV in bean plants. The culture filtrate treatments resulted in a statistically significant reduction in TNV disease. Although protection was observed with all the isolates, there was a consistent difference in resistance induced by application method: more resistance was induced when culture filtrates were provided to plant leaves (foliage treatment) than culture inoculation in soil (soil treatment). This suggests that a longer period of association between the microbial cultures and plant root may be necessary for the induction of resistance (Leeman *et a*l. 1995). It was also possible that the biologically active microbial metabolites are only weakly taken up by the root and transported to the site of infection (Wei *et al*. 1991). It has been reported that foliar treatment by the biocontrol agent is necessary before resistance can be realized (Yamaguchi *et al*. 1992): in our experiments, when culture filtrates were applied directly to plant leaves, they were faster in triggering the host defence response and consequently reduced the number of local lesions.

Among the four different rhizosphere microbial isolates that were tested, *Pseudomonas fluorescens* and *Streptomyces gibsonii* suppressed the viral disease to a greater extent than *Bacillus globisporus* and *Candida glabrata* isolates. The high antiviral activity of culture filtrates of *Pseudomonas fluorescens* and *Streptomyces gibsonii* may happen because of the existence of active metabolites (antibiotics, lipopolysaccharides, pyoverdine, etc.) which suppress viral infection (Bakker *et al*. 2003).

There is a clear suggestion that the ability of the four isolates to inhibit TNV infection was correlated with their ability to interfere with metabolism of host plant. Electrophoretic analysis of the extractable proteins from treated bean leaves revealed the appearance of new synthesized host proteins. These induced proteins have low molecular weights, and hence they are mostly candidates as pathogenesis-related proteins that could contribute to the induction of resistance against TNV (Van Loon *et al*. 1998).

Certain biochemical and serological studies on the pathogenesis-related proteins of bean have been conducted by Awade *et al*. (1989), who identified them as enzymatic proteins. β-1,3-glucanase activity was approximately equal to the control in all treatments except for *Pseudomonas fluorescens*, which exhibited both the highest enzymatic activity and viral inhibitory activity. Maurhofer *et al*. (1994) also observed the induction of β-1,3-glucanase in the intracellular fluid of plant leaves grown in presence of the CHAO strain of *Pseudomonas fluorescens,* as in the intracellular fluid of plant leaves previously immunized by TNV inoculation. Porat *et al*. (1999) confirmed also the role of β-1,3-glucanase in inducing resistance against different pathogens.

Peroxidase is an important enzyme in the reinforcement of plant cell walls, and helps in protein extension to generate a firmer matrix material to be a part of the activated defence response (Jabs *et al,* 1996). Increases in its activity have been found after infection of a hypersensitive plant with TMV, and this aids the plant in deterring any additional pathogens; moreover, there were certain peroxidase isozymes that increased specifically in response to TMV infection in inoculated leaves (Weststeum 1976). Recently, Kortekamp & Zyprian (2003) recorded that higher induction of peroxidase activity in response to fungal challenge enhanced disease resistance. In the light of these results, we can assume that there is a correlation between the high peroxidase activities and the relatively high induced resistance of bean against TNV in the *Bacillus*-treated plants of our experiment, as between the low peroxidase activity and weak induced resistance in *Candida*-treated plants.

The resistance induced by the four different microbial isolates are non-specific in affording enhanced protection against TNV. Accumulation of new proteins may indicate a

general response in resistant plants. The biological enzymatic functions of these proteins are not absolutely correlated with the resistance of the plant to viral infection. Finally, there are different mechanisms of disease suppression by these isolates; the combination of these modes of action may well lead to improved efficacy of biological control.

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