

INDUCTION OF DEFENSE RELATED ENZYMES AND PHENOLS IN GERBERA JAMESONII BY BACILLUS SPP. AGAINST ROOT KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

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ABSTRACT

Bacillus spp. (BG42, BG37 and B4) isolates were identified as potential bacterial antagonists against Root Knot Nematode, *Meloidogyne incognita* based on hatching and mortality studies. These three isolates were screened for their ability to induce defense related enzymes like peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, super oxide dismutase and phenols against *M. incognita* in gerbera plants of Valletta variety. Among the bacterial antagonists tested, liquid formulation of *B. subtilis* BG42 showed the higher activities of PO (2.93 change in OD min-1g-1 compared to control 1.23), PPO activity (1.22 change in OD min-1g-1 compared to control 0.76), PAL (5.49 change in OD min-1g-1 compared to control 3.23) and total phenols (3.43 µg mg-1 compared to control 1.57) after 7 days of application in gerbera plants inoculated with *M. incognita*. Native PAGE analysis revealed that liquid formulation of *B. subtilis* BG42 treated gerbera roots showed three isoforms indicating the higher induction of SOD. The other treatments showed similar trend but the enzyme activities and SOD content were less compared to liquid formulation of *B. subtilis* BG42. These results indicated that the liquid formulation of *B. subtilis* BG42 was capable of inducing systemic resistance against *M. incognita* by accumulation of defence enzymes and phenols.

INTRODUCTION

Gerbera plants are subjected to infection by many plant pathogens including the root knot nematodes, *Meloidogyne* spp. which are considered as the most important nematode species worldwide. Many effective control measures were used to manage root knot nematodes. To reduce the dependence on chemical crop protectants in agriculture, bioagents antagonists to pests are receiving increasing attention. Biological control agents manage the soil borne phytopathogens in an environment friendly approach compared to hazardous fungicides (Saleem *et al.*, 2000 and Anu Rajan *et al.*, 2013).

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria which have the ability to colonize the roots and either promotes plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth, 1978). They improve plant growth through mechanisms like antagonism effect against plant pathogens, improving host nutrition and stimulating plant host defense mechanisms (Choudhary and Johri, 2009). They are associated with many plant species and are commonly present in varied environments. Strains with PGPR activity, belonging to genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas* and *Serratia* have been reported (Hurek and Reinhold-Hurek, 2003). Among these, species of *Pseudomonas* and *Bacillus* are the most extensively studied. These bacteria competitively

colonize the roots of plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both.

Induced systemic resistance is the enhancement of the plants defense response by PGPR and systemic acquired resistance (SAR) is the defense response of plant against pathogen attack and other elicitors (Choudhary *et al.*, 2007). *Pseudomonas* species are the prominent plant associated bacteria which are widely used to induce systemic resistance against many plant pathogens by secretion of antimicrobial metabolites (Asha *et al.*, 2011). The use of fluorescent pseudomonads for inducing systemic resistance against phytonematodes has been well documented (Siddiqui, 2006; Patricia *et al.*, 2009). Many *Bacillus* species like *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *etc.*, can also induce defense response and reduce disease incidence in different host pathogen combinations (Kloepper *et al.*, 2004). These bacteria's can activate plants defence mechanisms by enhancing the levels of defense related enzymes like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), Superoxide dismutase (SOD) and phenolic compounds and makes the plant resistant to nematode invasion.

Jonathan and Umamaheswari (2006) reported that *B. subtilis* strain EPB5+EPB31 increase plant growth coupled with reduction in nematode population by enhanced activity of defence enzyme responsible for induction of systemic resistance such as peroxidases, polyphenol oxidase, phenylalanine ammonia lyase and *etc.* This paper deals with

two isolates of *Bacillus subtilis* strain (BG37 and BG42) and one isolate of *B. amyloliquefaciens* strain B4 were tested for their ability to induce defense related enzymes and phenolic content in gerbera plants against *M. incognita*.

MATERIALS AND METHODS

Ten isolates of *Bacillus* spp. were isolated from gerbera rhizosphere from four districts of Tamil Nadu namely Coimbatore, The Nilgiris, Salem and Krishnagiri. Bio efficacy of *Bacillus* isolates was assayed against root knot nematode by hatching and mortality tests. Among the ten isolates screened, highest inhibition in egg hatching and highest per cent mortality of *M. incognita* juveniles was observed at *Bacillus* isolate BG42 followed by BG37. The partial 16S rDNA sequences of the isolated strains BG37 and BG42 showed 99% identity with *B. subtilis* and were deposited in GenBank under accession numbers KM454178 and KM588210 respectively (Manju and Subramanian, 2015). Existing strain *B. amyloliquefaciens* B4 reported to be effective against plant pathogens was obtained from the Centre for Plant Protection studies, Department of plant pathology, Tamil Nadu Agricultural University, Coimbatore. Talc formulation (Vidhyasekaran and Muthamilan, 1999) with a inoculum density of 1×10^8 cfu/g and liquid formulation with a inoculum density of 10^8 cfu/ml of above three strains were prepared.

These three promising isolates of *Bacillus* spp. (*B. subtilis* BG37, *B. subtilis* BG42 and *B. amyloliquefaciens* B4) antagonists were tested for their efficacy to induce defense related enzymes like Peroxidase, Polyphenol oxidase, Phenylalanine ammonia-lyase, Superoxide dismutase and total phenolic compounds against *M. incognita* in gerbera. Gerbera seedlings (*var. Valletta*) were planted in Earthen pots of 30×20×18 cm size filled with 5 kg sterilized pot mixture containing red soil:sand:FYM, 2:1:1 (v/v). Seedlings were planted in the above pots at the rate of one plant / pot. The experiment was laid out in Completely Randomized Design (CRD) with eight treatments and three replications.

Each set of gerbera plants were treated with T1- Soil drenching of liquid formulation of *B. subtilis* BG42 @ 1%/m²; T2: Soil application of talc formulation of *B. subtilis* BG42 @ 1%/m²; T3 : Soil drenching of liquid formulation of *B. subtilis* BG37 @ 1%/m²; T4 : Soil application of talc formulation of *B. subtilis*

BG37 @ 1%/m²; T5: Soil drenching of liquid formulation of *B. amyloliquefaciens* B4 @ 1%/m²; T6 : Soil application of talc formulation of *B. amyloliquefaciens* B4 @ 1%/m²; T7- Soil application of Carbofuran (3.3g/m²). Gerbera plants without any treatment served as control.

Biochemical analysis

Root samples were collected at different time intervals (0, 3, 7, 12 and 18 hours after treatment) for enzyme assays. Fresh roots were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored in a freezer (-70°C) until used for biochemical analysis.

Determination of peroxidase (PO)

Root samples (1 g) maintained at -70°C were homogenized in 2mL of 0.1 M phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 16,000 g at 4°C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5mL of enzyme extract and 0.5 mL of 1 per cent H₂O₂. The reaction mixture was incubated at room temperature (28 ± 2°C). The changes in absorbance at 420 nm were recorded at 30s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min⁻¹ g⁻¹ protein (Hammerschmidt *et al.*, 1982).

Determination of polyphenol oxidase (PPO)

PPO activity was determined as per the procedure given by Mayer *et al.* (1965). The freeze dried root samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 μL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction 200 μL of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min⁻¹ g⁻¹ protein.

Determination of phenylalanine ammonia lyase (PAL)

Root samples (1g) stored at -70°C were homogenized in 3 mL of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16,000 g for 15 min. The

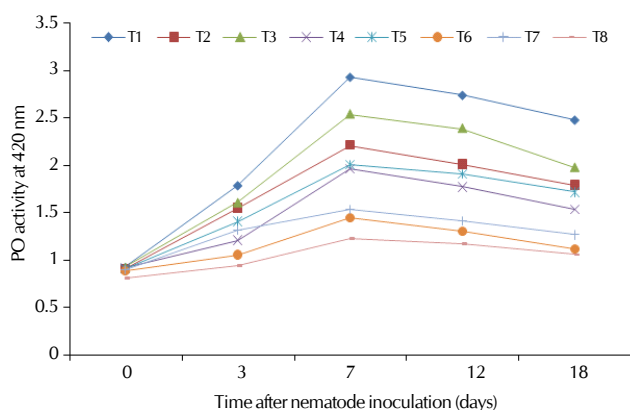


Figure 1: Peroxidase (PO) activity in different treatments

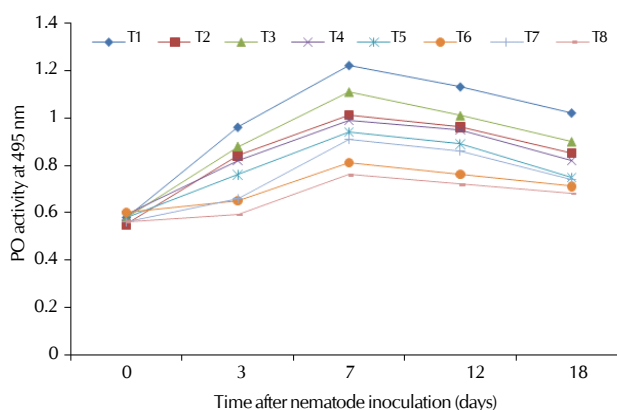


Figure 2: Polyphenol oxidase (PPO) activity in different treatments

supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984). A sample containing 0.4 ml of enzyme extract was incubated with 0.5 mL of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated.

Determination of phenol

Root samples (1 g) stored at -70°C were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C (Zieslin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 mL of distilled water and 250 μ L of Folin-Ciocalteu reagent (1/V) and the solution was kept at 25°C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725nm. Catechol was used as the standard. The amount of phenolics was expressed as μ g catechol g^{-1} protein.

Native page analysis

Superoxide dismutase

Native polyacrylamide gel electrophoresis was carried out to visualize the SOD activity in the samples. Equal amounts of protein (40 μ g) were loaded on to each lane. SOD activity was determined on native PAGE gels as described by Beauchamp and Fridovich (1971) and modified by Azevedo *et al.* (1998). The native PAGE gel was transferred into 0.05M potassium phosphate buffer (pH-7.8) and solution was added with a pinch of 0.1mM nitroblue tetrazolium and 1mM EDTA and riboflavin (0.05mM). Then 200 μ L of TEMED (0.3% v/v) was added and incubated in dark for 30 min. at the end of this period, the gel was rinsed with deionized water and placed in deionized water then the gel was exposed to UV light for reaction and development of bands. Later the SOD activity in the gel was photographed and was exposed to UV light for 5 to 10 min at room temperature until the development of colourless bands of SOD activity in a purple stained gel was visible.

Statistical analysis

The data generated were subjected to statistical analysis by following the standard statistical procedures (Gomez and Gomez, 1984).

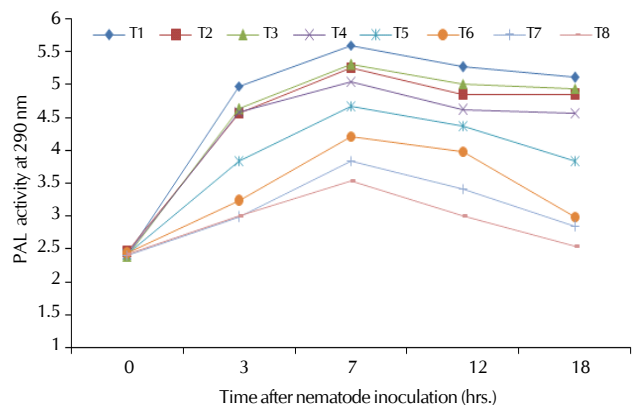


Figure 3: Phenyl alanine ammonia lyase (PAL) activity in different treatments

RESULTS AND DISCUSSION

The bacterized plants expressed higher amounts of defense enzymes in gerbera plants inoculated with *M. incognita* when compared to plants without bacterization.

Peroxidase and polyphenol oxidase

The results showed increased level of peroxidase (2.93 $\text{min}^{-1}\text{g}^{-1}$ root) activity in gerbera plants treated with liquid formulation of *B. subtilis* strain BG42 seven days after treatment (Fig. 1). The peroxidase activity was recorded as 1.23 $\text{min}^{-1}\text{g}^{-1}$ root in untreated control plant. Significant increase in the activity of polyphenol oxidase (1.22 $\text{min}^{-1}\text{g}^{-1}$) was observed in plants treated with liquid formulation of *B. subtilis* strain BG42 (Fig. 2).

Peroxidases have been found to play a major role in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, cross linking of extension monomers, oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran *et al.*, 1997). Enhanced activity of polyphenol oxidase by the application of *B. subtilis*, *B. firmus* and *B. coagulans* has been reported in eggplants against *M. javanica* (Abbasi *et al.*, 2014).

Phenylalanine ammonia lyase (PAL)

Similar trend was observed with PAL activity. Nematode challenged treatment liquid formulation of *B. subtilis* strain BG42 showed higher PAL activity (5.49 $\text{min}^{-1}\text{g}^{-1}$ root) compared to untreated control (3.23 $\text{min}^{-1}\text{g}^{-1}$ root) on 7th day. Significantly higher PAL activity was observed in treated plants compared to untreated control throughout the experiment. Plants treated with nematode alone showed increase in PAL activity for a period of 7 days and thereafter the activity declined significantly. PAL is the first enzyme in phenylpropanoid metabolism involved in the production of phenolics and phytoalexins that prevent establishment of the pathogen (Mariutto *et al.*, 2011). This enzyme induces the formation of necrosis, which resulted in the localization of the juveniles and prevented their further movement. The present investigation revealed increased activity of PAL due to *B. subtilis* treatment, which might have prevented the establishment of the nematode within the roots. Enhanced activity of PO, PPO, PAL and chitinase by the application of *P. fluorescens* Pf1 has been reported in rice against *M. graminicola* (Anita and

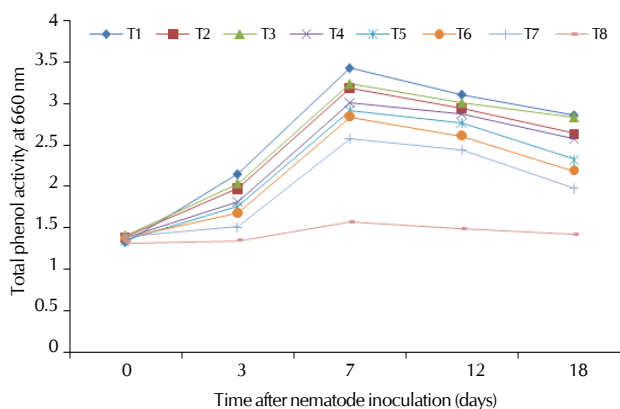
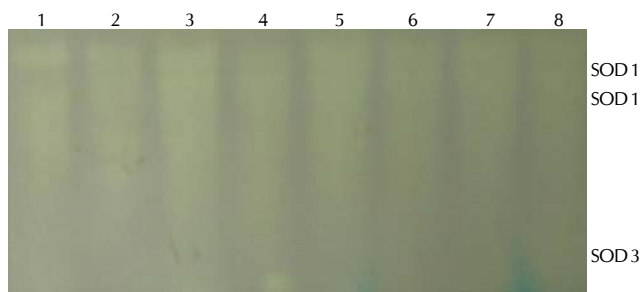


Figure 4: Total phenol activity in different treatments



T1-Untreated control
 T2- Soil application of Carbofuran (3.3g/m²)
 T3- Soil drenching of liquid formulation of *B. subtilis* BG42 @ 1%/m²
 T4 : Soil application of talc formulation of *B. subtilis* BG42 @ 1%/m²
 T5 : Soil drenching of liquid formulation of *B. subtilis* BG37 @ 1%/m²
 T6 : Soil application of talc formulation of *B. subtilis* BG37 @ 1%/m²
 T7: Soil drenching of liquid formulation of *B. amyloliquefaciens* B4 @ 1%/m²
 T8 : Soil application of talc formulation of *B. amyloliquefaciens* B4 @ 1%/m²

Plate 1: Superoxide dismutase activity induced in *Bacillus* spp. treated gerbera roots infested with *M. incognita* under pot culture condition

Samiyappan, 2012). Earlier and higher accumulation of these defense enzymes in rice root tissue resulting in significant reduction in nematode infection.

Total phenol

Studies on induction of defense mechanisms revealed higher accumulation of phenolics in bacterized gerbera roots inoculated with *M. incognita*. Accumulation of phenolics was observed seven days after inoculation with nematode. The highest accumulation of total phenols was observed with the soil drenching of liquid formulation of *B. subtilis* strain BG42 (3.43 μg catechol mg⁻¹ protein) on seventh day after application compared to untreated control (1.57 μg catechol mg⁻¹ protein) (Fig. 4). Phenolic compounds are known to play a major role in the defense mechanism of plants against various external infectious agents. The phenol is providing resistance to nematodes in plants either by repelling the juveniles or by adversely affecting the development of nematode juveniles according to Singh *et al.* (1983). Accumulation of phenols in plant system is imparting resistance to invading plant pathogens including nematodes. Accumulation of phenolic compound by the application of *Bacillus* spp. isolate B27 has been reported in grapevine (Ben Maachia Sihem *et al.*, 2010).

Superoxide dismutase (SOD)

Native PAGE analysis revealed that superoxide dismutase isoforms designated as SOD1, SOD2 and SOD3 were observed in liquid formulation of *B. subtilis* strain BG42 treated gerbera root tissues challenged with the *M. incognita* and the expressed isoforms showed higher intensity compared to other treatments (Plate. 1). The expressions of isoforms were more density revealing more induction of SOD activity treated gerbera plants infested with *M. incognita*. The least enzyme induction was observed in untreated control plants.

These findings coincidence with the findings of Zahra Oraghi *et al.* (2011) have reported that *P. fluorescens* CHA0 significantly induced peroxidase, polyphenoloxidase and superoxide dismutase activities in tomato root tissue. In the present study the strong induction of isoforms of SOD in liquid formulation of *B. subtilis* strain BG42 and liquid formulation of *B. subtilis* strain BG37 treated plants, induced systemic

resistance which alternately reduced the development of *M. incognita*.

The present study has helped in identifying an isolate of *B. subtilis* BG42 with higher ability to induce defense related compounds in gerbera plants against *M. incognita*. In addition to these, isolate being a spore forming organism, has better surviving ability in nature when used as a biocontrol agent in the field. Hence, the biocontrol potential of this isolate can be further tested in field and exploited for the management of *M. incognita* in gerbera.

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