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Research Article

Induced defense response in brinjal plants by *Bacillus megaterium* NBAII 63 against bacterial wilt pathogen, *Ralstonia solanacearum*

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ABSTRACT: *Bacillus megaterium* strain NBAII 63 was identified as a potential bacterial antagonist against brinjal bacterial wilt pathogen, *Ralstonia solanacearum*. It was tested for its ability to induce defense related enzymes *viz.*, peroxidase (PO), polyphenoloxidase (PPO) and total phenols against *R. solanacearum* in brinjal plants. Brinjal plants treated with *B. megaterium* challenge inoculated with *R. solanacearum* showed higher levels of defense related enzymes and phenols compared to antagonist alone, pathogen alone and untreated plants. *B. megaterium* strain NBAII 63 showed the higher activities of total phenols (173 µg g-1 of tissue compared to control 121), PO (2.75 change in OD min-1g-1 of tissue compared to control 0.75) and PPO activity (0.91 change in OD min-1g-1 compared to control 0.13) in brinjal plants treated with *R. solanacearum*. The present study clearly indicated that *B. megaterium* strain NBAII 63 has the ability to induce the defense related enzymes in the brinjal plants against *R. solanacearum*.

KEY WORDS: Induced defense, brinjal, *Bacillus megaterium*, bacterial wilt, *Ralstonia solanacearum*

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INTRODUCTION

Bacterial wilt disease caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. (Syn *Pseudomonas solanacearum* E. F. Smith) is one of the most devastating diseases of solanaceous vegetable crops in India and affects more than 450 plant species across the World. There is a typical browning of vascular tissues in roots, stems and tubers. Different races and biovars exists in the bacterium. In extreme cases the yield losses due to the disease in egg plant and tomato has been reported as high as 80 and 90 per cent, respectively (Sivakumar *et al*., 2011). Due to its wide distribution, wide host range and soil borne nature, it is difficult to control with chemicals and cultural practices alone (Grimault *et al*., 1993). Plant growth-promoting rhizobacteria (PGPR) improve plant health through mechanisms like antagonism, improving host nutrition and stimulating plant host defense mechanisms (Choudhary and Johri, 2009). Many *Bacillus* species like *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, etc., can induce defense response and reduce disease incidence in different host-pathogen combinations (Kloepper *et al*., 2004). These bacteria can activate plant defence mechanisms by enhancing the levels of defense related enzymes like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and phenolic compounds and make the plant resistant to pathogen. Phenols have been known to occur in all the plants investigated so far. Some of the phenolic enzymes occur constitutively, whereas others are formed in response to pathogen ingress and associated as part of an active defense response in the host (Nicholson *et al*., 1992). The constitutive phenolics are known to confer resistance either directly or indirectly through activation of post infection responses in the hosts (De Vecchi *et al*., 1989). The antagonism and bioefficacy of promising *B. megaterium* strain NBAII 63 against *R. solanacearum* was proved under green house (Sivakumar *et al*., 2011) and field (Sivakumar and Rangeshwaran, 2013) conditions. In the present study, this strain was tested for its ability to induce defense related enzymes and phenolic content in brinjal plants against *R. solanacearum*.

MATERIALS AND METHODS

Preparation of talc formulation of *Bacillus megaterium*

Bacillus megaterium strain NBAII 63 was grown in nutrient broth for 48 h in shake culture at 150 rpm at room temperature (28 ± 2°C) which was used for the preparation of talc formulation. The talc powder was autoclaved for

45 min at 137.3 kPa (kiloPascal) pressure and its pH was adjusted to 6.5-7 by using calcium carbonate ($CaCO_3$). Five hundred milliliters of nutrient broth having bacterial cells $9x10^8$ cfuml-1 was added to 1 kg of sterilized talc powder and mixed well under sterile conditions. The formulations were air-dried to 20% (w/v) moisture content, packed in polythene bags and incubated at 28 ± 2°C. The population of bacteria in talc formulation was estimated by serial dilution plate technique on nutrient agar medium.

Preparation of pathogen

Ralstonia solanacearum was isolated from the infected brinjal plants. The collar portion of the infected plants were cut into two to three pieces and put into sterile water. The bacterial ooze coming out of the cut end was streaked in the triphenyl tetrazolium chloride (TTC) medium. The typical wild type colonies which formed an irregularly-round, fluidal, white colony with a pink centre were collected and stored in sterilized water.

Analysis of defense related enzymes and total phenols

The antagonist *B. megaterium* strain NBAII 63 was tested for their efficacy to induce defense related enzymes *viz*., peroxidase (PO), Ppolyphenol oxidase (PPO) and total phenols against *R. solanacearum*. The following were the treatments. T1: Root dipping with talc formulation of *B. megaterium* strain NBAII 63 at 10g L^{-1}; T2: Root dipping with talc formulation of *B. megaterium* strain NBAII 63 and drench inoculated with *R. solanacearum* (culture suspension 2 x 10^8 cfu ml^{-1}) evenly at 30 ml pot^{-1} after 24 hrs of antagonist treatment; T3: Plants inoculated with the pathogen alone; T4: Untreated control plants.

The experiment was conducted under glass house condition and the pots were arranged in a completely randomized design. Brinjal seedlings were raised using the seeds of susceptible cultivar VK-1. Seedlings were treated with antagonistic talc formulation (10g in 1000ml sterile water) by root dipping for 10 min and then transplanted in plastic pots filled with sterilized coir pith. The plants were challenge inoculated with *R. solanacearum* after 24hrs of antagonist treatment. Four replications were maintained and each treatment had four pots.

Analysis of phenolic enzymes

Shoot and leaf samples were collected at different time intervals (2, 4, 6, 8, 10 and 12 days after pathogen inoculation) for enzyme assays. One gram of sample from each treatment was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle under ice cold condition. The homogenate was centrifuged for 15 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying PO, PPO activity and 80% ethanol extracts were used for assaying total phenolic content (modified from Anand *et al*., 2010).

Assay of total phenols

An aliquot of 0.1ml of ethanol extract was evaporated in hot water bath. After complete evoporation of ethanol, 6 ml water was added and shaken well before addition of 0.5 ml Folin-Ciocalteau reagent (1 N). After 5 min, 2 ml of 20% sodium carbonate solution was added and incubated for 30 min in dark condition at room temperature. Absorbance was recorded at 660 nm in a spectrophotometer HITACHI U2910 and the phenol content in the sample was calculated using pyrocatechol as standard. The quantity of total phenols was expressed in μg g^{-1} of fresh plant weight (Malik and Singh, 1980).

Assay of peroxidase (PO)

The reaction mixture consisted of 100μl enzyme extract, 1.5ml of 0.05M pyrogallol in 0.1M sodium phosphate buffer (Ph 6.5) and 0.5ml of 1% hydrogen peroxide. Boiled ezyme preparation served as blank. The change in absorbance at 420 nm was recorded at 30 sec interval for 3 min in spectrophotometer. The enzyme activity was expressed as change in the absorbance of the reaction mixture $min^{-1}g^{-1}$ of fresh plant weight at 420nm (Hammerschmidt *et al*., 1982).

Assay of polyphenol oxidase (PPO)

Polyphenol oxidase activity was assayed by the change in color intensity of catechol oxidation products. The reaction mixture consisted of 100μl enzyme extract and 1.5ml of 0.1M sodium phosphate buffer (pH 7.0), the reaction started when 200μl of 0.01M catechol was added. The change in the absorbance was recorded at 30 sec interval for 3 min at 495 nm and the enzyme activity was expressed as changes in absorbance at 495nm min^{-1} g^{-1} of fresh plant weight (Mayer *et al*., 1965).

RESULTS AND DISCUSSION

Peroxidase activity

All the treatments showed increase in the activity of the peroxidise upto 6 days after inoculation except control where the increase was gradual (Table 1). There was significant increase in peroxidase activity in the antagonist treated brinjal plants as compared to control and pathogen inoculated. Highest activity of peroxidise (2.75 changes in absorbance $min^{-1}g^{-1}$ of tissue) was

Table 1: Peroxidase activity in brinjal plants treated with *B. megaterium*

Treatments	Peroxidase (PO) activity (changes in absorbance/min/g of tissue)					
	Days after inoculation (DAI)					
	2	4	6	8	10	12
Root dipping of *B. megaterium*	1.41	1.51	2.01	1.72	1.61	1.55
Root dipping of *B. megaterium* + *R. solanacearum*	1.72	1.82	2.75	2.26	2.11	2.01
R. solanacearum	1.12	1.14	1.62	1.53	1.52	1.34
Control	0.54	0.72	0.75	1.03	1.15	1.21

observed 4 days after inoculation in the bacterized plants challenge inoculated with the pathogen as compared antagonist treated (2.01 $min^{-1}g^{-1}$), pathogen inoculated (1.62 $min^{-1}g^{-1}$) and control (0.75 $min^{-1}g^{-1}$).

Polyphenol oxidase activity

The maximum increase in polyphenol oxidase activity was observed in the bacterized brinjal plants with challenge inoculation of pathogen (0.91 change in absorbance $min^{-1}g^{-1}$ of tissue) followed by bacterized plants alone (0.77) pathogen alone (0.52) and control (0.13) 6 days after inoculation of the pathogen. In control plants there was not much variation in the activity. The activity of the enzyme declined significantly 6 days after the inoculation of the pathogen (Table 2).

Phenol content

There was significant increase in phenol content in the antagonist treated brinjal plants as compared to control. The phenol was increasing from 4th day after inoculation and attained maximum at 6 days after inoculation of the pathogen. The maximum increase in phenol content was observed from 149 to 173µg g^{-1} of plant tissue in the bacterized brinjal plants with challenge inoculation of pathogen followed by bacterized plants alone from 145 to 155 µg g^{-1}, pathogen inoculated plants alone from 142 to 149 µg g^{-1}. In control plants the phenol content was increasing gradually up to 10 days after inoculation and was declining afterwards (Table 3).

Table 2: Polyphenol oxidase activity in brinjal plants treated with *B. megaterium*

Treatments	Peroxidase (PPO) activity (changes in absorbance/min/g of tissue)					
	Days after inoculation (DAI)					
	2	4	6	8	10	12
Root dipping of *B. megaterium*	0.32	0.44	0.77	0.70	0.49	0.43
Root dipping of *B. megaterium* + *R. solanacearum*	0.68	0.70	0.91	0.59	0.57	0.50
R. solanacearum	0.25	0.39	0.52	0.31	0.25	0.15
Control	0.10	0.11	0.13	0.14	0.16	0.13

Table 3: Phenol content in brinjal plants treated with *B. megaterium*

Treatments	Peroxidase (PO) activity (changes in absorbance/min/g of tissue)					
	Days after inoculation (DAI)					
	2	4	6	8	10	12
Root dipping of *B. megaterium*	145	146	155	149	148	147
Root dipping of *B. megaterium* + *R. solanacearum*	149	150	173	164	153	154
R. solanacearum	142	143	149	114	143	142
Control	120	122	121	123	123	122

Induction of systemic resistance in plants by application of any bioagent is thought to be the best alternative for plant protection from pathogens. Our present study clearly proved that the bacterial antagonist *B. megaterium* induced the defense enzymes against *R. solanacearum*. Many studies have shown that members of bacterial genera can induce systemic resistance in different plants for control of soil-borne diseases (Nagorska *et al*., 2007). Some members of *Bacillus* are able to produce various lytic enzymes (e.g. chitinase and b-1, 3-glucanase) and antibiotics, along with induction of systemic resistance in plants, such as increasing the activities of plant defense related enzymes of peroxidase, polyphenol oxidase and phenylalanine ammonialyase (PAL) (Jayaraj *et al*., 2004). Oxidative enzymes such as PO and PPO, can catalyze the formation of lignin and other oxidative phenols, and contributes in formation of defense barriers by changing the cell structure defense system get activated against pathogens (Thilagavathi *et al*., 2007). These enzymes have been reported to correlate with the defense activities against pathogens in several plant species (Thilagavathi *et al*., 2007). The present study proved that there was increase in the activity of peroxidase and polyphenol oxidase and phenol content in the brinjal plants bacterized with antagonist upon inoculation of pathogen. This results were in accordance with the earlier reports (Ramanujam *et al*., 2012: Nakkeeran

et al., 2006) which revealed that the application of bacterial antagonists *P. fluorescens*, *B. subtilis* increased the level of the defense enzymes peroxidase, polyphenol oxidase and phenylalanine ammonia lyase after 3-4 days of inoculation of pathogen. Elicitation of induced systemic response by use of *Bacillus* strains has been documented on tomato against fungal and bacterial diseases. *B. subtilus* strain FZB-G was shown to be effective to produce defence related biochemical in tomato against fusarium wilt disease (Gupta *et al*., 2000). Phenolic compounds have an important role in protection of plants against fungal pathogens. In the present study it was observed that when roots of brinjal seedlings treated with *B. megaterium*, an increased level of phenolic compounds was observed in brinjal plants as compared to control. Present study revealed that application of talc formulation of *B. megaterium* strain NBAII 63 increased the activity of phenolic enzymes and phenols in brinjal plants against bacterial wilt pathogen. This bacterial strain could effectively be utilized for the management of bacterial wilt disease of brinjal.

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