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ORIGINAL ARTICLE

Use of an abscisic acid-producing *Bradyrhizobium japonicum* isolate as biocontrol agent against bacterial wilt disease caused by *Ralstonia solanacearum*

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Abstract

Bacterial wilt disease in tomato (*Solanum lycopersicum*) is a serious threat in agriculture that significantly reduces the production. The aim of the present investigation was to search for an effective biocontrol measure against bacterial wilt disease in tomato. The causative agent (bacterial wilt disease) isolated from infested tomato plants was identified as *Ralstonia solanacearum* BR-001. *Ralstonia solanacearum* is considered as one of the most dangerous plant pathogens globally due to its wide host range. The disease progression caused by *R. solanacearum* is very fast and difficult to control. Several strategies like mineral oil, lytic phages, and antimicrobial metabolites produced by different virulent bacterial strains and medicinal plants have been reported to control the infection/ wilt disease caused by *R. solanacearum*. However, it is difficult to control the progression once it enters the xylem tissue. Furthermore, *R. solanacearum* produces two different types of DNases which help the bacteria to escape from the plant defense system. We also have isolated 43 bacterial candidates from the rhizospheres of few unaffected tomato plants from the same field. Interestingly, one out of 43 candidates exhibiting efficacy against *R. solanacearum* BR-001 *in-vivo* was identified as *Bradyrhizobium japonicum* BRC 2485. But no isolate was found to control disease progression effectively during *in-vitro* condition. To understand the biocontrol potential of *B. japonicum* BRC 2485, an *in-vivo* comparative study was conducted with one *Bradyrhizobium* type strain (MTCC 120) and one *Bradyrhizobium* reference strain (MCC 2940). The experimental evidence suggests that the priming of tomato plants with *B. japonicum* BRC 2485 limits the multiplication of *R. solanacearum* BR-001 therein. The *Bradyrhizobium* strains were tested for the production of siderophores, ethylene, and abscisic acid (ABA). All the experimental *Bradyrhizobium* strains were found to be negative for siderophore production and positive for ethylene production. However, only *B. japonicum* BRC 2485 was found to produce ABA, which plays a major role in triggering induced systemic resistance (ISR) in plants. To the best of authors' knowledge, this is the first report of a strain of *B. japonicum* with activity against bacterial wilt disease.

Keywords Biocontrol · *Bradyrhizobium* · Phytopathogens · Competition · Non-leguminous plant

Introduction

In order to meet the global demands of food for the ever-expanding human population, crop production needs to be doubled by 2050 (UN General Assembly 2009). However, pests and pathogens are creating serious problems to the crop production along with global climatic changes. Plant pathogens alone have been shown to reduce crop yield about 14% (Velasquez et al. 2018). To control bacterial plant pathogens (BPPs), the demand of biological control agents (BCAs) is increasing rapidly under the integrated crop management (ICM) practice (Chattopadhyay et al. 2017). Tomato is one of the major horticultural crops in the world. During 2016–2017, the tomato production was estimated

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to be around 19.7 million tones. In India, it is grown over an area of 0.497 million hectare with a production of 17.35 million tons (FAO/WHO 2006).

The bacterial wilt symptoms in tomato are characterized by initial wilting of upper leaves followed by complete wilting of the plants. Bacterial wilt management in tomato is difficult because the causative agent grows endophytically, survives in deeper layers of soil, and disseminates through water. Yuliar et al. have extensively reviewed the management of bacterial wilt disease with physical, chemical, biological, and cultural methods (Yuliar and Toyota 2015). The ideal candidate as BCA against bacterial wilt should have the following characteristics: (1) self-sustaining, (2) long-term disease suppression, (3) reduced input of nonrenewable resources, (4) safe for environment, and (5) economically cheap (Kulkarni et al. 2017).

Several bacterial BCAs have been reported to control bacterial wilt such as *Acinetobacter* sp. (Xue et al. 2009), *Bacillus thuringiensis* (Zhou et al. 2008), *Burkholderia* spp. (Nion and Toyota 2008), etc. In this present report, the BCA potential of bacterial isolates from the rhizosphere of healthy tomato plants in a wilt infected field has been investigated against the bacterial pathogen isolated from the infected tomato plants. The plant pathogen was identified to be a strain of *Ralstonia solanacearum. R. solanacearum* is considered as one of the top ranked plant pathogens that causes bacterial wilt disease in more than 200 plant species worldwide (Nion and Toyota 2015). It is also classified as 'quarantine organisms', 'bioterrorism', and 'double usage agents' by different regulatory authorities in USA and Europe (Cellier et al. 2007). The most potent BCA was identified as a strain of *Bradyrhizobium japonicum. B. japonicum* strains are well known for their biological nitrogen fixation property. *Bradyrhizobium* has been reported as an endophyte in the roots of the non-*Fabaceae* plant species like *Arabidopsis thaliana*, cotton, sweet corn, and tomato (de Matos et al. 2017; Msaddak et al. 2017). It has been shown to provide protection against a wide range of fungal pathogens like *Macrophomina phaseolina*, *Rhizoctonia solani,* and *Fusarium* spp. (Htwe et al. 2018). However, the potential of *Bradyrhizobium* as BCA against bacterial wilt was not clear till date. Thus, an attempt has also been made to elucidate the biocontrol mechanism of the *B. japonicum* isolate.

Materials and methods

Isolation of causative agent responsible for bacterial wilt in tomato

Field survey was conducted to determine the prevalence of bacterial wilt in tomato field plants from Tarapith (latitude: 24.114; longitude: 87.796), Birbhum district of West Bengal, India. Tomato plants showing typical wilt symptoms were taken, and a tentative diagnosis of *R. solanacearum* was made as a result of oozing from cut stems placed in water. Isolation was done on tetrazolium chloride (TZC) agar medium following a standard protocol (Kelman 1954).

Plant materials and growth conditions

Four-week-old tomato plants (*Solanum lycopersicum* cv. Pusa Ruby) were used for the experimental purpose. Seeds were surface-sterilized and germinated in aseptic conditions. Seedlings were grown in double autoclaved soil (loamy soil) under culture room conditions (temperature: 32 ± 2 °C, relative humidity: $\geq 75\%$, photoperiod 17 h, light intensity: 7200 ± 200 lx).

Pathogenicity tests

To check virulence, *in-vivo* pathogenicity tests were conducted in triplicate batches of 30 seedlings. The root tips of four-week-old tomato plants were excised to make entry points for bacteria. Excise root tips were dipped into a suspension of 2×10^7 CFUmL^{-1} for 24 h and observed until complete wilting of unprotected plants. Disease symptoms were scored according to Feng et al. (2012) following a 0–4 scale (0, no wilting; 1, 0–25%; 2, 25–50%; 3, 50–75%; 4, 75–100% of wilted leaves).

Isolation of rhizospheric bacteria

Soil samples were collected from rhizosphere of the live tomato plants from the same field from where the pathogen was isolated. One gram of soil was dissolved in 10 mL of sterile double distilled water, and serial dilution was done accordingly. 100 µL sample from each dilution was taken and plated on nutrient agar medium (HiMedia, India) plates (pH 7.2), followed by 24-h incubation period at 30 °C. Colonies were selected on the basis of distinctive morphology. Pure cultures were obtained through repeated streaking method. The cultures were stored at 4 °C for further use.

Pathogen density determination

Bacterial internal growth curves were performed according to the protocol described by Deslandes et al. (1998). Briefly, plants inoculated with the bacterial strain were weighed, sterilized with 250 mL of 70% ethanol for 3 min, rinsed three times in sterile water, and ground in a mortar after addition of sterile water (2.0 mL per g of fresh weight). Various dilutions of the ground material were then performed with sterile water and the bacteria spread on petri plates containing bacteria specific medium (TZC agar medium for

R. solanacearum) and grown at 30 °C. For each time point, triplicate assays were performed for each bacterial strain.

Screening for BCA

For screening of BCA, four-week-old tomato plants were root-inoculated with the rhizospheric isolates using a suspension of 5×10^8 CFUmL^{-1} in triplicate batches of 30 seedlings. Twenty-four hours after the first inoculation, plants were challenged by the virulent isolate at a concentration of 2×10^7 CFU mL^{-1}. The bacterial internal growth curves were performed according to Deslandes et al. (1998), whereas the disease symptoms were scored according to Feng et al. (2012).

Identification of the pathogen and BCA

Selected isolates (pathogen and BCA) were characterized through biochemical tests in accordance with Bergey's Manual (Smith et al. 1974). The species level identification of the isolated strain was done using 16S rRNA sequence analysis following the methods of Banerjee et al. (2018). The sequence was then edited and submitted to GenBank to obtain the NCBI accession number. The phylogenetic tree was constructed using the neighbor-joining method and validated with the bootstrap analysis (10,000 replicates) using Mega 6.0 software.

Reference strains and growth conditions

One type strain *B. japonicum* MTCC 120 (Singha et al. 2015) and one reference strain *Bradyrhizobium* MCC 2940 (Ojha, 2016) were used in this study for comparative analysis. Both strains were grown in 250-mL flasks containing 100 mL of yeast extract mannitol (YEM) medium at 30 °C in a shaker incubator until exponential growth phase [OD at 600 nm nearly 1, equivalent to 1.25×10^9 colony-forming units (CFU) mL^{-1} in YEM-agar, respectively]. Selective mediums were used for testing bacterial phytopathogens used in this study, viz. Hofers alkaline medium (HiMedia, M717) for *A. tumefaciens* MTCC 609 (Karwasara et al. 2011); boric acid peptone (BAP) agar (HiMedia, PHM001) for *P. syringae* MTCC 1604 (Sharma et al. 2008); and tryptone sucrose tetrazolium (TST) agar medium (HiMedia, M1217) for *X. campestris* NCIM 2961 (Palaniraj et al. 2011).

Antimicrobial assay

In order to check the BCA potential, each of the *Bradyrhizobium* strains was grown in yeast extract manitol (YEM) broth as shake flask (240 rpm) culture up to 1×10^8 CFU mL^{-1}(OD at 600 nm nearly 1). This fermented broth was then examined for antimicrobial activity against selected bacterial phytopathogens in nutrient agar (NA) medium by the cup plate method (Shetty et al. 2014). The zone of inhibition was measured by using a calibrated scale. To study the antimicrobial activity, solvent extraction followed by disc diffusion assay was also done following the method of Banerjee et al. (2018).

Competitive assay

BCA potential of the *Bradyrhizobium* strain might be the consequences of a direct physical competition for space within the xylem vessels. In order to check this hypothesis, *R. solanacearum* BR-001 and selected *Bradyrhizobium* strains were simultaneously co-inoculated in 1:1 ratio. Plant internal bacterial growth curves were performed according to the protocol described by Deslandes et al. (1998). Control plants were inoculated with *R. solanacearum* BR-001 only.

Measurement of siderophore, ethylene and ABA

Siderophore production was determined according to the protocol of Louden et al. by using the blue agar CAS (chrome azurol S) assay (Louden et al. 2011). In brief, plates were sown with 1 µl of YEM pure bacterial culture in halfway points of a Petri dish containing agar CAS medium, incubated at 30 °C and observed daily for the orange color formation around each colony for up to 7 days. Ethylene production was detected according to the protocol described by Strzelczyk et al. (1994). Briefly, air samples (500 µl) were taken from air-tight flasks after 7 days of inoculation in YEM. Air samples were then analyzed by gas chromatography (GC) with a flame-ionizing detector (FID). ABA production was estimated according to the protocol of Ali et al. (2013). Briefly, centrifuged broth of the 7 days old YEM culture were partitioned four times with the same volume of acetic acid–saturated ethyl acetate (1%, v/v). Then acidic ethyl acetate was evaporated at 36 °C, and the dried samples were diluted in 100 µL of acetic acid/methanol/water (1:30:70) and analyzed by High Performance Liquid Chromatography (HPLC) with an ultraviolet (UV) detector. A stainless-steel column (250 × 4.6 mm I.D.) packed with LiChrosphere RP-18e (5 µm) was applied for the separation. A mixture of 0.5% acetic acid (40 mL) and acetonitrile (60 mL) was used as mobile phase at a flow rate of 0.3 mL min^{-1}. Samples were analyzed using UV wavelength at 254 nm.

Exogenous ABA application

To confirm the role of ABA produced by the BCA in biocontrol, ABA was exogenously applied to the tomato plant and challenged with the pathogen (Hu and Bidochka

2021). Disease index and bacterial internal colonization was assessed. Briefly, three doses of ABA (0.02 µgmL^{-1}, 0.2 µg mL^{-1}, and 2.0 µg mL^{-1}) were prepared in 25% aqueous ethanol. Roots of four leaf stage plants were treated with 10 mL of ABA solution per plant with these three concentrations separately. Aqueous ethanol (25%) was used as the control. After 24 h of ABA treatment, plants were challenged by the isolated virulent candidate at a concentration of 2×10^7 CFU mL^{-1}.

Statistical analysis

Each experiment was performed at least 3 times unless stated otherwise. Standard deviation for each treatment was determined. Statistical significance was measured by using the data of the pathogenicity tests by a two-way analysis of variance (ANOVA). Means were separated using Duncan's multiple range test (DMRT; P = 0.05) using SPSS software.

Results

Identification of bacterial wilt causing agent

Morphological studies revealed that the isolated bacterial strain (BR-001) from the wilt infected tomato plants was Gram-negative, rod-shaped, non-capsulated, and non-spore forming (Table S1). The isolate grown on SMSA medium (Engelbrecht-Wiggans 1994) was highly fluidal, white-colored (with a light pink center) and round shape; typical characteristics of *R. solanacearum*. In pathogenicity tests, the isolate produced typical wilt symptoms on tomato within 5–6 weeks after inoculation using root injury (Table 1). The 16S rRNA sequence analysis of the isolate (GenBank Accession No. MH718793) showed 99% similarity with *R. solanacearum* strain UY031 with a max score 2601 (E value 0.0). The distinct phylogenetic position of the strain BR-001 along with *R. solanacearum* UY031 is presented in Fig. 1. Thus, based on 16S rRNA sequence, the isolate was identified as *R. solanacearum* BR-001.

Identification of biocontrol agents from the rhizospheric isolates

All together 43 isolates were obtained from the rhizospheric soil of the unaffected plants. None of these isolates was found to exhibit inhibition zones against *R. solanacearum* in *in-vitro* NA cup plate method. To check the potential of these strains *in-vivo*, four-week-old healthy tomato plants were first root inoculated with a rhizospheric isolates, followed by challenge with *R. solanacearum* BR-001 after 24 h of inoculation. Out of 43 rhizospheric isolates, BRC 2485 exhibited potential biocontrol activity (Table 2). The colony of BRC 2485 grown on YEM agar medium appeared as circular, small (≥ 1 mm in diameter), white, convex, and granular in texture within 5–7 days of incubation. Based on biochemical characterization (Table S2) and 16S rRNA sequence analysis (GenBank Accession No. MK377407), the rhizospheric isolate BRC 2485 was identified as *B. japonicum*. The distinct phylogenetic position of the strain BRC 2485 along with *B. japonicum* HMS-02 is also presented in Fig. 1.

Efficacy of *B. japonicum* BRC 2485 against *R. solanacearum*

To check the efficacy of BRC 2485 against bacterial wilt, a disease progression curve was plotted against time (Fig. 2). From the plot it is clear that BRC 2485 can control bacterial wilt of tomato effectively when challenged with *R. solanacearum*. In control set of experiments, *R. solanacearum* was found to induce heavy wilt (3.8 ± 0.2) at 9th day of inoculation. But tomato plants treated with BRC 2485 show a significant decrease (1.8 ± 0.25) in disease progression within the stipulated time. *B. japonicum* BRC 2485 did not show any in vitro antimicrobial activity against the experimental plant pathogen *R. solanacearum* BR-001 (Table S3), as well as other tested bacterial pathogens (viz. *A. tumefaciens*, *P. syringae*, and *X. campestris* (Table S3).

Table1 Defense responses of tomato plants against *R. solanacearum* BR-001 after eight days priming effect of different *Bradyrhizobium* strains (Each experiment was performed in triplicates with 30 plants in each set)

Experiment	Immunized with	Challenged with	Disease index
1	–	*R. solanacearum* BR-001	4.8 ± 0.45^{a}
2	*B. japonicum* BRC 2485	*R. solanacearum* BR-001	1.34 ± 0.05^{c}
3	*B. japonicum* MTCC 120	*R. solanacearum* BR-001	3.75 ± 0.25^{b}
4	*B. japonicum* MCC 2940	*R. solanacearum* BR-001	4.2 ± 0.35^{a}

0 = no wilting; 1 = 0–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100% of wilted leaves

Different lowercase letters indicate significant differences between groups at the same time (P < 0.05)

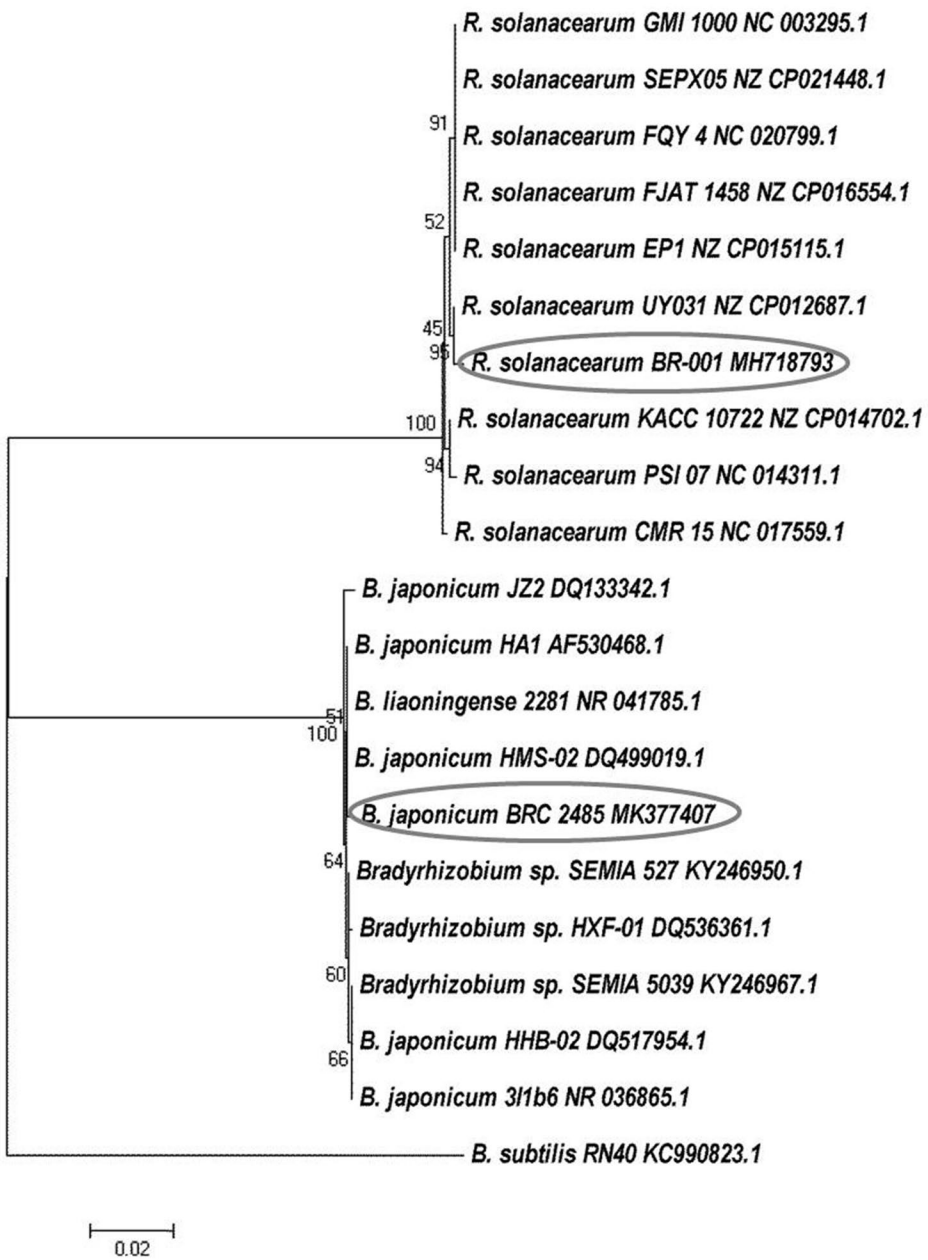

Fig. 1 Bootstrap consensus neighbor-joining tree based on 16S rRNA gene sequences presenting relationship of the pathogenic isolate *R. solanacearum* BR-001 and rhizospheric isolate *B. japonicum* BRC 2485 (marked with circles) with other bacterial strains. *R. solanacearum* BR-001 is showing maximum similarity with *R. solanacearum* UY031, whereas *B. japonicum* BRC 2485 is showing maximum similarity with *B. japonicum* HMS-02. *Bacillus subtilis* RN40 was used as an outgroup. Bootstrap values are presented at the nodes (1000 replications). The scale bar represents 2 substitutions per 1000 bases

A comparative analysis of biocontrol of *B. japonicum* BRC 2485 with reference strains

Four-week-old healthy tomato plants were first inoculated with *B. japonicum* BRC 2485 and two other *Bradyrhizobium* reference strains (MTCC 120 and MCC 2940) in three different sets of experiments, followed by challenge with *R.* solanacearum BR-001. In each experimental set, non-primed tomato plants inoculated with *B. japonicum* BRC 2485 have been taken as a control. Among the three strains used for priming, *B. japonicum* BRC 2485 was recorded to induce maximum protection (1.34 ± 0.05), followed by MTCC 120 (3.75 ± 0.25) and MCC 2940 (4.2 ± 0.35) against infection caused by *R. solanacearum* BR-001 (Table 1).

Priming of *B. japonicum* BRC 2485 limits multiplication of *R. solanacearum* BR-001

To determine whether the reduced disease symptoms are correlated with lower population of *R. solanacearum* BR-001 in plants primed with *B. japonicum* BRC 2485, endophytic bacterial growth was measured from tissue homogenates after 0, 3, 8, and 0 days of the administration of the pathogen (Table 3). Control plants were inoculated either with *R. solanacearum* BR-001 (positive control) or

Table 2 Biocontrol activities of bacterial isolates obtained from the rhizospheric soil of the unaffected tomato plants of the wilt infected field against *R. solanacearum* BR-001

Sr. No	Primed with	Challenged with	Disease index
1	–	BR-001	4.80 ± 0.45^{a}
2	BRC 2431	BR-001	3.80 ± 0.25^{b}
3	BRC 2432	BR-001	3.75 ± 0.25^{b}
4	BRC 2433	BR-001	4.20 ± 0.35^{a}
5	BRC 2434	BR-001	4.70 ± 0.45^{a}
6	BRC 2435	BR-001	3.75 ± 0.25^{b}
7	BRC 2441	BR-001	3.75 ± 0.25^{b}
8	BRC 2445	BR-001	3.85 ± 0.25^{b}
9	BRC 2449	BR-001	3.75 ± 0.25^{b}
10	BRC 2451	BR-001	4.65 ± 0.45^{a}
11	BRC 2452	BR-001	4.75 ± 0.45^{a}
12	BRC 2453	BR-001	3.75 ± 0.25^{b}
13	BRC 2456	BR-001	3.85 ± 0.25^{b}
14	BRC 2466	BR-001	3.65 ± 0.25^{b}
15	BRC 2475	BR-001	3.55 ± 0.25^{b}
16	BRC 2478	BR-001	3.85 ± 0.25^{b}
17	BRC 2479	BR-001	3.80 ± 0.25^{b}
18	BRC 2480	BR-001	3.55 ± 0.25^{b}
19	BRC 2481	BR-001	4.80 ± 0.45^{a}
20	BRC 2482	BR-001	3.75 ± 0.25^{b}
21	BRC 2484	BR-001	4.75 ± 0.45^{a}
22	**BRC 2485**	**BR-001**	$\mathbf{1.34 \pm 0.05^{c}}$
23	BRC 2486	BR-001	4.80 ± 0.45^{a}
24	BRC 2487	BR-001	4.65 ± 0.45^{a}
25	BRC 2488	BR-001	3.85 ± 0.25^{b}
26	BRC 2489	BR-001	4.60 ± 0.45^{a}
27	BRC 2490	BR-001	4.65 ± 0.45^{a}
28	BRC 2491	BR-001	3.80 ± 0.25^{b}
29	BRC 2493	BR-001	3.75 ± 0.25^{b}
30	BRC 2495	BR-001	3.85 ± 0.25^{b}
31	BRC 2498	BR-001	3.75 ± 0.25^{b}
32	BRC 2500	BR-001	3.85 ± 0.25^{b}
33	BRC 2519	BR-001	3.65 ± 0.25^{b}
34	BRC 2520	BR-001	3.55 ± 0.25^{b}
35	BRC 2528	BR-001	4.80 ± 0.45^{a}
36	BRC 2529	BR-001	3.75 ± 0.25^{b}
37	BRC 2530	BR-001	3.65 ± 0.25^{b}
38	BRC 2531	BR-001	3.85 ± 0.25^{b}
39	BRC 2541	BR-001	3.75 ± 0.25^{b}
40	BRC 2542	BR-001	4.75 ± 0.45^{a}
41	BRC 2543	BR-001	4.75 ± 0.45^{a}
42	BRC 2544	BR-001	4.80 ± 0.45^{a}
43	BRC 2545	BR-001	4.65 ± 0.45^{a}

Based on the disease index, we have selected this strain for further study (in bold)

0 = no wilting; 1 = 0–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100% of wilted leaves

Different lowercase letters indicate significant differences between groups at the same time ($P < 0.05$)

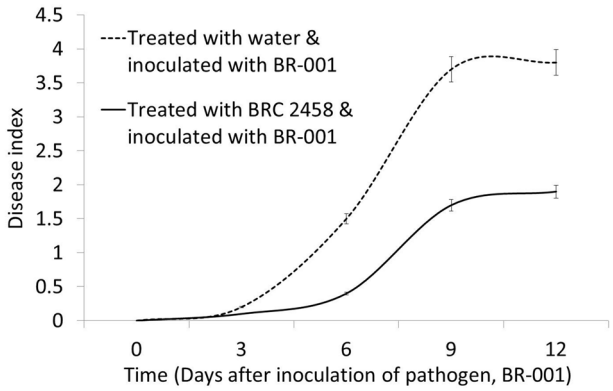

Fig. 2 Disease symptom development curves: Inoculated *B. japonicum* BRC 2485 protects tomato plants from a subsequent challenge with *R. solanacearum* BR-001. Plants were scored daily using a scale between 0 and 4. Means and standard errors (SE) were calculated from a total of 30 plants per assay (from three independent experiments)

with *B. japonicum* BRC 2485 (negative control). When tomato plants primed with *B. japonicum* BRC 2485 were challenged with *R. solanacearum*BR-001, the BR-001 population remained low (4.10 ± 0.55Log CFU g FW^{-1}) in comparison to positive control (12.25 ± 2.25 Log CFU g FW^{-1}) after 8 days of challenge (Table 3). Even, co-inoculated (1:1) plants also showed difference in *R. solanacearum* BR-001 population (4.50 ± 0.55 Log CFU g FW^{-1}) after 8 days of co-inoculation (Table 3). After 10 days of challenge all the plants of the positive control died due to disease progression. Therefore, to demonstrate the effect of *B. japonicum* BRC 2485 on *R. solanacearum* BR-001 *in-vivo*, endophytic populations of both strains were plotted after 8 days of challenged period (Fig. 3). There is a huge difference in the population count of positive (inoculated only with *R. solanacearum* BR-001) and negative (inoculated only with *B. japonicum* BRC 2485) control (Fig. 3a). However, the endophytic population of *B. japonicum* BRC 2485 does not vary much after challenge with *R. solanacearum* BR-001 even in comparison with negative control. But, the endophytic population of *R. solanacearum* BR-001 remains drastically low in plants primed with *B. japonicum* BRC 2485 (Fig. 3a). The trend remains the same even when both strains were co-inoculated in a ratio of 1:1. The endophytic population of *R. solanacearum* BR-001 varies from 45 to 48% in case of immunized and co-inoculated plants, respectively (Fig. 3b).

Production of siderophore, ethylene, and ABA by experimental *Bradyrhizobium* strains

Siderophores secreted by pathogens regulate the production of many virulence factors (e.g., exotoxin, endoprotease, pyoverdine, etc.), which are major contributors to the ability of

Table 3 Effect of priming of tomato plants with *Bradyrhizobium* MCC 2485 on multiplication of *R. solanacearum* BR-001 inside plants (Each experiment was performed in triplicates with 30 plants in each set)

Sr. No	Primed with	Challenged with	Multiplication of *R. solanacearum* BR-001 (log CFUg FW^{-1}) inside plant			
			Day 0 after challenge	Day 3 after challenge	Day 8 after challenge	Day 10 after challenge
1	BRC 2485	–	0	0	0	0
2	BRC 2485	BR-001	2.35 ± 0.45^{d}	3.05 ± 0.55^{c}	4.10 ± 0.55^{b}	6.28 ± 0.75^{a}
3	–	BR-001	4.28 ± 0.50^{c}	6.10 ± 0.75^{b}	12.25 ± 2.25^{a}	Not detected (due to death of all plants)
4	Co-inoculated BRC 2485: BR-001 (1:1)	2.50 ± 0.45^{d}	3.45 ± 0.55^{c}	4.50 ± 0.55^{b}	5.78 ± 0.75^{a}	

Different lowercase letters indicate significant differences between groups at the same time ($P < 0.05$)

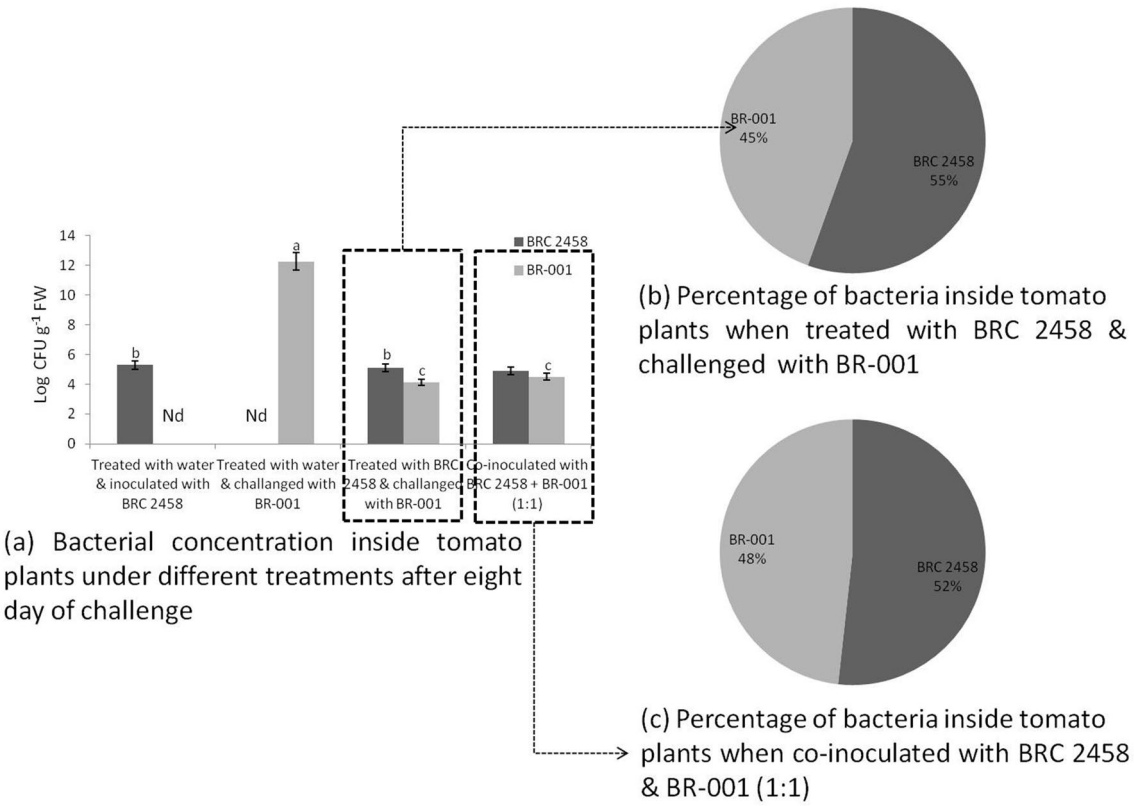

Fig. 3 Effect of *B. japonicum* BRC 2485 on multiplication of *R. solanacearum* inside tomato plants: **a** Bar diagram showing different multiplication pattern of *R. solanacearum* and *B. japonicum* in control, in *B. japonicum* BRC 2485 inoculated plant, and in co-inoculated (1:1) plants; **b** pie chart sowing competitive growth of *R. solanacearum* and *B. japonicum* in *B. japonicum* BRC 2485 treated plants; **c** pie chart sowing competitive growth of *R. solanacearum* and *B. japonicum* in co-inoculated (1:1) plants

the pathogen to disease establishment. *B. japonicum* BRC 2485 and the reference *Bradyrhizobium* strains were unable to produce siderophores. But all these strains were able to produce ethylene. The production of ethylene in liquid cultures was confirmed by GC–MS analysis (Fig. 4). The ethylene production was recorded highest in MTCC 120 (8.1 ng mL^{-1} h^{-1}), followed by *B. japonicum* BRC 2485 (3.5 ng mL^{-1} h^{-1}) and MCC 2940 (2.0 ng mL^{-1} h^{-1}) on 8th day of inoculation (Table 4). ABA production was measured through a HPLC–UV detection system. Only *B. japonicum* BRC 2485 was found to have the unique property of ABA production (Fig. 5). The ABA concentration (0.019 µg mL^{-1} h^{-1}) was measured on 8th day of inoculation (Table 4).

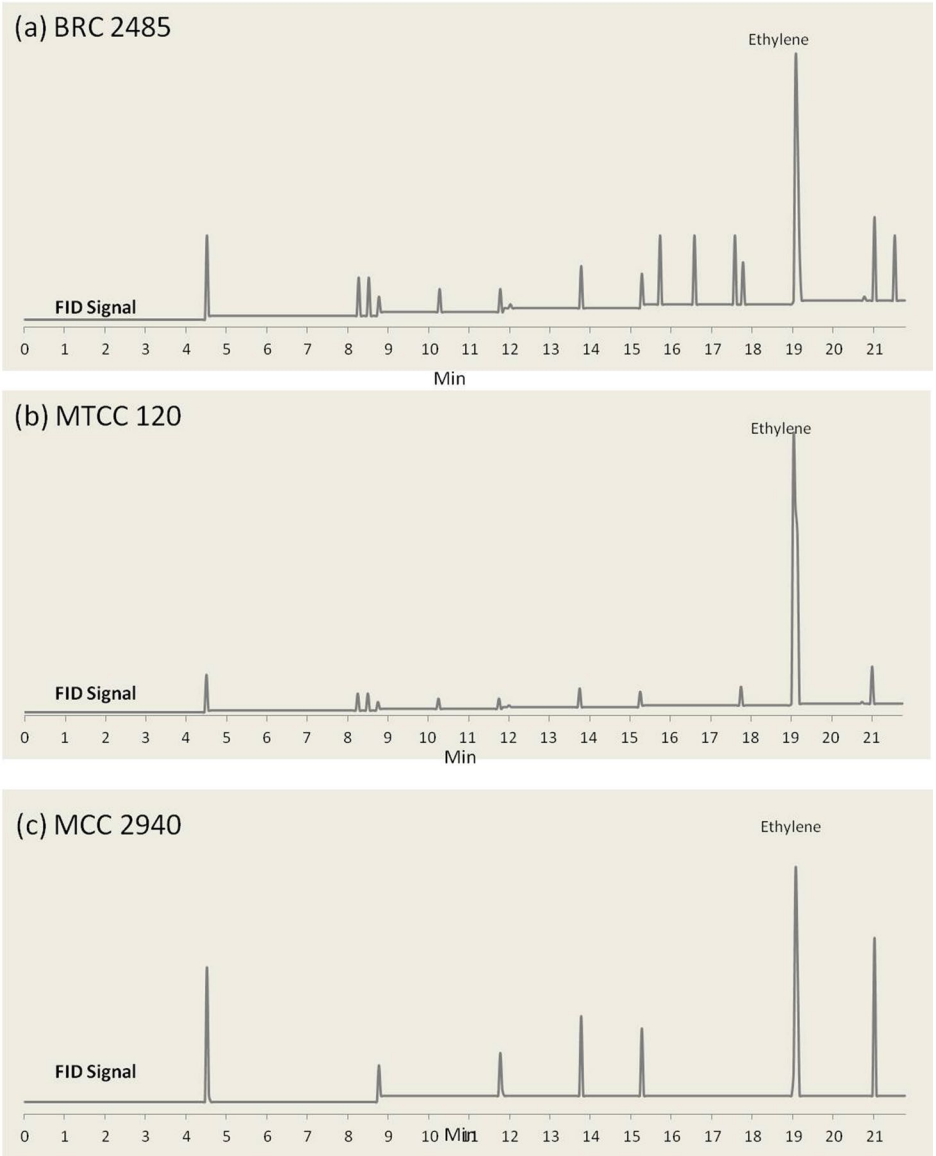

Fig. 4 Detection of ethylene by GC-FID analysis of 7-day-old YEM cultures of *B. japonicum* BRC 2485 along with two reference *Bradyrhizobium* strains (MTCC 120, and MCC 2940). **a**–**c** represent detection curves from three strains, respectively

Table 4 Production of siderophore, ethylene, and ABA by experimental *Bradyrhizobium* strains up to 8th day of fermentation

Bradyrhizobium strains	Siderophore production	Ethylene production (ng mL^{-1} h^{-1})	ABA production (µg mL^{-1} h^{-1})
BRC 2485	Not detected	3.5 ± 0.24	0.019 ± 0.002
MTCC 120	Not detected	8.1 ± 0.50	Not detected
MCC 2940	Not detected	2.0 ± 0.24	Not detected

Effect of exogenous ABA on wilt control

To check the effect of exogenous ABA against bacterial wilt, disease index and internal colonization of the pathogen were studied against three different concentrations (Table 5). From the table it is clear that exogenous ABA (2.0 µgmL^{-1}) effectively reduces the disease index and internal colonization of the virulent strain. However, a lower concentration (0.02 µg mL^{-1}) of exogenous ABA could not effectively control the disease progression.

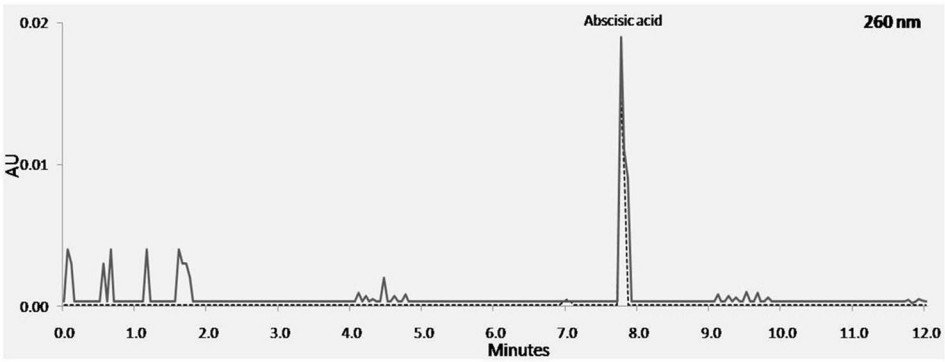

Fig. 5 Detection of ABA by HPLC–UV analysis in f 7-day-old YEM cultures of *B. japonicum* BRC 2485. The dotted line represents the standard ABA procured from Sigma-Aldrich (A1049). ABA production was not detected in YEM cultures of MTCC 120, and MCC 2940

Table 5 Effect of priming of tomato plants with exogenous ABA on disease index and multiplication of *R. solanacearum* BR-001 inside plants at 8th day of inoculation (Each experiment was performed in triplicates with 30 plants in each set)

Treatment	Disease Index	Multiplication of *R. solanacearum* BR-001 (log CFUg FW^{-1})
Control (25% aq. ethanol)	4.8 ± 0.45^{a}	12.25 ± 2.25^{a}
ABA 0.02 µg mL^{-1}	3.55 ± 0.25^{a}	10.25 ± 1.25^{b}
ABA 0.2 µg mL^{-1}	2.25 ± 0.25^{a}	8.50 ± 1.25^{b}
ABA 2.0 µg mL^{-1}	1.75 ± 0.5^{b}	5.25 ± 0.5^{c}

Different lowercase letters indicate significant differences between groups at the same time ($P < 0.05$)

Discussion

Bradyrhizobium is a well-known bacterial genus for biological nitrogen fixation. It is already known that *Bradyrhizobium* strains can colonize in root nodules of Fabaceae family plants including soybean, kidney bean, and cowpea (de Matos et al. 2017; Pena-Cabriales and Alexander 1983). *B. japonicum* was also reported to be endophytic in the roots of non-modulating plants like sugarcane, sweet corn, and cotton (de Matos et al. 2017; McInroy and Kloepper 1995). Different *Bradyrhizobium* strains are known to control disease caused by fungal phytopathogens including *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium* spp. (Ehteshamul-Haque and Ghaffar 1993). Rhizobial protection against *Fusarium* wilt was previously reported on chickpea (Arfaoui et al. 2006). Only a few reports are available about the antagonistic activity of *Bradyrhizobium* strains against bacterial phytopathogens (Gross and Vidaver 1978; Kohlmeier et al. 2015). In the present investigation, for the first time a *B. japonicum* strain was reported to control bacterial wilt disease effectively.

In general, there are three possible suppression mechanisms which are commonly used by different BCAs against phytopathogens: (1) production of antimicrobials and siderophores, (2) competition, and (3) induced systemic resistance (ISR). Few *Bradyrhizobium* strains are known to produce bacteriocin (Kohlmeier et al. 2015). On the other hand, few *Bradyrhizobium* strains have been reported to produce siderophores to overcome iron starvation (Plessner et al. 1993). However, in the present investigation, *B. japonicum* BRC 2485 along with reference *Bradyrhizobium* strains does not show any antimicrobial activity. All the strains were also negative for siderophore production.

Previously, it was reported that BCA and pathogenic strains compete to invade xylem vessels in tomato (Etchebar et al. 1998; Lace and Ott 2018). Bacterial wilt disease progression in tomato can be easily correlated with colonization of the phytopathogen. In the present investigation, priming effect of *B. japonicum* BRC 2485 limits the multiplication of *R. solanacearum* BR-001 within the host plant. The present experimental evidence suggests that the *B. japonicum* BRC 2485 provides good competition to *R. solanacearum* BR-001 for colonization within the plant host.

The mechanism of ISR within the host plant may differ from one species to another. For example, ISR against *R. solanacearum* by many plants growth-promoting rhizobacteria (PGPR) is mediated through the jasmonic acid and ethylene dependent signaling pathways (Hyakumachi et al. 2013). But ISR by *Bacillus thuringiensis* against *R. solanacearum* is mediated through the salicylic acid-responsive defense-related genes (Heil and Bostock 2002). On the other hand, ISR against *R. solanacearum* in *Arabidopsis thaliana* was achieved by the Δ*hrpB* mutant of *R. solanacearum* through ABA-responsive defense-related genes (Feng et al. 2012). In the present investigation, the rhizospheric isolate, *B. japonicum* BRC 2485 was found to have the unique property to produce ABA. Boiero et al. also have reported ABA synthesis by *B. japonicum* USDA110 (Boiero et al. 2007). Therefore, there may be a probability that the ABA produced by *B. japonicum* BRC 2485 may trigger ISR of tomato plants. In the present investigation, exogenous ABA (2.0 µg mL^{-1}) effectively reduced the disease index and internal colonization of the virulent strain. These results may be correlated

with the findings of Hu and Bidochka (2021). They have reported ABA as key player in differential responses to endophytic colonization by *Metarhizium* and pathogenic colonization by *Fusarium*. However, ABA production by *B. japonicum* BRC 2485 *in* vitro was found to be as low as 0.0 19 ± 0.002 µg mL^{-1}(0.02 µg mL^{-1} approx.). Application of exogenous ABA of the same concentration failed to demonstrate the same effect produced by *B. japonicum* BRC 2485. ABA-mediated immune response of tomato plants is still not fully elucidated, and the present investigation could not provide any insight into the plant immunity aspect.

Conclusion

In this present study, biological control of bacterial wilt using an ABA-producing *B. japonicum* BRC 2485 has been demonstrated. The observed biocontrol activity in tomato plants may be explained by competition or ABA production or both by the *Bradyrhizobium* strain. However, its effectiveness should be investigated at different agro-climatic conditions. Further investigation on ISR against *R. solanacearum* in tomato plants through ABA-responsive defense-related genes will be important. We strongly believe that this information will be helpful to develop new-generation biocontrol agents.

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Declarations

Conflict of interest None of the authors have any conflict of interest.

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