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Burkholderia contaminans: a potential soil-borne antagonist against *Rhizoctonia solani* causing black scurf and stem canker in potato

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ABSTRACT

Antagonistic bacteria provide a greener alternative to synthetic fertilizer that also promote the plant growth in addition to preventing soil-borne fungus. In the present study, 10 bacterial isolates were isolated from the stem or leave and tuber of the potato from the research field of Gazipur Agricultural University (GAU), Gazipur, Bangladesh. The *in vitro* dual culture assay confirmed the antagonism of three isolated bacteria against potato black scurf and stem canker fungi *Rhizoctonia solani*. The selected antagonistic bacteria were characterized with different biochemical tests. The bacterial strains were Gram negative rods. Positive results in protease activity, potassium solubilization, phosphate solubilization, starch solubilization, and urease tests signified their potentiality as contributors to solubilize protein, potassium, phosphorus, starch, and ammonium in soil. The positive result during indole acetic acid (IAA) test indicated the growth promotion activities of the three isolates. Molecular data identified the bacterial strains B_2 (MZ396406), B_3 (MZ396407), and B_4 (MZ396408) as *Burkholderia contaminans*. Among the three strains, B_4 (MZ396408) significantly improved plant growth, suppressed the specific disease symptom, and increased potato yield during field and pot trials. The maximum plant height (29.75 cm), branching (4.73), leaf number per plant (57.3), yield (999.67 g/5p) was recorded in the B_4 (MZ396408) treated plot in the field experiment. Whereas, minimum plant height (16.00 cm), branching (3.2), leaf number per plant (31.23), yield (523.00 g/5p) was recorded in negative control in the field experiment. The highest severity of stem canker (65%), black scurf (30%) and presence of sclerotia percentage (80%) was recorded in *R. solani* inoculated plot, whereas the lowest (0%) stem canker, black scurf and presence of sclerotia were found in the treatment B_4 (MZ396408) treated plot. This strain can be used as reference strain for further study.

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Abbreviations: IAA, Indole Acetic Acid; RS, *Rhizoctonia solani*; PDA, Potato Dextrose Agar; YPDA, Yeast Extract Peptone Dextrose Agar; NA, Nutrient Agar; YPD, Yeast Peptone Dextrose; PI%, Percent growth inhibition; PCR, Polymerase Chain Reaction; BARI, Bangladesh Agricultural Research Institute; RCBD, Randomized Complete Block Design; PCNB, Penta-Chloro-Nitrobenzene; DAP, Days After Planting.

Introduction

Rhizoctonia solani Kühn [teleomorph *Thanatephorus cucumeris* (Frank) Donk] is a destructive soilborne pathogen associated with severe damage to many agricultural crops, including potato (Sneh *et al.*, 2013, Kotba *et al.*, 2018, Parmeter, 2023). This fungus is one of the most important phytopathogenic agents due to its wide host range, large geographical distribution, and adaptability to different environmental conditions (Sneh *et al.*, 2013). On potato (*Solanum tuberosum*), this pathogen causes brown to black sunken canker lesions (on sprouts, stems, stolons, and roots); leaf rolling, purple leaf pigmentation as foliar symptoms; and black scurf disease in all potato-growing areas worldwide (Lehtonen *et al.*, 2008, El Khaldi *et al.*, 2016,). Besides, the fungus can produce necrotic lesions on sprout tips that may inhibit or delay the emergence of the sprout, develop cracked or malformed tubers, and sometimes the development of green aerial tubers on stems above the soil (Hicks *et al.*, 2014, El Khaldi *et al.*, 2016) which cause marketable yield losses (up to 30%). In addition, sclerotia on tubers are considered to be a very important inoculum as they contribute to the long-distance dispersal, can remain active in the soil for several years under adverse environmental conditions and infect potato plants more rapidly.

Rhizoctonia diseases of potato are commonly controlled using combinations of cultural methods such as crop rotation, reduced tillage, certified (disease-free) seed potatoes, and chemical control of inoculum (Hicks *et al.*, 2014). Chemical control of *R. solani* can effectively reduce tuber-borne inoculum, but the soil-borne inoculum is difficult to control with fungicides in case of high level of initial inoculum (Wilson *et al.*, 2008). Therefore, chemical control is no longer considered as the most effective tool to limit the pathogenic growth and distribution. Moreover, the continuous use of expensive chemical fertilizers and fungicides has adversely impacted human health and negatively impacted the environment (Zaman *et al.*, 2021).; hence, the safe use of microorganisms that improve soil fertility, enhance plant growth, and limit the growth of phytopathogenic fungi has been receiving immense attention from researchers (Adesemoye *et al.*, 2009).

Recently, most of the species of *Burkholderia* widely used as a potential biocontrol agent against a wide range of phytopathogens, including fungi, bacteria, and yeast in many diversified crops such as tomato, grapevine, and maize (Elshafie and Camele, 2021). This microorganism is a well-known producer of

many bioactive metabolites like bacteriocins, alkaloids, lipopeptides, and polypeptides which draw the attention of several researchers to assess as a biocontrol agent (Winkelmann, 1998). As some genera, such as *B. cepacia* complex, are ubiquitous in the environment and capable of infecting people with cystic fibrosis (Savi *et al.*, 2019), it is needed to carefully studies before use this bacterium. However, the virulence mechanisms of *Burkholderia* are not completely revealed yet. Moreover, studies have shown that antifungal compound producer *Burkholderia* species have not been identified as pathogenic potentiality (Eberl and Vandamme, 2016).

Previously, commercial biological control products were assessed for the control of *R. solani* on potato with some success (Brewer and Larkin 2005, Wilson *et al.*, 2008), although none effectively controlled stem canker and black scurf (Hicks *et al.*, 2014). In this context, the present investigation was undertaken to isolate, identify, and evaluate the biocontrol efficacy of three antagonistic bacteria (isolated from potato tuber, stem or leaves) against *R. solani* of potato.

Materials and Methods

Collection, isolation, and identification of potato stem canker fungi

Black scurf and stem canker symptoms (from the leaves, stems, and tubers) were collected from the potato fields at GAU, Bangladesh, and kept in a sterilized plastic bag, then stored in the refrigerator (4°C) until further use. The target pathogen was isolated according to prior references (Carling and Leiner, 1986, Goswami *et al.*, 2010) with slight modifications. Briefly, a surface sterilized thin layer of infected tissues was placed onto a Petri dish (90-mm diameter) containing water agar (1% agar) and incubated at 25°C for 72 h. Later, pure culture was obtained by hyphal tip method and cultured onto a PDA plate. A total of 2 isolates (RS1 & RS2) were identified as *R. solani* on the basis of morphological character such as and preserved at -18°C until further study.

Pathogenicity tests of Rhizoctonia solani

A highly susceptible Potato cultivar "Diamant" was used for pathogenicity tests and further experiments (Khandaker *et al.*, 2006). Two isolates (RS1 & RS2) were used in the pathogenicity test using potato seedlings (grown in a 20 cm plastic pot) filled with sterilized soil. The *R. solani* inoculum was prepared in wheat seed according to Rubayet and Bhuiyan (2016). Then, inoculum of the *R. solani* isolate was prepared on autoclaved moist wheat grains in 500 ml Erlenmeyer flask. Before using, wheat grains were soaked in water for 12 hours. After soaking excess water was drained out and water-soaked grains were poured into 500 ml Erlenmeyer flask. Five-millimeter diameter mycelial discs were cut from the edge of three days old PDA cultures in Petri dishes. Five to seven mycelial discs of pathogen were added to autoclaved wheat grains in the flasks and incubated at 25°C for 21 days. It was shaken by hand at 2-3 days interval for proper colonization. The colonized wheat grains were air dried for two days and stored at 10^0 C for further use.

The inoculum of the two isolates was prepared separately, incubated at 25°C for 7 days, and 5 pots were inoculated with each isolate inoculum separately. Each pot (containing 2 kg of sterilized soil) was inoculated with 10 g of wheat inocula from each of two isolates separately. Five pots (approximately 2 kg sterilized soil/pot) were inoculated with each isolate inoculum separately. Two potato seedlings of 2 weeks old were planted in each *R. solani* inoculated pots. There were three replicates for each isolate, and the experiment was repeated three times. The symptoms of stem canker were observed at 2 weeks after the inoculation and evaluated according to Tsror *et al.* (2001) and López-Corrales *et al.* (2023). The more pathogenic isolate was selected for the *in vitro* and *in vivo* experiments.

Collection, isolation, and preservation of antagonistic bacteria

The isolation of antagonistic bacteria from healthy potato plant parts (leaves, stems, and tubers from susceptible cultivar Diamant) was carried out according to Berg *et al.* (2005) with slight modifications. The leaf and stem samples were collected aseptically using sterile plastic bags and kept at 4°C until further processing. Three grams of sample from each of the leaves and stems were transferred separately to 27 ml of a 0.85% NaCl solution in sterile Erlenmeyer flasks and shaken in a rotary shaker for 10 min. The tuber samples were cleaned, disinfected (1% NaOCl for 20 m), washed thrice (with sterile distilled water), dried (for 1 h in a laminar air flow), cut into small slices (approximately 2×2 cm^2), homogenized with mortar/pestle, and mixed in isotonic solution (0.85% NaCl). Aliquots of 100 µl of each suspension were spread onto Yeast extract Peptone Dextrose Agar (YPDA) medium and incubated for 5 d at 25°C. The bacterial colonies from each plate were separated on the basis of different colony characteristics such as colony size, form, colour, texture, and margin. Finally, the purified colonies were stored at -70°C containing 70% glycerol. Sixteen bacterial strains were collected from the potato stems, leaves, and tubers

Evaluation of in vitro antagonism of the selected bacteria against Rhizoctonia solani

The competitive interaction of selected bacteria antagonists against the selected pathogenic fungi *R. solani* (RS1) was assessed through the dual culture method, according to Xu and Kim (2014) with slight modification. Briefly, the bacterial isolates were grown on YPDA plates for 24 h at 25°C. Pathogenic *R. solani* (RS1) was cultured separately on PDA plates for 5 d at 25°C. A mycelial disc of RS1 (1cm diameter) was cut from the edge of 5 d old colony and placed at the edge of a freshly prepared PDA plate. Three bacterial isolates (B_2, B_3 and B_4) were selected for the *in vitro* antagonism test. Selected bacterium isolate was separately inoculated through streaking by a sterilized wire-loop at 1.5 cm distance from the fungal disc and from the opposite edge of the same plate. Control plates were inoculated with *R. solani* isolate and streaked with distilled water instead of bacterial antagonists. All plates were incubated at 25°C for 7 d. There were three replicates for this experiment, and the experiment was

repeated three times. The antagonistic effect was determined by observing the inhibition zone formed on plates during the incubation period. The percent growth inhibition (PI) of pathogenic fungi was assessed according to Lahlali and Hijri (2010)

$$P1(\%) = \frac{Rc - Ri}{Rc} \times 100$$

Where,

Ri = The radial mycelial growth of *R. solani* towards the antagonistic bacteria

Rc = The radial mycelial growth of *R. solani* on a control plate

Later, more antagonistic bacterial strains were selected for further studies.

Molecular characterization of strain

DNA extraction, PCR amplification, and sequencing

Genomic DNA of bacterial strains was extracted using the alkaline lysis method (Sambrook and Russell, 2001) with minor modifications. Extracted DNA was stored at -20°C. The universal bacterial forward primer 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and reverse primer 1541r (5′-AAG GAG GTG ATC CAG CC-3′) were used to amplify 16S rRNA gene in a thermal cycler (Yang *et al*., 2016). A total of 35 PCR cycles were performed consisting of 40 seconds at 94°C, 40 s at 55°C, and 60 s at 72°C was followed by a final extension step at 72°C for 7 min. The PCR products were visualized by gel electrophoresis. PCR products were sent to the National Institute of Biotechnology, Savar, Bangladesh, for cleanup and sequencing.

Phylogenetic analysis

The nucleotide sequences were edited in Geneious v10. Basic Local Alignment Search Tool (BLAST) search was used to search for similar sequences in the National Centre for Biotechnology Information (NCBI) database (Tatusova *et al.*, 2014). Multiple alignments of the nucleotide sequences along with reference strains were done using a multiple sequence alignment program MAFFT v7.2.8 in the Geneious v10 Plugin (Katoh and Standley, 2013). The maximum-likelihood phylogenetic tree from nucleotide alignment was constructed using RAxML in the Geneious v10 plugin. The phylogenetic tree was visualized by Tree Graph. The annotated sequence information of isolates B_2, B_3 and B_4 was deposited in NCBI's GenBank under the accession numbers MZ396406, MZ396407, and MZ396408, respectively.

Biochemical and physiological characterization of bacterial antagonists

A number of tests were conducted to assess the biochemical and physiological characteristics of two days old bacterial culture (grown on YPDA). The gram reaction of the selected antagonist bacterial isolates was carried out as described by Vincent and Humphrey (1970), while the activity of oxidase, catalase, and KOH solubility test were performed based on Bergey's Manual of Systematic Bacteriology (Bergey, 1994). Other biochemical tests, such as the oxidative fermentative (O-F) test (Lemos

et al., 1985), gelatin hydrolysis test (Schaad *et al.*, 2001), and protease test (Sharmin *et al.*, 2005) were performed according to the reference.

Plant growth promoting activities of bacteria

Potassium (K) solubilization

To screen for K-solubilizing rhizobacteria, isolates were grown on Aleksandrov agar medium [per liter: 5.0 g glucose, 0.5 g $MgSO_4.7H_2O$, 0.005 g $FeCl_3$, 0.1 g $CaCO_3$, 2 g $Ca_3(PO_4)_2$, 2 g $AlKO_6Si_2$ (potassium aluminum silicate, a source of insoluble inorganic potassium), and 15 g agar]. For control, each bacterial strain was marked onto $AlKO_6Si_2$-deficient Aleksandrov agar plates. Inoculated plates were incubated at 28°C for 7 d. The appearance of a clear zone around the colonies indicated a positive potassium solubilization (Haque *et al.*, 2020).

Phosphate solubilization

Qualitative phosphate (P) solubilization ability was assessed according to a previously described method. In brief, 2 µL of overnight-grown culture (10^7 CFU mL^{-1}) was spotted (1 loop/plate) onto National Botanical Research Institute's phosphate (NBRIP) agar [per liter: 10 g glucose, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar] plates containing 0.5% $Ca_3(PO_4)_2$ or 0.8% rock phosphate. For control, each bacterial strain was dotted onto $Ca_3(PO_4)_2$ free NBRIP agar plates. The phosphate solubilization index (SI) of bacteria was calculated as the ratio between the diameter of the halo and the diameter of the colony (Tagele *et al.*, 2018).

Starch solubilization

For screening starch solubilizing bacteria, isolates were grown on Starch agar medium [per liter: 3.0 g beef extract, 3.0 g starch, 5.0 g tryptone, 1000 ml distilled water, and 15 g agar, adjusting the p^H to 7]. Inoculated plates were incubated at 28°C for 7 d. The appearance of a yellow halo around the colonies indicated positive starch solubilization. After that, the Petri dish were treated with Lugols iodine solution (10 g potassium iodide, 5 g iodine, and 1000 ml distilled water).

Indole production

A single colony of each bacterium was inoculated in 5 ml Luria-Bertani (LB) broth (1% of tryptone, 0.5% of yeast extract, 0.5% of NaCl, pH was adjusted to 7.0) supplemented with 0.2% of L-tryptophan (Bio Basic Inc., Canada) and incubated at 28°C under shaking conditions (160 rpm). After 48 h incubation, 1 ml culture was collected and centrifuged at 14000 rpm for 10 min. Then, 500 µL supernatant was transferred to a sterile glass test tube and mixed with 1 ml Salkowski reagent (98 ml 35% perchloric acid and 2 ml 0.5 M $FeCl_3$). The test tubes were incubated at room temperature for 45 min in the dark. The development of a reddish-orange color indicated a positive for auxin production (Haque *et al.*, 2020).

Urease production

A modified yeast salt (YS) broth was prepared by adding 0.5 g $NH_4H_2PO_4$, 0.5 g K_2PO_4, 0.2 g $MgSO_4.7H_2O$, 5.0 g NaCl, 1.0 g yeast extract,

and chresol red 0.016 g dissolved in 1000 ml distilled water. The medium was autoclaved, and filter sterilized urea solution was added to that medium to give it 2% final concentration. 5 ml of the medium was poured into each sterile test tubes and were inoculated with bacterial cells of fresh culture and incubated at 30℃. An increase in alkalinity indicated by the magenta (pink) color was evidence of rapid-urease-positive organisms within 24 h (Schaad *et al.*, 2001).

Effect of different cultural conditions of the bacterial isolates

Growth on both NA and YPDA

The growth test of the selected isolates was performed as described by Schaad *et al.* (2001). Five specific growth mediums, such as NA, YPDA, NA + 6% NaCl, YPDA + 6% NaCl, and YS broth, were used for this test. Twenty-four-hour old bacteria culture on NA medium was used as an inoculum source for this experiment. 3 g beef extract, 5 g peptone, and 15 g agar were mixed in 1000 ml distilled water to prepare NA media. 3 g dextrose, 0.6 g peptone, 3.0 g yeast extract, and 20 g agar were dissolved in 1000 ml distilled water to prepare YPDA media. Freshly prepared YPDA and NA media were sterilized and poured into petri dishes, then allowed to solidify. One loopful of bacterial suspension (water containing bacteria) was streaked on the solidified NA and YPDA medium for each plate. The plates were incubated at 4°C for 48 h. A pair of similar experiments was set again with two distinguished incubation temperatures such as at 28°C, 36°C and 45°C.

In addition, both NA and YPDA media were supplemented with 6% NaCl to check their influence on bacteria growth. One loopful of bacterial inoculum was inoculated with this 6% NaCl supplemented media. The growth was observed at 28°C for 2 to 7 d.

Growth in YS broth

YS broth was used by adding 0.5 g $NH_4H_2PO_4$, 0.5 g K_2PO_4, 0.2 g $MgSO_4.7H_20$, 5.0 g NaCI, and 5.0 g yeast extract dissolved in 1000 ml of distilled water and the pH of the solution was adjusted at 7.0. Five ml of freshly prepared YS broth medium was poured into a test tube, plugged, and autoclaved at 121°C for 20 min. Freshly cultured (24 h) bacterial suspension was inoculated into the tubes with a wire loop. Then, the tubes were placed in a running hot water bath and maintained a temperature of 36°C or 28 °C or 45°C.

The bacterial growth was recorded as positive growth (+) or negative growth (-) in different cultural conditions (media, temperature, and incubation period).

Morphological changes of Rhizoctonia solani (RS1) against antagonistic bacteria using microscopy

A mycelial disc of RS1 (1cm) was cut from the edge of the inhibition zone between the fungi and antagonistic bacteria. Only three selected antagonistic bacteria were used in this study. The mycelial disc was mounted on a glass slide containing 70% glycerol. Then, the morphological changes due to the antagonistic effect of the selected bacterium were recorded

under a compound light microscope (Zeiss 3116017489; Heidelberg, Germany) at 400× magnification and compared the morphological changes with the mycelia of *R. solani* grown in the control plate.

In vitro antagonism through volatile compound production in a dual culture plate

This experiment was conducted as 'sandwich plate method' according to Ebadzadsahrai *et al.* (2020) with some modifications. A single Petri plate containing PDA was inoculated with 0.5 cm disc (collected from the edge of 3 days old culture) of the selected *R. solani* isolate (RS1). A second Petri plate containing YPDA medium was streaked with a single bacterial antagonist (collected from 24 h old culture on YPDA) isolate. Later, the lid of the RS 1 and bacterial antagonist inoculated plates are opened and placed in an inverted manner. Finally, the plates were sealed with a sterilized parafilm. For each bacterial isolate, 3 replications were maintained and the experiment repeated three times. The control was prepared using the same experimental setup, except for the bacterial isolate in the second petri plate. All plates were incubated at 25°C. The inhibition rate of each antagonist against pathogenic fungus was calculated based on the above-mentioned formula Lahlali and Hijri (2010) then the data were statistically analyzed.

In Planta antagonism test of selected antagonistic bacteria in pot trial

Collection and preparation of plant material and soil

The seed tubers (cultivar Diamont) were collected from BARI, Gazipur, Bangladesh, for this study. The soil used for the pot experiment was collected from the upper 30 cm of the crop field soil in GAU, Gazipur, and was autoclaved at 121°C for 1 hr before use. The pots (20 cm) were sterilized with 70% ethanol and filled with 600 g steam-sterilized soil. One seed tuber was sown per pot. The pots were grown in the temperature range from 25 to 35 °C and watered with sterile water. The potato tubers were surface sterilized with 70% ethanol for 30 sec followed by rinsing with sterilized distilled water twice. In each pot, the soil was fertilized with 2 g Urea, 2g TSP, and 0.5 g MOP.

Inoculation of Rhizoctonia solani in the pot soil

As both the isolates (RS1 and RS2) were pathogenic to potato tubers the more pathogenic isolate (RS1) was used for this study. The inocula of *R. solani* were prepared in the wheat grain and inoculated in the pot soil (50 g/pot) following the same process mentioned above. The soil treated with non-colonized PDA discs served as the negative control.

Preparation of bacterial inocula

Three bacterial isolates were inoculated into Yeast Peptone Dextrose broth (YPD) liquid medium and incubated at 28°C on a rotary shaker at 150 rpm for 48 h in the dark. After

incubation, the broth containing bacteria was placed for centrifuge at 8000 rpm, maintaining 20°C temperatures for 5 min. The soil near the root zone was drenched with 10 ml of a bacterial suspension (10^8 CFU/ml) at 15 d intervals (Tagele *et al.*, 2018).

The treatments used in the pot trials were T_1= Negative control 1 (no soil inoculation), T_2= Negative control 2 (soil inoculation with *R. solani* at 50 g/pot), T_3= T_2 + B_2 (Bacterial strain 1, at 10 ml suspension/plant), T_4 = T_2 + B_3 (Bacterial strain 2, at 10 ml suspension/plant), T_5= T_2 + B_4 (Bacterial strain 3, at 10 ml suspension/plant), T_6 = Positive control or soil treatment with Penta cholro nitrobenzene (PCNB) fungicide at 0.2 g/ 100 g soil. There were seven pots for each treatment.

In Vivo antagonism test of selected antagonist bacteria in field trial

Field preparation and treatment application

The experimental plot was prepared accordingly. The plot was divided into different blocks (100 × 100 cm^2/block), and the block soil was fertilized according to BARI recommendation. The experiment was laid out in Randomized Complete Block Design (RCBD) with the above mentioned six treatments, and three replications per treatments. The soil was inoculated with *R. solani* at 50 g/plot. The antagonistic bacteria (10 ml suspension/plant) were applied after 15 days of pathogen inoculation in the field. After 7 days of bacteria inoculation, the tubers were sown in the field. For positive control, PCNB was applied as 6g/m^2. Different intercultural operations such as stalking, tagging, weeding, rouging, irrigation, and pesticide application were conducted as per requirement. There were three replicates for each isolate, and the experiment was repeated three times.

Assessment of disease incidence and severity

Three specific diseases symptoms or sign data were recorded during *in vivo* field experiments after harvest. The incidence (%) and severity (%) of canker on infected stems or stolons, was recorded and estimated according to a 0 - 5 scale (Tsror, L. and Peretz-Alon, I. 2005). The density of sclerotia on tubers (after harvest) was recorded in 0-3 (Tsror and Peretz-Alon, 2005);

The disease severity index (DSI) was calculated according to (Tsror *et al.*, 2001).

$$\text{Disease severity index DSI} = \frac{\sum(n \times x)}{N}$$

Where,

- n= number of stems, either symptomless or low-level symptoms or moderate-level symptoms or high-level symptoms.
- x = respective disease scale (0-5 as mentioned above).
- N= total number of infected plant parts

Data collection and analysis from in planta and in vivo experiments

Data for morphological parameters, namely, plant height, branch number, and leaf number per plant were measured every 15 d. Plants were harvested at 90 DAP (days after planting),

and yield data was taken. In addition, leaf total chlorophyll content (SPAD unit) in pot trail was determined with chlorophyll-meter SPAD 502 (Konica Minolta, Japan) from the youngest fully expanded leaf of each plant at 30 DAP (Dihazi *et al.*, 2012, Tagele *et al.*, 2018). Data were analyzed using Minitab software.

Results

Pathogen identification and Pathogenicity test

Both isolates RS1 and RS2 were identified as *R. solani* on the basis of colony and morphological characters according to Moni *et al.*, (2016) and Kotba *et al.*, (2018). Both the isolates (RS1 and RS2) of *R. solani* produced stem canker lesions similar to the original symptom on potato stems grown in the pot. However, all five tested plants demonstrated stem canker symptom for RS1, and only three plants demonstrated the desired symptom for RS2. Therefore, isolate RS1 was selected for this study.

Isolation of antagonistic bacterial strains and in vitro pathogen inhibition assays

Ten bacterial strains obtained from the phyllosphere and rhizosphere soil of healthy potato plants were used in the present study (Table 1).

Table 1. Antagonistic bacteria with their sources and Radial growth of *R. solani* in dual culture assay (cm) (mean ±SE) and the percentage (%) of mycelial growth inhibition (PI) of *R. solani* towards the antagonistic bacterial strains at 1 DAI, 3 DAI, and 5 DAI

Treatments	Source of bacteria	Radial growth of Rhizoctonia in dual culture assay (cm) (mean ±SE)			PI (%) of R. solani against antagonistic bacteria		
		1 DAI	3 DAI	5 DAI	1 DAI	3 DAI	5 DAI
B_1	Leaf/stem of Potato	1.45 ef±0.03	3.48de±0.04	4.23bc±0.03	3.33%	3.33%	3.86%
B_2	Leaf/stem of Potato	0.40 a ± 0.01	0.98a ± 0.01	1.35a ± 0.02	73.33%	72.77%	69.31%
B_3	Leaf/stem of Potato	0.41 a± 0.00	1.01a ± 0.03	1.43 a± 0.04	72.67%	60.27%	67.50%
B_4	Tuber of Potato	0.40 a± 0.01	0.94 a± 0.01	1.33a ± 0.04	73,33%	63.05%	69.78%
B_5	Tuber of Potato	1.25 b±0.03	3.25bc±0.04	4.26cd±0.05	16.67%	3.06%	3.18%
B_6	Tuber of Potato	1.35 cd±0.03	3.28bc±0.04	4.18bc±0.04	10.00%	8.89%	5.00%
B_7	Tuber of Potato	1.43 def±0.04	3.32bc±0.04	4.13bc±0.05	4.67%	7.77%	6.13%
B_8	Leaf/stem of Potato	1.40 de±0.04	3.18b±0.04	4.10b±0.05	6.67%	11.67%	6.81%
B_9	Leaf/stem of Potato	1.28 bc±0.03	3.39cd±0.03	4.1b±0.04	14.67%	6.11%	6.81%
B_{10}	Tuber of Potato	1.28 bc±0.03	3.45d±0.03	4.2b±0.05	14.67%	4.17%	4.55%
Control		1.50 f± 0.00	3.60e ± 0.01	4.40d ± 0.03			

*Mean of 4 replications; *SE = Standard Error

Means followed by the same letter (s) are not statistically different at a 5% level of significance. Mean separation was conducted using Tukey HSD. B_2= Bacterial strain 1, B_3= Bacterial strain 2, B_4= Bacterial strain 3, Control= *R. solani*.

The growth of the *R. solani* isolate was significantly suppressed by the three bacterial strains (B_2, B_3, and B_4) compared to the control. The radial growth of the *R. solani* against B_2, B_3, B_4 was 0.40-0.41 cm on the first day after inoculation (DAI), and it reached at 1.33- 1.43 cm after 5 DAI. Whereas in control, it was 4.4 cm after 5 DAI (Table 1). At 3 DAI, the radial growth of the fungi against B_2, B_3, B_4 was 0.94-1.01 cm, and 3.6 cm, respectively. The highest *in vitro* growth suppression (69.78%) of *R. solani* was achieved by the B_4 strain at 5 DAI (Table 1).

Biochemical and physiological characterization of bacterial antagonists

All 3 bacterial isolates [B_2 (MZ396406), B_3 (MZ396407), B_4 (MZ396408)] developed a bluish violate color at the smear within 10s which indicated oxidase positive (Figure 1A). The gram staining reaction of the 3 bacterial isolates [B_2 (MZ396406), B_3 (MZ396407), B_4 (MZ396408)] showed pink color, and the shape was rod (Figure 1B). All three bacterial isolates B_2 (MZ396406), B_3 (MZ396407), and B_4 (MZ396408) were KOH test positive as thread-like string occurred within the first 30 seconds after mixing the bacteria in KOH solution (Figure 1C). However, the positive oxidative reaction, pink colour in gram staining, and development of thread in the

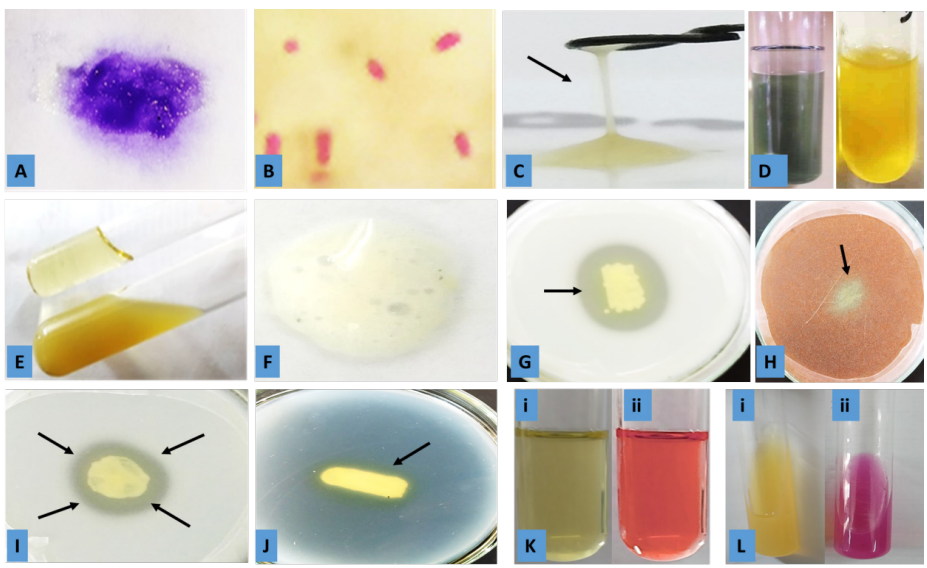


Figure 1. Different biochemical responses of selected bacterial antagonists. A) Oxidase test (+); B) Gram staining test (+); C) KOH test (+); D) Oxidative fermentative test (+); E) Gelatin liquefaction test (+); F) Catalase test (+); G) Protease test (+); H) Potassium solubilizing test (+); I) Phosphate solubilizing test (+), arrowhead showing halo zone surrounding bacterial colony); J) Starch solubilization test (+); K) Indole acetic acid (IAA) test i) control and ii) (+); L) Urease test i) control and ii) (+). The symbol inside the parenthesis (+) indicates the positive reaction of all the tests.

KOH test indicated that all three bacteria were gram-negative rods.

All 3 bacterial isolates [B_2 (MZ396406), B_3 (MZ396407), B_4 (MZ396408)] showed a positive result in the oxidative fermentation (O-F) test, gelatin liquefaction test, catalase test, and protease test. Oxidative fermentation test positive was indicated by the developed yellow colour in the medium (Figure 1D). The possible reason behind this reaction may be the fermentation or acidification of specific carbohydrates (glucose) by the bacterial strains. The gelatin liquefaction test positive was indicated by hydrolyzing the gelatin, whereas the control medium remained solid (Figure 1E). The bacterial isolates may be able to produce extracellular proteolytic enzymes (gelatinases) that liquified gelatin. Bubbles produced by the bacteria confirmed that they were catalase positive (Figure 1F). The probable cause of bubble formation may be the respiring habit of the bacterial strains as anaerobic bacteria that characteristically produce catalase enzymes. Protease tested positive as the formation of a clear zone around the colony was observed (Figure 1G). The formation of a clear zone around the colony on the milk casein agar may be due to the ability of the bacterial strains to produce some proteolytic enzymes such as proteases, pepsin, and papain.

Plant growth promoting activities of bacteria

All three bacterial isolates [B_2 (MZ396406), B_3 (MZ396407), and B_4 (MZ396408)] exhibit positive results in the potassium solubilizing test (Figure 1H), phosphate solubilization test (Figure 1I), starch test (Figure 1J), IAA production (Figure 1K) and urease production (Figure 1L). Positive results in potassium solubilization, phosphate solubilization, and starch test was indicated by a clear zone around the bacterial colonies produced by the three bacteria in the respective medium. IAA production and urease production confirmed by orange to red color and magenta color production in the medium by the bacteria, respectively.

A possible reason for positive potassium solubilization might be due to the conversion of the insoluble potassium to soluble forms. In the phosphate solubilizing test, the zone diameter was increased over the incubation period. The increment of zone diameter from 15.71 mm, 15.59 mm, and 16.11 mm at 3 DAI to 21.89 mm, 21.85 mm, and 21.94 mm at 7 DAI in B_2 (MZ396406), B_3 (MZ396407), B_4 (MZ396408), respectively. Consequently, the phosphate solubilization index (SI) of the bacterial strain increased with the increase in the incubation period. The possible cause for the development of the halo zone may be attributed due to the production of phosphatase enzymes or other chemicals, such as polysaccharides and organic acids. The hydrolysis reaction of urea to ammonium might be catalyzed by urease. Thus, ammonium can be easily absorbed by plants.

Molecular characterization of bacterial isolates

All 3 isolates showed amplification with an

amplicon size of 16S ribosomal partial gene was 525 bp. To assess the definitive status of species of isolate B_2, B_3, B_4 the 16S ribosomal partial gene sequence of strains was aligned to other known sequences deposited in GenBank. The phylogenetic analysis revealed that strains B_2, B_3, B_4, and strains of *Burkholderia contaminans* clustered within a group and well separated from other species (Figure 2). It is therefore considered that strain B_2, B_3, B_4, should be identified as *Burkholderia contaminans*. This result revealed that phylogenetic analysis of the gene sequence is discriminatory and possibly can place an isolate within a named or novel *Burkholderia contaminans* group.

Different cultural conditions of the bacterial isolates

All three bacterial strains [B_2 (MZ396406), B_3 (MZ396407), B_4 (MZ396408) showed positive growth on NA, YPDA, NA + 6% NaCl, YPDA + 6% NaCl, and YS broth at different temperatures from 28 °C to 45°C (Table 2). However, the growth response was negative at an incubation temperature of 4 °C.

After observing the media and temperature effects on the 3 isolates of the bacterial antagonists, the optimum temperature for the growth of the bacterial isolates was 28°C on NA, YPDA supplemented with 6% NaCl, and YS broth.

Interaction with antagonistic bacteria

The microscopic observation of interactions between *R. solani* and bacterial strains showed a change in mycelial structure in the form of blunted hyphae, swollen mycelial tip, excessive branching, and deformation at the inhibition zone (Figure was not provided). However, the hyphae of the *R. solani* were

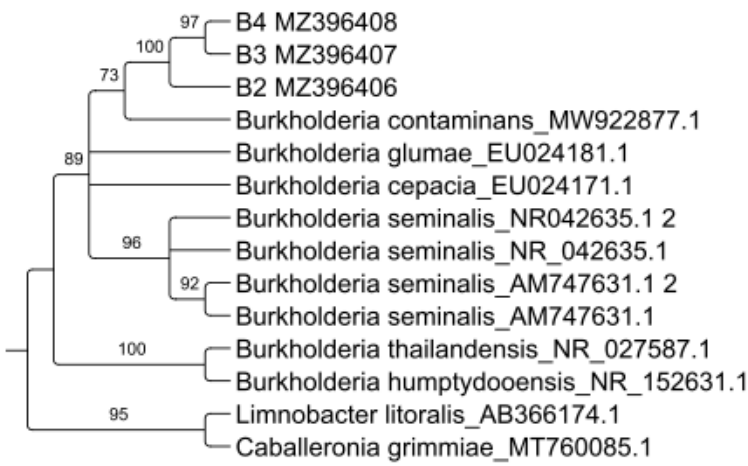


Fig. 2. Molecular characterization of *Burkholderia contaminans*. Maximum likelihood phylogenetic tree derived from the 16S rRNA partial gene sequence analysis of *Burkholderia* species, with two outgroups, *Limnobacter litoralis* and *Caballeronia grimmiae*.

Table 2. Effect of different growth mediums, incubation temperature, and incubation period on the growth of the antagonistic bacteria

Media	Incubation Temp (°C)	Incubation Period (h)	Result
NA	4°C	48 h	-
	28°C	24 h	+
	36°C	24 h	+
	45°C	24 h	+
YPDA	4°C	48 h	-
	28°C	24 h	+
	36°C	24 h	+
	45°C	24 h	+
NA + 6% NaCl	4°C	48 h	-
	28°C	24 h	+
	36°C	24 h	+
	45°C	24 h	+
YPDA + 6% NaCl	4°C	48 h	-
	28°C	24 h	+
	36°C	24 h	+
	45°C	24 h	+
YS broth	4°C	48 h	-
	28°C	24 h	+
	36°C	24 h	+
	45°C	24 h	+

Note: All observations were made after the mentioned incubation period and continued up to 7 days; + indicates positive growth; - indicates negative growth

normally branched with no abnormalities in the case of control plates.

Effect of volatile compounds against R. solani

The radial growth of *R. solani* (RS1) was significantly reduced by the volatiles released by the bacterial strains. The lowest radial growth of *R. solani* was 1.42 and 3.0 cm at 2 and 5 DAI, respectively by the effect of B_4 (MZ396408) but the suppression of radial growth of B_2 (MZ396406), B_3 (MZ396407) was 1.67-2.00 cm, and 3.99-4.2 cm at 2nd and 5th DAI, respectively. In contrast, the radial growth of *R. solani* at the control plate reached 4.5 cm at 5 DAI.

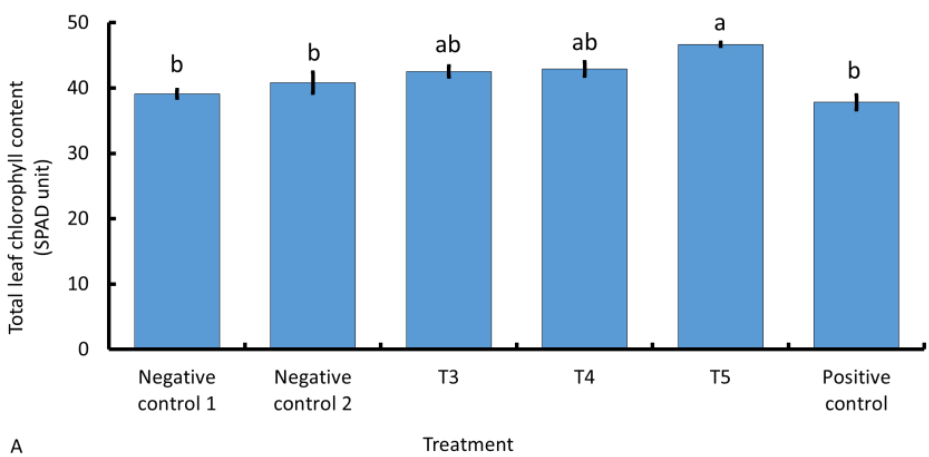
In planta antagonism test of selected antagonist bacteria in pot trial

Three bacterial antagonists significantly affected the yield contributing characters and yield of potato during the *in planta* pot trial (Figures 3 and 4).

The highest chlorophyll content (46.67) was found in T_5 (soil treated with B_4 (MZ396408)), which was statistically similar to T_3 (42.53, soil treated with B_2 (MZ396406)), and T_4 (42.93, soil treated with B_3 (MZ396407)). However, the lowest chlorophyll content (37.83) was found in the positive control (37.83, soil treated with chemical fungicide), and this was statistically similar with negative control 1 and 2 (39.10 and 40.83 at natural soil and pathogen inoculated soil, respectively) (Figure 3A). The maximum plant height (23.60 cm) was found in treatment T_5 (soil treated with B_4 (MZ396408)), which was similar to T_3 (21.70 and 21.27 cm, soil treated with B_2 and B_3 (MZ396407), respectively). However, the minimum plant height (15.93 cm) was observed in T_2 (negative control 2, where the soil was inoculated with *R. solani*) (Figure 3B). The highest branch number (5.50) was observed in T_3 and T_4 (soil treated with B_2 and B_3 (MZ396407)), and it was statistically similar with all treatments (3.75 to 4.00) except T_2 (3.63, *R. solani* inoculated soil) (Figure 3C). The number of leaves significantly varied according to different treatments (Figure 3D). The maximum leaf number (78.75) was recorded from B_3 (MZ396407) inoculated soil (T_4), and this is statistically different with B_2 (MZ396406), and B_4 (MZ396408) inoculated soil (64.75 and 58.75, respectively). The lowest leaf number was found in the positive control (49.25), which was statistically similar to both the negative controls (49.50 to 50.50). The maximum yield (95.75g / p) was measured in B_4 (MZ396408) treated soil (T_5) followed by B2 (MZ396406) and B_3 (MZ396407) treated soil (59.38 and 55.50 g/p, respectively) (Figure 4). The lowest yield (39.00 g/ p) was obtained in *R. solani* inoculated soil (T_2) (Figure 4).

Antagonism test in the field experiment

Analysis of yield and yield contributing characters in the field experiment



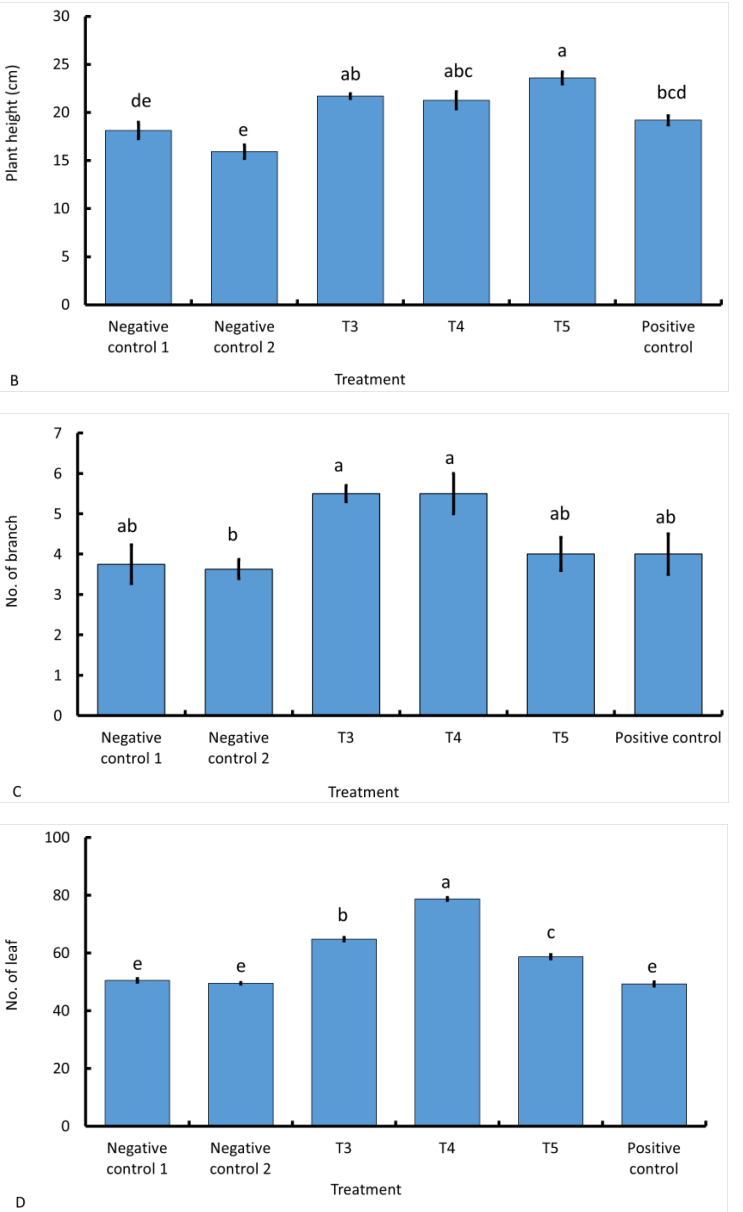


Fig. 3. Effect of selected bacterial antagonists on yield contributing character and on yield of potato (var. Diamant) *in planta* pot experiment. A) Effect of antagonists on chlorophyll content of potato; B) Effect of antagonists on plant height (cm) of potato; C) Effect of antagonists on the number of branch; D) Effect of antagonists on the number of leaf. Means followed by the same letter (s) are not statistically different at 5% level of significance. Mean separation was conducted using Tukey HSD. T1= Negative control 1 or no soil inoculation), T2= Negative control 2 or soil inoculation with R. solani, T3= T2 + B2 (MZ396406), T4 = T2 + B3 (MZ396407), T5= T2 + B4 (MZ396408), T6 = Positive control or soil treatment with PCNB) fungicide at 2 g/ 100 g soil.

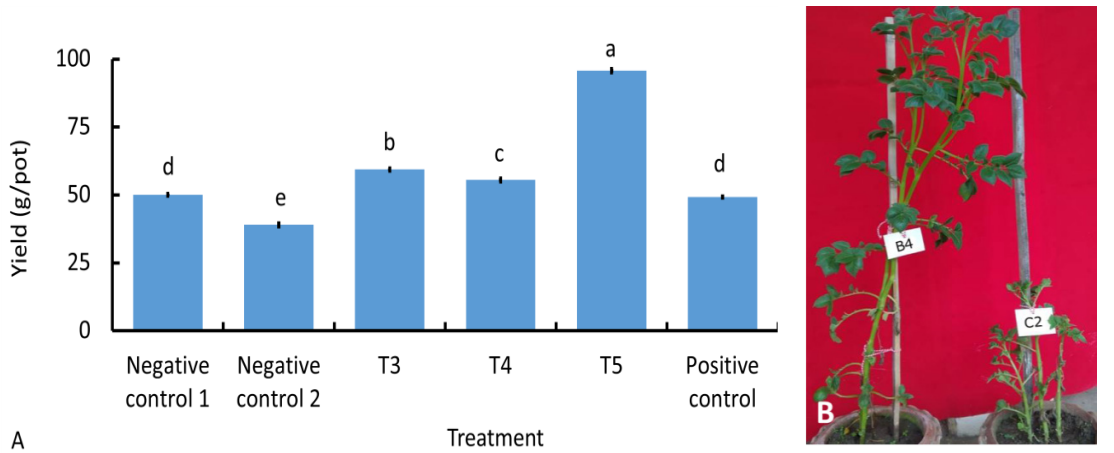
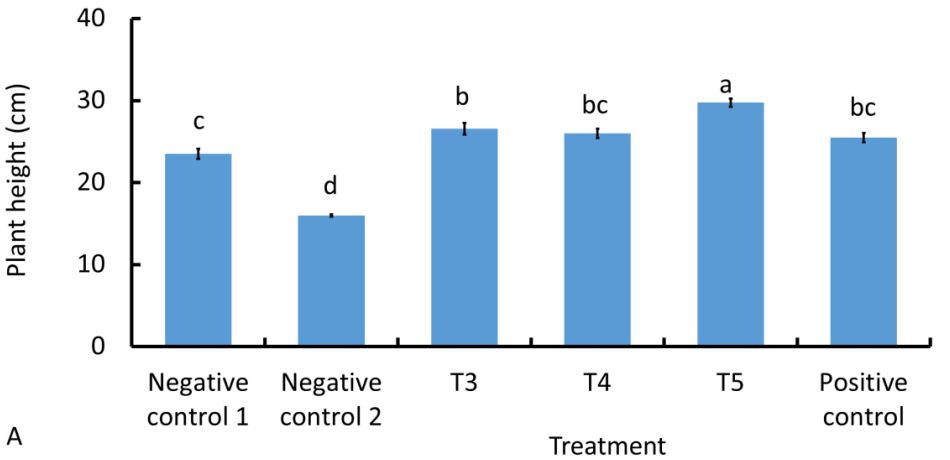


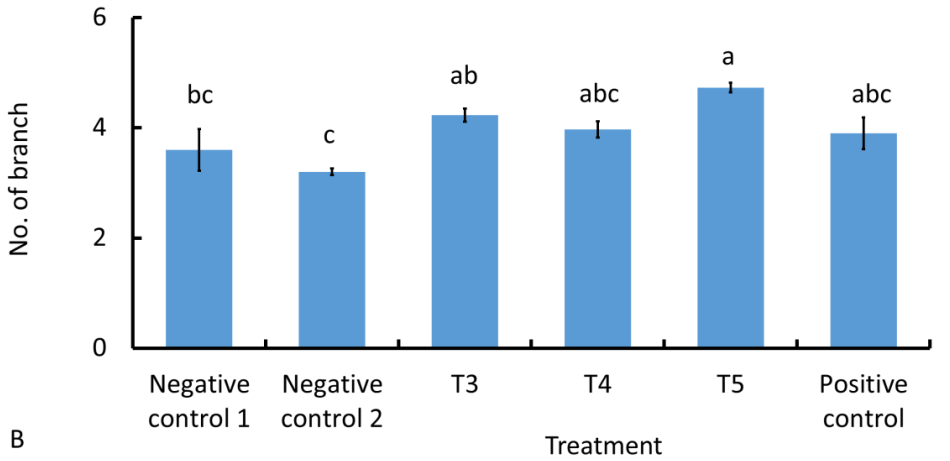
Fig. 4. Effect of selected bacterial antagonists on yield of potato (var. Diamant) *in planta* pot experiment. A) Effect of antagonists on yield (g/pot) of potato. B) Effect of antagonists on yield contributing characters of potato. Means followed by the same letter (s) are not statistically different at 5% level of significance. Mean separation was conducted using Tukey HSD.

Significant variations were recorded for yield and yield contributing characters between the antagonist treated, and not treated plots of potato (Figure 5). The maximum plant height (29.75 cm) was recorded from B_2 (T_5) treated plot, which was significantly different from all the treatments (Figure 5A). However, the minimum plant height (16.00 cm) was observed in *R solani* (Negative control 2) treated plot. The plant height varied from 25.50 to 26.57 cm for other treatments (Figure 5A). The maximum branching (4.73) occurred in treatment T_5 (Figure 5B). This branching number was statistically similar to other treatments (3.77 to 4.23) except for the negative controls (T_1, and T_2). The lowest branch number (3.20) was observed in Negative control-2 (Figure 5B). The leaf number per plant was significantly varied with all the treatments. The highest leaf number (57.30) was observed in T_5, whereas the lowest leaf number (31.23) was found in T_2 (Figure 5C).

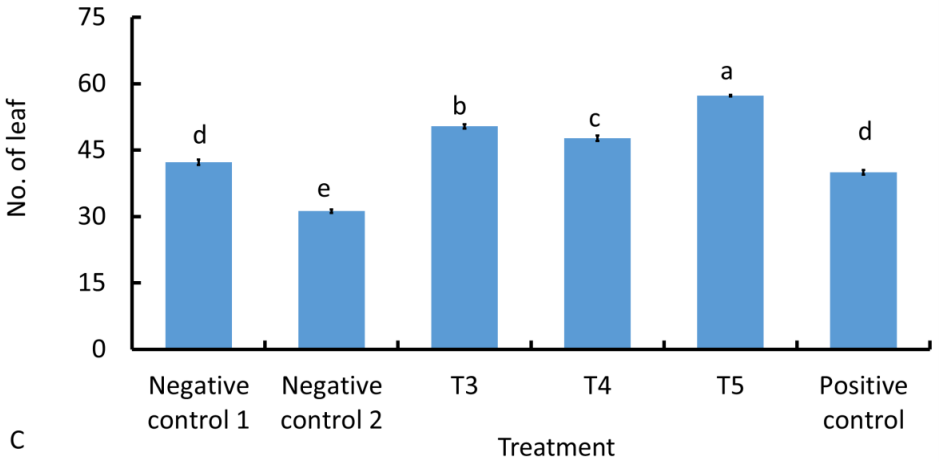
The maximum yield (999.67 g/5p) was obtained in T_5 followed by T_3 (899.67 g/5p), T_4 (789.33 g/5p), Positive control (699.33 g/5p), and Negative control-1 (650.67 g/5p). The lowest yield was obtained in Negative control- 2 (523.00 g/5p) (Figure 5D).


Plant height (cm)
40
30
20
10
0
c
d
b
bc
a
bc
Negative control 1
Negative control 2
T3
T4
T5
Positive control
Treatment

A


No. of branch
6
4
2
0
bc
c
ab
abc
a
abc
Negative control 1
Negative control 2
T3
T4
T5
Positive control
Treatment

B


No. of leaf
75
60
45
30
15
0
d
e
b
c
a
d
Negative control 1
Negative control 2
T3
T4
T5
Positive control
Treatment

C

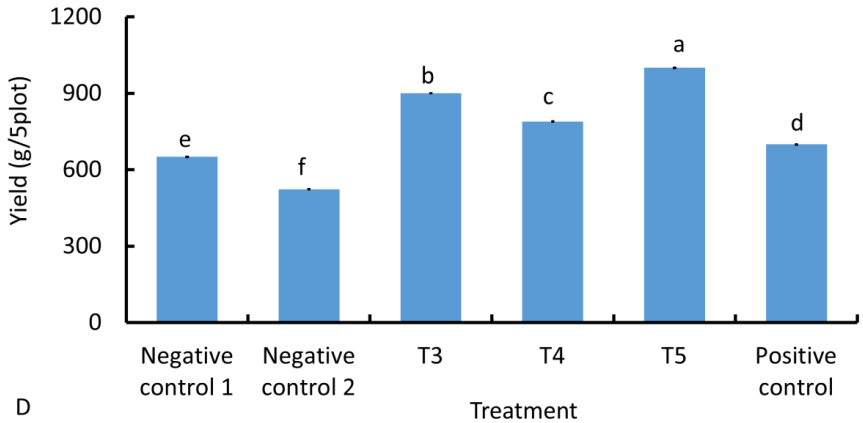


Fig. 5. Effect of selected bacterial antagonists on yield contributing character and on yield of potato (var. Diamant) *in vivo* field experiment. A) Effect of antagonists on plant height (cm) of potato; B) Effect of antagonists on the number of branches; C) Effect of antagonists on the number of leaves; D) Effect of antagonists on yield (g/5 plot) of potato. Means followed by the same letter (s) are not statistically different at 5% level of significance. Mean separation was conducted using Tukey HSD. T_1= Negative control 1 or no soil inoculation), T_2= Negative control 2 or soil inoculation with *R. solani*, T_3= T_2 + B_2 (MZ396406), T_4 = T_2 + B_3 (MZ396407), T_5= T_2 + B_4 (MZ396408), T_6 = Positive control or soil treatment with fungicide PCNB at 6g/m².

Disease severity

The disease severity of the stem canker and black scurf caused by *R. solani* were evaluated by the disease severity index (DSI).

All three antagonist strains successfully reduced stem canker, and black scurf symptom along with visible sign of sclerotia in potato (Figure 6, 7 and 8). All three symptoms developed in the artificially inoculated plot (Figure 7). The highest severity percentage (65) of stem canker was recorded in *R. solani* (T_2) inoculated plot, whereas the lowest (0%) was in the treatment B_4 (MZ396408) treated plot (T_5) (Figure 6). The other two antagonist strains B_2 (MZ396406) and B_3 (MZ396407)) provided 5 to 8% disease severity. The chemical fungicide treated plot was observed with 10% disease severity, whereas 15% disease severity was recorded from the naturally infected plot (Negative control- 1) (Figure 6).

The severity of the black scurf percentage, along with presence of sclerotia percentage followed the similar trend (Figure 6 and 8). Antagonist B_4 (MZ396408) completely inhibits the development of both symptoms *in vivo*. The highest severity was observed in the Negative control-2 (30% and 80% for black scurf and sclerotia, respectively). and lowest in treatment T_5 (0%) followed by T_3 (2%), T_4 (2.7%), T_6 (2.8%), T_7 (4%) and T_1(15%). Sclerotia density on tubers was observed as maximum in treatment T_2 (80%) and minimum in treatment T_5 (0%).

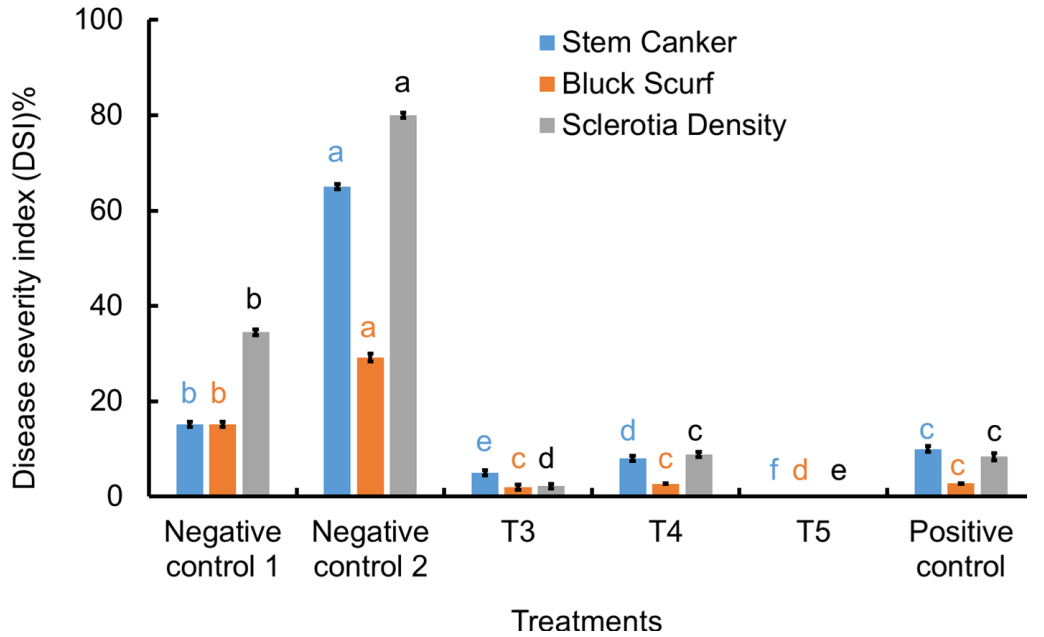


Fig. 6. Severity and incidence of different symptoms caused by *R. solani* in potato in field experiment

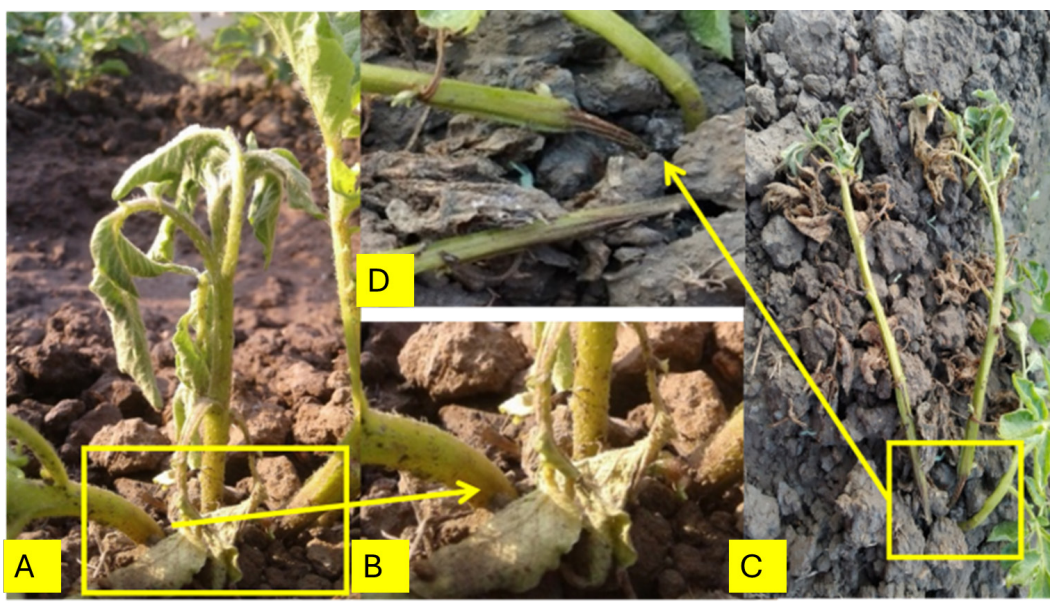


Fig. 7. Severity and incidence of different symptoms caused by *R. solani* in potato *in vivo* field experiment. A, B *Rhizoctonia* wilt; C and D) Stem canker symptom

Fig. 8. Severity and incidence of different symptoms caused by *R. solani* in potato *in vivo* field experiment. A) Dense sclerotial mass (80%) on potato tuber; B) Sclerotia free (0%) Potato tuber.

Discussion

This study evaluated the antagonistic potential of three strains of *B. contaminans* recovered from a specific ecological niche, such as potato growing soil of GAU field. All three isolates of *B. contaminans* successfully reduced the *in vitro* radial growth of *R. solani* in the laboratory. Antifungal activity of other *Burkholderia* species, such as *B. cepacia*, *B. bryophila*, *B. megapolitana, B. gladioli*, against different phytopathogens (*Botrytis cinerea*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium digitatum*, *P. expansum*, *Sclerotinia sclerotiorum*, *Phytophthora cactorum, Verticillium dahlia, Rhizopus stolonifera*, *Colletotrichum gloeosporioides*) was also reported previously (Vandamme *et al.*, 2007, de Los Santos-Villalobos *et al.*, 2012; Elshafie *et al.*, 2012; Ghosh *et al.*, 2016; Elshafie *et al.*, 2017; Elshafie and Camele, 2021). *B. contaminans* caused four specific structural deformities in the *R solani* (such as blunted hyphae, swollen mycelial tip, excessive branching, and deformation at the inhibition zone) *in vitro*. It was hypothesized that volatile organic compounds (VOC) from the selected antagonistic bacteria might be responsible for these structural deformations. According to Giorgio *et al.* (2015), antagonistic bacteria can produce diffusible and volatile metabolites that affect the fungal growth in dual culture assay. This observation was consistent with previous reports (Cuong *et al.*, 2011; Tagele *et al.*, 2018), who stated that the morphological defects of *R. solani* caused by *B. contaminans*, and *Bacillus pumilus*. A number of authors discussed the morphological alteration due to bacterial VOCs

of different fungi other than *R. solani* such as; shortening of conidiophores (*Aspergillus giganteus*), thin-walled vesicle-like swellings (*Fusarium oxysporum* fsp. *conglutinans*), abnormal swelling and deformation of hyphae (*Phoma* sp., *Colletotrichum* sp., *P. cinnamomi*, *F. oxysporum* f.sp. *melonis* and *S. lycopersici*), hyphae cytoplasm granulation and ultrastructural alterations at cell organelles (*S. sclerotiorum*), conidial deformations (*A. alternate*) (Giorgio *et al.*, 2015; Tagele *et al.*, 2018; Ebadzadsahrai *et al.*, 2020). Therefore, it is evident that bacterial volatile could alter the growth, sporulation, or germination of fungi.

This antagonist also reduced the *in planta* symptoms in potato seedlings after artificial inoculation of the mentioned pathogen in a pot trial. Moreover, the minimum severity of stem canker and black scurf in the field was recorded when this antagonist were added in the field soil. This result was consistent with a number of previous *in vitro* experiments, where *B. contaminans* inhibited the growth of a number of plant fungal pathogens in dual culture, namely *G. boninense*, *Nigrospora sphaerica*, *Xylaria* spp., *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium oxalicum*, and *R. solani* (Yurnaliza *et al.*, 2020; Zaman, N. R. *et al.* 2021). It was hypothesized that spatial and nutrient completion could be the main reason for this *in vitro* antifungal antagonism of *B. contaminans* (Yurnaliza *et al.*; 2020). Moreover, *Burkholderia* strains were also reported synthesizing versatile antifungal metabolites and hydrolytic enzymes to inhibit the growth of their competitors *in vitro* (Gu *et al.*. 2011; Kandel *et al.*, 2017). Previously, *B. contaminans* was reported to produce Occidiofungin (a unique glycopeptide) with antifungal properties (Gu *et al.*, 2011; Deng *et al.*, 2016). Therefore, it could be assumed all these three mechanisms were simultaneously involved in the *in vitro* and *in planta* antagonism of three *B. contaminans* isolates in the present investigation.

However, reports regarding the control of potato stem canker and black leg symptoms with *B. contaminans* are limited according to the author's knowledge. This is the first successful *in vitro*, or *in planta* report of *B. contaminans* antagonism against potato black scurf disease or *R. solani* in Bangladesh.

In the present study, *B. contaminous* successfully increased leaf chlorophyll content, plant height, and branch number along with yield in both *in planta* pot and field experiments. This observation was consistent Zaman *et al.*, (2021), who presented significant growth promotion in jute by *B. contaminans* NZ in the *in vivo* pot experiments. A number of mechanisms are involved in plant growth promotion following the inoculation of beneficial bacteria. The growth promotion was the combined response of nitrogen fixation, IAA, siderophore production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Zaman *et al.*, 2021). Of all the mechanisms, the potential of IAA production and P solubilization is important as these two can be synergistically involved

in plant growth promotion (Prashanth and Mathivanan, 2010; Mehta *et al.*, 2014). Moreover, it was reported (Alemneh *et al.*, 2021) that different genera of phosphate solubilizing bacteria (PSB) are capable of producing a wide range of IAA concentrations (4.1 and 67.2 µg mL^{-1}) *in vitro*. The present study demonstrated that all three isolates of *B. contaminans* were capable of P solubilization, IAA production with other growth promoting characters. In addition, other species of *Burkholderia*, such as *B. gladioli B. tropica*, *B. unamae* and *B. cepacia* also successfully evaluated for their growth promotion activities in different crops (apple, common grape vine, common club moss, *Arabidopsis thaliana*) recently (Karakurt and Aslantas 2010, Groenhagen *et al.*, 2013; Ghosh *et al.*, 2016, Elshafie and Camele, 2021). However, characterization of volatile compounds responsible for plant growth promotion or suppression of fungal growth in the present study needs to be conducted in the future.

Conclusion:

The present work reports the biocontrol potential of three *B. contaminans* isolates against of *R. solani* on potato. Our results clearly demonstrated the ultra-structural changes of *R. solani* due to bacterial antagonism, important biochemical attributes of the proposed biocontrol agent, and *in vitro/ in planta* disease suppression. Further experiments are required to determine the survival ability of the laboratory cultured *B. contaminans*, its appropriate dose, the appropriate formulation of the carrier material, mechanisms involved in this disease suppression in field application, along with human health risk. The desired practical use of these isolates depends not simply on crop disease suppression but also on human health safety. Therefore, the antagonistic effect of *B. contaminans* metabolites or culture filtrate needs to be tested also. It will be necessary to evaluate the health risk of metabolites or culture filtrate with the bacteria along with crop improvement.

Conflict of Interest

All the authors state that they have no conflict of interest.

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