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Isolation and evaluation of rhizobacteria against *Ralstonia solanacearum* the incitant of bacterial wilt of tomato

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is the world's most economically important destructive disease of crop plants. In the current study, we aimed to evaluate the novel bacterial isolates from tomato rhizosphere for biocontrol of *Ralstonia solanacearum.* One eighty bacterial strains were isolated from the tomato rhizosperic soils collected from different regions of Uttarakhand state and evaluated for their biocontrol activity against *R. solanacearum* under *in vitro* conditions. Among them, six isolates were found to be highly effective in inhibiting the growth of *R. solanacearum.* The isolate GP2NA8 produced the highest inhibition zone followed by that of GP1NA2 and GP3NA6.

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INTRODUCTION

Tomato is one of the most popular and important vegetable in many countries worldwide because of its nutritive and economic importance (Khoso, 1994). It ranks second in importance next to potato, known for its edible fruits which can be consumed either raw or cooked (Kirankumar, 2007). It is rich in different vitamins *viz.,* A, B and C. It is also used in developing value added products like soup, juice, ketchup and powder through processing. The major tomato growing countries in the world are China, India, USA, Egypt, Turkey, Italy, Iran, Brazil and Spain. It occupies an area of about 4.73 million hectares with a production of 163.96 million tonnes in the world (FAO, 2016). Tomato ranks third in priority after potato and onion in India but ranks second after potato in the world. India ranks second in the area as well as in production of tomato.

Diseases are a major limiting factor for tomato production. Tomato is susceptible to more than 200 diseases. Yield losses due to diseases may be as high as 70 per cent to 95 per cent. The major diseases of tomato are: Bacterial wilt, Early Blight, Damping off, *Fusarium* wilt, bacterial stem and fruit canker, etc. Among all these diseases, bacterial wilt is usually the most damaging

disease which causes about 60-70 per cent yield loss (Chen *et al.,* 2009). Bacterial wilt caused by *Ralstonia solanacearum,* a non-spore forming, rod-shaped (0.5-1.5µm), aerobic, gram negative, nitrate-reducing, ammonia-forming, bacteria with one polar flagellum. It invades the roots of diverse plant hosts from the soil and aggressively colonizes the xylem vessels, causing bacterial wilt disease. It is a devastating plant disease most commonly found in tropical, subtropical and warm-temperate regions of the world (Buddenhagen and Kelman, 1964 and Hayward, 1991).

For the management of soil and seed borne bacterial diseases such as bacterial wilt of tomato, various physical, cultural and chemical management strategies have been employed, however, none of these strategies has been able to manage the disease successfully due to the genetic diversity and the broad host range of the pathogen, Chemical pesticides have conventionally been used to combat bacterial diseases but long-term usage of these chemicals induce resistance in pathogens which ultimately makes the pathogen tolerant to these chemical applications (Cooksey, 1990; McManus *et al.,* 2002 and Ma and Michailides, 2005). Moreover, pesticides lose effectiveness with time and most of these treatments are relatively expensive and pose risks to humans and the environment. Every year, there are about 10,000 pesticide poisoning cases reported in India which are great concern for human and animal health (Centre for Science and Environment, 2020). Because the pathogen invades the inner parts of the plants, the conventional chemical products such as copper may not provide adequate control for the disease. Hence, biological control has an immense potential as an alternative strategy in management of bacterial wilt. Biological control agents exhibit a number of beneficial features that have increased their use in preference to the use of chemicals. Such features include reduced input of non-renewable resources, their potential to be self sustaining and spread after initial establishment and the capability to afford long-term disease suppression (Mamphogoro *et al.,* 2020).

One of the basic idea in biological control is to employ a rhizosphere colonizer that would antagonize *R. solanacearum* at the root infection site. The region of soil surrounding and including the plant root (rhizosphere) is of vital importance for plant health and nutrition. The plant rhizosphere is an important soil ecological environment for plant microbe interactions as it involves colonization by a variety of microbes in and around the roots which may result in either symbiotic, neutralistic, associative or parasitic relations within the plant depending on the type of micro-organisms, defence sysem, soil nutrient status and soil environment (Verma *et al*., 2010). The objective of the present study is to isolate, identify and evaluate the efficacy of different native isolates (isolated from different regions of Uttarakhand) of antagonistic *Rhizobacteria* against bacterial wilt disease caused by *Ralstonia solanacearum* in the tomato plant.

MATERIAL AND METHODS

Isolation of *R. solanacearum* from bacterial wilt affected tomato plant:

Whole infected plants were brought to the lab in a sterile bag for the isolation of the pathogen. Bacterial wilt infected plant was identified in the field by observing physical symptoms, such as wilting of young leaves, discolored tissue at the dissected part of the stem base, and white, slimy ooze when the dissected part of the plant when immersed in the glass of water.

In the laboratory the discolored vascular tissues of plant samples were sliced into small bits measuring 4-5 mm in length and the tissue was surface sterilized by immersing bits in one per cent sodium hypochloride for 30 seconds followed by repeated washing in autoclaved distilled water for 5 minutes to remove traces of sodium hypochloride. These surface sterilized bits were then suspended in the 10ml of sterile distilled water taken in test tube for ten minutes. After the water in the test tube becomes turbid due to oozing of bacterial cells from cut ends of diseased tissue, the bacterial suspension was serially diluted in 9 ml sterile distilled water. One hundred microliter of the diluted bacterial suspension was poured onto the surface of solidified Triphenyl Tetrazolium Chloride agar (TZC) medium (casein hydrolysate 1g/l, peptone 10 g/l, glucose 5g/l, agar 15g/l amended with 5 ml of a 1 per cent stock solution of 2, 3, 5-tripheny tetrazolium chloride) in sterilized petri plates (Kelman, 1954). The bacterial suspension was spread on the surface of TZC medium with a sterilized spreader. The inoculated plates were inocubated at 28°C for 48 hours. At the end of the incubation period, the plates were observed for the development of colonies of *R. solanacearum*. The virulent colonies of *R. solanacearum* are of irregularly shaped, fluidal, dull

white colonies with slight red center. Whereas, avirulent colonies with small, round, convex, butyrous with large red pigment and narrow bluish margins as described by Kelman (1954).

Purification of pathogen isolated from wilted plants:

Typical virulent colonies of *R. solanacearum* on TZC medium were picked and streaked separately on TZC medium in sterilized petri plates. The plates were incubated at 28°C for 48 hours. The well separated virulent colonies of *R. solanacearum* was picked up with sterile inoculated loop and suspended in sterile distilled water culture collection tubes and stored at 4°C in refrigerator for further use as stock culture.

Isolation and *in vitro* evaluation of tomato rhizobacteria against *Ralstonia solanacearum*.

Tomato rhizospheric soil samples were collected by visiting different tomato growing areas of Uttarakhand. The samples were collected essentially from healthy tomato plants compared to nearby sites where the bacterial wilt was severely present in the same field. Healthy and young seedlings of tomato were gently uprooted and were carried to lab in sterile polythene bags. Each sample was stored in refrigerator at 4^0C till further processing. The rhizospheric soil of tomato was removed and air dried. Rhizobacteria were isolated by serial dilution technique from the collected soil samples, for this 10g of rhizosphere soil was taken into a 250 ml of conical flask and 90 ml of sterile distilled water was added to it. The flask was shaken for 30 min on a rotary shaker. 1 ml of suspension was added to 10 ml vial and shaken for 2 min. Serial dilution and pour plating technique was performed upto 10^{-7} dilution. An aliquot (0.1 ml) of this suspension was spread on the plates containimg Nutrient agar (NA) as well as King's B medium. Plates were incubated for 3 days at 28 ± 2^0C to observe the colonies of bacteria. A total of 180 isolates were purified by sub-culture technique, for this well isolated single colony was picked up and re-streaked to fresh NA plates and incubated thus, obtained were preserved at -20°C in 25 per cent glycerol for further studies.

After isolating tomato rhizobacteria these were further evaluated for their efficacy against *R. solanacearum* by inhibition zone assay method *i.e* Agar well plate method (Balouiri *et al.,* 2016). Heavy suspension (3 day old) of *R. solanacearum* multiplied in nutrient broth (20ml) was mixed with luke warm nutrient agar medium (1000 ml) contained in conical flask. Fifteen to twenty ml of seeded medium was poured into the sterilized Petri plates and allowed to solidify. A well was made in the solidified media using sterile cork borer, 20µl of 3 day old antagonistic organism multiplied in nutrient broth was inoculated in well in the centre of the Petri plates containing the seeded medium. A positive and negative controls were maintained separately by adding antibiotic and autoclaved distilled water each to two different wells, respectively. Plates were incubated at 28^0C for 72 h and observations were recorded for the zone of inhibition produced by antagonistic bacteria around the well against the growth of the pathogen.

Characterization and identification of isolates

The identification and classification of bacteria are of crucial importance for this all the effective isolates were studied for their morphological characters *viz.,* cell form, Gram staining, colony pigmentation and production of UV-fluorescent pigments. The best-selected strain with maximum degradation ability was further identified on the basis of complete 16S rRNA gene sequence analysis.

RESULTS AND DISCUSSION

The colonies of *R. solanacearum* obtained were of fluidal, irregularly round, white colonies with pink

Table 1: Rhizobacterial isolates, identified species and corresponding zone of inhibition formed by them (Antagonistic activity)

Sr No.	Isolate code	Name of the species identified	Zone of inhibition formed (radius in mm)
1.	GP1NA2	*Bacillus subtilis* subsp. *spizizenii*	18
2.	GP2NA8	*Bacillus velezensis*	24
3.	GP3NA6	*Bacillus subtilis*	16
4.	GP4NA5	*Bacillus subtilis* subsp. *spizizenii*	15
5.	GP5KB6	*Bacillus stratosphericus*	13
6.	GP6NA6	*Bacillus velezensis*	10

centers on 2,3,5-triphenyltetrazolium chloride-amended (TZC) medium (Kelman, 1954). Gram staining and observation using a microscope showed that the bacteria were gram negative, rod-shaped and non-spore forming, which further confirmed that the bacteria was *R. solanacearum*.

After *in vitro* evaluation of efficacy of all the 180 isolated rhizobacteria, six rhizobacterial isolates showed antagonistic effect against *R. solanacearum*, with inhibition zone radii ranging from 10 to 24mm (Table 1). Isolate GP2NA8 isolated from rhizospheric soil collected from Garampani area was most potent compared to other isolates. GP6NA6 isolated from rhizospheric soil collected from Haldwani area was the least effective among these potent species. However, the inhibitory activities of all these isolates were satisfactory in terms of inhibition zone shown. Based on morphological, biochemical and molecular analysis (complete 16S rRNA gene sequence), these isolates were found to belong to different *Bacillus* spp. (Table 1).

Similar results were also reported by other workers like Kelman (1953) observed that *B. subtilis, B. prodigiosus, Bacillus megatierium* and *B. mesentricus* when added to bacterial wilt sick soil, the disease failed to spread. Gallardo *et al.* (1989) reported the inhibition of *R. solanacearum in vitro* by using *P. fluorescens* strain BC-8. Ciampi-Panno *et al.* (1989) shown that both *in vitro* as well as growth chamber assay of biological agent designated as BC-6 caused strong inhibition of *R. solanacearum*. Anuratha and Gnanamanickam (1990) tested 52 strains of non-fluorescent bacteria and 125 strains of fluorescent against the *R. solanacearum* initially in the laboratory for their antibiosis and evaluated strains of B33 and B36 of *Bacillus* species under green house and field conditions. Kumar and Sood (2001) attributed significant reduction in bacterial wilt incidence to the rhizobacteria (*P. fluorescens* and *B. cereus*) antagonist incorporated in the soil. Samuel *et al.* (2019) reported two promising bacterial biocontrol strains *viz., Pseudomonas fluorescens* and *Bacillus velezensis* having *in vitro* antagonistic activity against *R. solanacearum,* upon testing in field conditions they observed development of both rhizosphere competence as well as priming of plant defense against the pathogen. A new *B. velezensis* strain FJAT-46737 confirmed to have strong antibacterial activity against the bacterial wilt pathogen *R. solanacearum* by both *in vivo* and *in vitro* experiments. Moreover, suppressive effects of of this strain was associated with lipopeptide secretion *viz.,* fengycins (Chen *et al.,* 2020).

Conclusion:

Biological disease control is an attractive alternative strategy for the control of plant diseases. Mean while, it also provides practices compatible with the goal of a sustainable agricultural system. Therefore, above mentioned rhizobacterial isolates could be used as new sources of potential biocontrol agents against bacterial wilt incited by *R. solanacearum*.

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