Diversity and antagonistic potential of apoplastic bacteria against *Ralstonia pseudosolanacearum* race 4 causing bacterial wilt of ginger

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**Abstract:** Bacterial wilt caused by *Ralstonia pseudosolanacearum* race 4 is a devastating disease of ginger, for which almost all control measures met with limited success. In this study, 150 bacteria isolated from the apoplastic fluid of ginger were screened for antagonism against *R. pseudosolanacearum* both *in vitro* and *in planta* and shortlisted six isolates which were further characterized for biocontrol and plant growth promoting traits. The promising isolates were identified as *Bacillus subtilis* (IISRGAB 5), *B. marisflavi* (IISRGAB 43), *B. licheniformis* (IISRGAB 107), *Agrobacterium tumefaciens* (IISRGAB24), *Micrococcus luteus* (IISRGAB 48) and *Staphylococcus haemolyticus* (IISRGAB 146). Green house evaluation against *R. pseudosolanacearum*, by seed priming and soil drenching showed that *B. licheniformis* strain GAP107–MTCC 12725, was able to reduce bacterial wilt incidence up to 67%. Hence, this bacterium was identified as a suitable candidate for developing a potential biocide for the management of bacterial wilt in ginger.

**KEYWORDS:** Apoplastic *Bacillus licheniformis*, bacterial wilt, ginger, *Ralstonia pseudosolanacearum* race 4

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**INTRODUCTION**

Ginger (*Zingiber officinale* Rosc.) is one of the valuable spice crop cultivated in India and outside for the spicy green ginger and dried ginger. Bacterial wilt caused by *Ralstonia pseudosolanacearum* race 4 is a severe threat to ginger cultivation in all the ginger growing tracts (Kumar and Hayward, 2005). The pathogen is both soil and seed borne and the perpetuation of the disease is through seed and soil borne inoculum. Different management strategies are in practice but none of them met with satisfactory disease control. Since organic farming is gaining momentum, ecofriendly biological control measures are highly preferable to chemical control which poses serious environmental threats. Several rhizobacteria, endophytic bacteria, actinomycetes, phages etc were evaluated for bacterial wilt management in many economically important crops ((Lemessa and Zeller, 2007; Yamada et al., 2007; Ramesh et al., 2009; Barretti et al., 2012; Achari and Ramesh, 2014; Yuan et al., 2014)). In ginger, the common strategies adopted for bacterial wilt management include selection of disease free rhizomes, selection of fields with no history of bacterial wilt, seed treatment using chemicals, strict phytosanitary measures to avoid the carryover of pathogen inoculum through field workers or irrigation water and crop rotation with non-host plants (Kumar and Hayward, 2005).

Endophytes may confer resistance against pathogens, by induction of defence reactions, production of antagonistic substances or through competition for colonization of sites and nutrients (Kloepper et al., 2004). *Ralstonia pseudosolanacearum* being a xylem inhabitant, moving through the intercellular spaces, endophytes residing in the apoplast can be a suitable source of antagonists to explore. So, the objective of the present study was to exploit the antagonistic potential of apoplastic bacteria against *R. pseudosolanacearum* race 4 causing bacterial wilt of ginger.

**MATERIALS AND METHODS**

**Isolation of ginger apoplastic microorganisms plant material**

Ginger plants were collected from different ginger growing tracts of Kerala and Karnataka and from different germplasm accessions maintained at ICAR-Indian Institute of Spices Research experimental farm Peruvannamuzhi,
Kozhikode, Kerala. Fresh and healthy ginger plants were collected in fresh polythene bags and sealed properly, labelled and brought to the laboratory at ICAR-IISR for further processing.

**Extraction of apoplastic fluid**

The collected ginger plants were thoroughly washed with tap water, the leaves and pseudostems were cut individually into bits of 7-10 cm length and surface sterilized by immersing in 70% ethanol in a sterile polyethylene bottle for 10 minutes and transferred to 5% H₂O₂ for 5 min. These were then washed four times in sterile distilled water (Asis et al., 2003). Apoplastic fluid was extracted from surface sterilized leaves and pseudostems by vacuum infiltration and centrifugation (Nouchi et al., 2012) with slight modifications. Leaf/ pseudostem bits (5-8 bits) were immersed in sterile distilled water in a glass beaker and placed in vacuum desiccator. The vacuum was applied for 15 minutes for leaves and 30 minutes for pseudostems at 40 kPa pressure by releasing and re-applying vacuum for 5 minutes interval. After vacuum infiltration, the leaves and pseudostem bits were transferred into sterile filter paper and surface dried inside a laminar air flow cabinet. After drying, 3-4 leaves were rolled vertically and placed inside a centrifuge tube with conical bottom in such a way that the petiole is facing towards the bottom of the tube and centrifuged at 3000g for 10 minutes at 4°C using Beckman coulter Avanti J-301 centrifuge. Similarly, vacuum infiltration and centrifugation was applied for pseudostems also. After centrifugation the leaves/pseudostems were removed and the remaining fluid at the bottom was taken as the apoplastic fluid.

**Isolation of culturable microbes from apoplastic fluid**

The extracted apoplastic fluid was either directly plated (depending on the quantity extracted) or serially diluted up to 10⁶ and 0.1ml was plated on to selective media, viz. Tryptic Soy Agar (TSA) for bacteria, Rose Bengal Agar (RBA) for fungi and Actinomycetes Isolation Agar (AIA) for actinomycetes. TSA and RBA plates were incubated at 28°C for five days at day- night intervals and AIA plates were incubated at 28°C for 15 to 20 days. Based on the morphological difference, bacteria were selected, purified and maintained in 40% glycerol at -80°C. Working stock was maintained at -30 °C.

**Characterization of apoplastic bacteria**

**Morphological characterization**

The bacteria were sub-cultured on to tryptic soy agar and morphological characteristics of bacterial colonies such as colour, shape, size, elevation, surface, texture, opacity etc., were observed. Pure colonies were subjected to Gram staining and biochemical tests. Gram staining for differentiating the bacteria into gram positive or gram negative was done using standard staining procedure. The stained slides were air dried and observed under oil immersion objective of Leica DM 5000B microscope.

**Biochemical characterization**

The basic biochemical characterization the apoplastic bacteria were done using catalase test and carbohydrate fermentation test. It is essential for differentiating catalase-positive from catalase-negative bacteria for classification in the genus level. Catalase test was done using 3% H₂O₂ (Clarke and Cowan, 1952). In carbohydrate fermentation test, the ability of the apoplastic bacteria to utilize different carbohydrate was tested. Nutrient broth containing appropriate sugars at a concentration of 0.5% (glucose, lactose, sucrose, mannitol and sorbitol) was used and phenol red (0.018mg in 100 ml) was added as the pH indicator dye (MacFaddin, 2000). To the media the test bacteria were inoculated and incubated at 30°C for 24 h. The change in colour of the dye from red to yellow was recorded as positive. If the colour remains same, the test bacteria could not utilize the respective sugar source and was recorded as negative.

**In vitro screening of apoplastic bacteria**

The 150 isolates of apoplastic bacteria extracted from ginger and maintained as glycerol stocks were retrieved in pure culture (Prameela, 2016) and tested for their antagonism to R. pseudosolanacearum by cross streak method (Lemos et al., 1985) using Kings’ B (KB) agar medium. The plates were incubated at 28°C for 48h. After incubation, the plates were streaked with the race 4 biovar 3 virulent strain of R. pseudosolanacearum GRs Mnt2 as parallel lines without touching the apoplastic bacterial line in the middle. The plates were again incubated for 48h. at 28°C and the zone of inhibition was measured. The experiment was repeated twice.

**In planta screening**

Potting mixture containing soil, sand and farmyard manure (3:1:1) was filled in plastic basins of 15 × 7 cm and planted with three ginger rhizome bits (~10g each) (variety- IISR Varada). After germination, (30 days old), the apoplastic bacterial inoculum at a concentration of 1×10⁸CFU ml⁻¹ was poured around the base of the plants @100ml basin⁻¹. After two weeks of inoculation, all the plants were challenged with 50 ml of OD 0.1 (absorbance at 600nm=0.1) culture of R. pseudosolanacearum strain GRs Mnt2. A set of plants inoculated with pathogen alone served as the positive control and another set without pathogen or apoplastic bacteria served as absolute control. After inoculation, the plants were observed for typical green wilt symptoms such as downward
drooping of leaves and wilting of pseudostem.

**In vitro antagonism by agar well diffusion test**

Six apoplastic bacteria after preliminary evaluation were screened for *in vitro* antagonism against *R. pseudosolanacearum* by agar well diffusion method (Ramesh *et al.*, 2009) using KB agar medium. The plates were incubated at 28°C for 48 hours and the zone of inhibition was recorded by measuring the radius (mm) from the outer edge of the well. The experiment was repeated twice.

**Biocontrol traits**

The isolates were screened for the production of siderophore on solid siderophore Chrome azurol S (CAS) blue agar plates as described by Schwyn and Neilands, 1987, production of hydrogen cyanide was checked using the method of Lorck, 1948 and acetoin production as per Cappuccino and Sherman, 2005.

**Plant growth promoting traits**

The growth promoting traits viz. Ammonia production by Nessler’s reagent method (Cappuccino and Sherman, 1992), IAA production by Salkowsky’s reagent method (Sarwar and Kremer, 1995) and Phosphate solubilization using Pikovskayas agar medium (Gaur, 1990), were tested for the selected isolates using standard procedures.

**Extracellular enzyme production**

Extracellular enzymes like production of α-amylase was tested in starch agar (Anjea, 2003), protease in Skim milk agar (HiMedia Laboratories) (Vermelho *et al.*, 1996), lipase in tributyrin agar containing of tributyrin (Collins *et al.*, 1995) and cellulase in Carboxymethyl Cellulose (CMC) agar plates (Apun *et al.*, 2000) as per standard procedures.

**Molecular identification of promising isolates**

Genomic DNA was extracted from the six bacteria using Qiagen blood and tissue mini kit (Quagen, Germany). 100ng DNA was used for the PCR amplification of 16S rDNA using universal primers (pAF-5’AGAGTTTGATCCTGCGGCTAG 3’ and pHR- 5’ AAGGAGGTGATCCAGCCGCA 3’). PCR amplification of was done using GoTaq PCR reagents (Promega corporation, USA) and the temperature profile include initial denaturation at 96°C for 9 min followed by 30 cycles of 95°C for 1 min, 48°C for 1 min, 72°C for 1 min 30 s with a final extension of 72°C for 10 min. All PCR products were electrophoresed through a 1.0% agarose gel and purified using the Gel Elution kit (Sigma Aldrich, USA). The purified PCR products were sequenced bi-directionally on an ABI PRISM Genetic Analyzer by the same PCR conditions and the same primers used for PCR amplification. The sequences were assembled in DNA baser software package (DNA Sequencer v3 (2013), Heracle BioSoft, www. DnaBaser.com) and sequences were analyzed.

**Pot culture evaluation of apoplastic bacteria against Ralstonia pseudosolanacearum race 4**

Pot experiment was conducted for the evaluation of six apoplastic bacteria to test their biocontrol efficacy against bacterial wilt. Besides, its colonization in the rhizosphere and apoplastic fluid of ginger plants was also studied.

**Seed priming and planting**

To assure the apoplastic colonization, ginger seed rhizomes were pre-primed by soaking in apoplastic bacterial suspension. Briefly, the 48h grown bacterial culture was pelleted by centrifugation at 8000 rpm for 5 min at 4°C and re-suspended in sterile distilled water so as to get an optical density of 1.0 at 600 nm which is equivalent to 10⁶ CFU ml⁻¹. The ginger seed rhizomes (cv-Rejatha) were soaked in these apoplastic bacterial suspensions for about 45-60 min and air dried on a blotting paper. Approximately 25g pre primed ginger bit were planted in pots containing potting mixture of soil, sand and farm yard manure in a ratio 1:1:1. The experiment was in CRD with 19 treatments (Table 3) and 3 replications with three pots/replication. The treatments were four apoplastic bacteria individually (IISRGAB 5, IISRGAB 43, IISRGAB 48 and IISRGAB 107, their combination in two, three and four, copper oxychloride (0.25%), streptomycin sulphate (200ppm), positive control with pathogen alone and absolute control. After 45 days of planting, the plants were challenged with *R. pseudosolanacearum* GRs Mnt2 (10⁸ CFU/ml). Disease incidence was recorded at specific intervals. The tiller count of each plant was recorded on the day of pathogen inoculation and the plants were observed for the typical wilting symptoms. The experiment was repeated in the successive year also.

**Study on soil physical properties**

pH, electrical conductivity and dehydrogenase enzyme activity was analysed using standard protocols besides pathogen population and population of introduced bacteria. pH was measured using a calibrated pH meter (Mettler Toledo, Switzerland). Electrical conductivity was measured using Cyberscan Con II conductivity/TDS/°C meter (Eutech Instruments, Singapore). Dehydrogenase activity of soil was tested according to the protocol of Casida *et al.*, 1964.

**Estimation of pathogen population and introduced apoplastic bacteria from the soil**

The population level of *R. pseudosolanacearum* in the soil was estimated by serial dilution plating in SMSA (Engelbrecht, 1994). Serially diluted soil samples were plated in tryptic soy agar to compare natural bacterial population with that of apoplastic bacteria treated soil.
Colonization of bacterial population in ginger roots, rhizomes, pseudostems and leaves

To compare the colonization of introduced apoplastic bacteria in ginger roots and rhizomes, the roots and rhizomes were washed in tap water and surface sterilized with 1% sodium hypochlorite for 5 minutes and then washed in sterile distilled water. It is then immersed in 70% ethanol for 5 min and washed thrice in sterile distilled water. From this 0.1 g of the tissue was ground in phosphate buffer of pH 7.0. This suspension was serially diluted and plated in Tryptic Soy Agar (TSA) and TSA amended with NaCl (7%). The plates were incubated for 3-4 days at 28°C and observed for the bacterial colonies. The apoplastic fluid of pseudostems and leaves was extracted by vacuum infiltration and centrifugation method (36). This apoplastic fluid was plated in TSA and TSA amended with NaCl (7%) and incubated for 3 to 5 days and observed for the presence of typical colonies.

RESULTS AND DISCUSSION

Isolation of ginger apoplastic microorganisms

When apoplastic fluid was plated in three different selective media for isolation of fungi, bacteria and actinomycetes, colonies appeared only in bacterial media. No fungal or actinomycetes colonies appeared in their respective media even after 20 days of incubation. From the bacterial medium (Tryptic Soy Agar) a total of 150 bacterial colonies differing in colony morphology were isolated, purified and maintained as glycerol stock in 40% glycerol at -80°C (Fig. 1). The yield of apoplastic bacteria ranged from $3 \times 10^1$ to $3 \times 10^3$ in the pseudostems and $2 \times 10^2$ to $2 \times 10^3$ in the leaves from plants collected from different ginger growing tracts, whereas the yield ranged from $3 \times 10^1$ to $3 \times 10^3$ in and $1 \times 10^1$ to $9.9 \times 10^2$ respectively in the rhizomes and in the leaves of plants collected from different ginger germplasm accessions.

It is interesting to note that two of the germplasm accessions CLT-G-0139 and CLT-G-0413 did not show the presence of any culturable bacteria from apoplastic fluid on tryptic soy agar, so also, the leaves of ginger plant from Peruvannamuzhi, Kozhikode and also the germplasm accessions CLT-G-0089, CLT-G-0144, CLT-G-0187, CLT-G-0201, CLT-G-0204, CLT-G-0224, CLT-G-0249, CLT-G-0276. The isolates were named by giving the prefix IISRGAB where GAB stands for Ginger Apoplastic Bacteria and IISR stands for Indian Institute of Spices Research. They were numbered serially from 1 to 150. The details of apoplastic bacteria isolated are given in Table 1 and Table 2.

Diversity of apoplastic bacteria

A great diversity was obtained in the population of apoplastic bacteria isolated from ginger (Supplementary fig. 1). A maximum of 15 diverse bacteria were obtained from a place called Nanminda in Kozhikode district of Kerala. A maximum of 8-7 diverse types were obtained from cultivars like Maran, Mahima Varada, Rejatha, Rio de Janeiro etc. But in the germplasm accessions maximum diversity was observed in accession number CLT-G-0171, which was originally collected from Wayanad, Kerala. But from the other germplasm accessions only 1-4 diverse isolates could be obtained.

Table 1. Details of apoplastic bacteria isolated from ginger fields

| Sl. No. | Place          | Latitude & Longitude | Variety | CFU ml-1 (Pseudostem) | CFU ml-1 (Leaf) | No. of Bacterial isolate |
|--------|----------------|----------------------|---------|-----------------------|----------------|-------------------------|
| 1      | Peruvannamuzhi, Kozhikode, Kerala | 11°35’N & 75° 49’E | Mahima | 4.2× 102 | No bacteria | 8 (IISRGAB,1,2,3,4,5,6,7 & 8) |
| 2      | Nanmanda, Kozhikode, Kerala | 11° 24’N & 75° 49’E | Varada | 5.6× 102 | 6.6× 102 | 15 (IISRGAB 43 to 57) |
| 3      | Ambalavayal, Wayanad, Kerala | 11° 37’N & 76°12’E | Maran | 3× 101 | 2.5× 103 | 5 (IISRGAB58,59,60, 61&62) |
| 4      | Pulpally, Wayanad, Kerala | 11° 47’N & 76°9’E | Maran | 3.24× 103 | 2× 102 | 7 (IISRGAB 3, 64, 65, 66, 67, 68&69) |
| 5      | Suntikoppa, Karnataka | 12° 28’N & 75° 49’E | Riode Janeiro | 7.2× 102 | 1.24× 103 | 7 (IISRGAB35,36,37, 38,39,40,41&42) |
| 6      | Appangala, Kodagu, Karnataka | 12° 26’N & 75° 45’E | Mahima | 3.6× 102 | 8.4× 102 | 7 (IISRGAB21,22,23, 24,25,26&27) |
| 7      | Appangala, Kodagu, Karnataka | 12° 26’N & 75° 45’E | Suravi | 2.4× 102 | 3.2× 102 | 7 (IISRGAB 8,29,30, 31, 32,33 &34) |
| Sl. No. | Ginger accession no | Habitat | Collection place | CFU ml-1 (pseudostem) | CFU ml-1 (Leaf) | No. of bacterial isolates obtained |
|--------|---------------------|---------|------------------|----------------------|----------------|-----------------------------------|
| 1      | CLT-G-0089          | Cultivated | No data available | 1.3× 10^2            | No bacteria    | 2 (IISRGAB 9& 10)                 |
| 2      | CLT-G-0098          | Cultivated | Khasi Hills, Meghalaya | 2.1× 10^2            | 4.6× 10^2      | 2 (IISRGAB 102 & 103)             |
| 3      | CLT-G-0103          | Cultivated | Karbhi Anglong, Assam | 2.1× 10^2            | 2.7× 10^2      | 4 (IISRGAB 104, 105, 106 & 107)   |
| 4      | CLT-G-0127          | Cultivated | Mudigere, Karnataka | 3 × 10^1             | 7× 10^1        | 2 (IISRGAB 70 & 71)               |
| 5      | CLT-G-0139          | Cultivated | No data available | No bacteria          | No bacteria    | No bacteria                       |
| 6      | CLT-G-0144          | Cultivated | No data available | 1.6× 10^2            | No bacteria    | 2 (IISRGAB 11 & 12)               |
| 7      | CLT-G-0160          | Velliyur | Kozhikode, Kerala | 1.04× 10^3           | 1.9× 10^2      | 5 (IISRGAB 13, 14, 72,73 & 74)    |
| 8      | CLT-G-0165          | Cultivated | Wayanad, Kerala | 1.0× 10^2            | 1× 101         | 3 (IISRGAB 15, 75 & 76)           |
| 9      | CLT-G-0171          | Cultivated | Wayanad, Kerala | 4.5× 10^2            | 3.8× 10^2      | 6 (IISRGAB 77, 78,79,80,81&82)    |
| 10     | CLT-G-0187          | Cultivated | Assam | 1.2× 10^2          | No bacteria    | 1 (IISRGAB 15)                    |
| 11     | CLT-G-0201          | Cultivated | Angamali market, Eranakulam | 3× 10^1            | No bacteria    | 1 (IISRGAB 16)                    |
| 12     | CLT-G-0203          | Cultivated | Kottayam, Kerala | 1.7× 10^2            | 9.9× 10^2      | 3 (IISRGAB 83,84&85)              |
| 13     | CLT-G-0204          | Cultivated | Thodupuzha, Kerala | 2.3× 10^2            | No bacteria    | 3 (IISRGAB 86,87&88)             |
| 14     | CLT-G-0211          | Cultivated | Chengannur, Alappuzha, Kerala | 1.2× 10^2 | 1.2× 10^2 | 2 (IISRGAB 89 & 90) |
| 15     | CLT-G-0219          | Cultivated | Nagercoil, Tamil Nadu | 5× 10^1             | 3× 10^2        | 5 (IISRGAB 91, 92,93,94&95)      |
| 16     | CLT-G-0224          | Cultivated | Sooranad, Kollam, Kerala | 9× 10^1            | No bacteria    | 1 (IISRGAB 17)                    |
| 17     | CLT-G-0227          | Cultivated | Angamali, Eranakulam, Kerala | 1.0× 10^2 | 1.4× 10^2 | 3 (IISRGAB 96, 97 & 98) |
| 18     | CLT-G-0238          | Cultivated | Pottangi, Orissa | 3.22× 10^3           | 7.6× 10^2      | 2 (IISRGAB 99, 100 & 101)        |
| 19     | IISR-240            | wild | Silent Valley, Palakkad, Kerala | 1.3× 10^2 | 1.7× 10^2 | 2 (IISRGAB 117 & 118)            |
| 20     | IISR-246            | wild | Sabarimala, Kerala | 2× 10^1             | 1.6××10^2     | 4 (IISRGAB 126,127,128 & 129)     |
| 21     | CLT-G-0249          | Cultivated | Jorhat, Assam | 1.1× 10^2            | No bacteria    | 1 (IISRGAB 18)                    |
| 22     | CLT-G-0253          | Cultivated | Shillong, Meghalaya | 1.5× 10^2            | 1.9× 10^2      | 1 (IISRGAB 119)                   |
| 23     | CLT-G-0254          | Cultivated | Howrah, West Bengal | 2.2× 10^2            | 3.5× 10^2      | 1 (IISRGAB 120)                   |
| 24     | CLT-G-0255          | Cultivated | Agarthala, Tripura | 3.2× 10^2            | 4.6× 10^2      | 4 (IISRGAB 130, 131, 132 & 133)   |
| 25     | CLT-G-0261          | Cultivated | ICAR Complex, Shillong, Meghalaya | 1.8× 10^2 | 1.2× 10^2 | 1 (IISRGAB 121)                  |
| 26     | CLT-G-0276          | Cultivated | Agarthala, Tripura | 3× 10^1             | No bacteria    | 2 (IISRGAB 19 & 20)              |
| 27     | CLT-G-0296          | Cultivated | Pathanamthitta, Kerala | 1.20× 10^3           | 2.9× 10^2      | 2 (IISRGAB 108 & 109)            |
| 28     | CLT-G-0413          | Cultivated | Visakhapatnam | No bacteria          | No bacteria    | No colonies                       |
| 29     | CLT-G-0428          | Cultivated | Jamaica | 2.0× 10^2            | 1.8× 10^2      | 4 (IISRGAB 110,111,112 & 113)    |
| 30     | CLT-G-0441          | Cultivated | Jamaica | 6.4× 10^2            | 2.3× 10^2      | 1 (IISRGAB 122)                   |
Apoplastic bacteria against *Ralstonia pseudosolanacearum* race 4

More than 88.67% of ginger apoplastic bacteria produced the enzyme catalase which is indicated by the bubble formation immediately on adding H$_2$O$_2$. Based on the carbohydrate utilization pattern, the 150 isolates form 22 groups, out of which 5.33% can utilize all five tested carbohydrates while 26% cannot utilize any one of the five carbohydrates tested. 22.67% can use glucose, sucrose and mannitol and 14% can use glucose and sucrose. None of the bacteria utilize mannitol alone, whereas it was found that seven isolates can utilize glucose alone, five isolates sucrose alone and two isolates sorbitol alone (Table 3).

**Biochemical diversity**

![Image](image_url)

Fig. 1. (a) Apoplastic fluid extracted from ginger leaf. (b) apoplastic fluid extracted from ginger pseudostem. (c) Bacterial colonies on tryptic soy agar.

*Rhizobiaceae* (2.66%), *Aeromonadaceae* (3.33%) and *Moraxellaceae* (3.33%) (Table 4; Supplementary Fig. 2).

**Table 3. Carbohydrate utilization of apoplastic bacteria**

| Sl. No. | Sugar utilization pattern | Apoplastic bacterial isolates (IISRGAB) | No. of bacteria vs. Percentage |
|--------|--------------------------|----------------------------------------|------------------------------|
| 1      | All five sugars          | 26,27,33,37,39,40,41,44                | 8 (5.33%)                    |
| 2      | No sugars                | 1,2,8,9,10,11,12,24,31,32,51,60,62,65,66,67,69,70,71,72,73,74,75,76,77,78,79,80,81,82,86,92,93,94,95,105,108,132,149 | 39 (26%)                     |
| 3      | Glucose alone            | 54,56,85,88,89,100,107                 | 7 (4.67%)                    |
| 4      | Sucrose alone            | 47,64,87,137,138                       | 5 (3.33%)                    |
| 5      | Lactose alone            | 6,7,14                                 | 3 (2.0%)                     |
| 6      | Mannitol alone           | No bacteria -                          | -                            |
| 7      | Sorbitol alone           | 16,63                                  | 2 (1.33%)                    |
| 8      | Glucose & sucrose        | 29,57,59,90,97,101,109,111,12,116,117,119,121,127,128,133,140,141,142,146,148     | 21 (14.0%)                   |
| 9      | Glucose & lactose        | 5,15                                   | 2 (1.33%)                    |
| 10     | Glucose & mannitol       | 104,123,124,125,131                    | 5 (3.33%)                    |
| 11     | Glucose & sorbitol       | 46,50,68                               | 3 (2.0%)                     |
| 12     | Glucose, Sucrose & mannitol | 4,23,28,30,42,43,45,48,52,53,55,58,84,91,96,99,102,103,106,110,113,114,115,118,120,129,130,134,135,136,139,143,144,147 | 34 (22.67%)                 |
| 13     | Glucose, sucrose & sorbitol | 126                                   | 1 (0.67%)                    |
| 14     | Sucrose & lactose        | 13,17                                  | 2 (1.33%)                    |
| 15     | Sucrose & mannitol       | 61,145                                 | 2 (1.33%)                    |
| 16     | Sucrose, lactose, mannitol, sorbitol | 3,19,36                               | 3 (2.0%)                     |

**In vitro and in planta Screening**

Among the 150 bacteria isolated from apoplastic
fluid and tested, only three bacteria showed inhibition to *R. pseudosolanacearum*. The inhibition zone ranged from 2 to 10 mm. The highest inhibition was shown by IISRGAB 5 (10mm) followed by IISRGAB 146 (5mm) (Fig. 2).

All the 150 bacteria were also tested *in planta* for disease suppression. In *in planta* screening, infection was observed as typical bacterial wilt symptom from 10th day onwards after inoculation. The wilting percentage ranged from 0-100% (Supplementary Table 1). The time taken for infection varied from 10-30 days. All the isolates except IISRGAB 24, IISRGAB 48 and IISRGAB 107 showed more than 75% wilting/infection within 30 days of inoculation. One isolate IISRGAB84 showed infection within 10-15 DAI. Sixty-three isolates (42%) showed infection in 16-30 days and three isolates showed ≥50% infection in 30 days which include IISRGAB 43 (20%), IISRGAB 5 (40%) and IISRGAB 146 (50%). But three isolates viz. (IISRGAB 24, IISRGAB 48 and IISRGAB 107) did not took infection/wilting till the end of the experiment (Fig. 3) when compared to positive control where total collapse of plant was observed within 10 days of inoculation. The isolates that showed *in vitro* antagonistic effect is found ineffective under *in planta* evaluation, indicating that *in vitro* evaluation alone is insufficient to test the antagonistic ability of the organisms against soil borne pathogens.

**Characterization of apoplastic bacteria for potential traits**

The isolates that showed *in vitro* antagonistic effect as well as that showed *in planta* biocontrol potential were characterized for biocontrol traits. *In vitro* antibiosis by agar well diffusion method clearly showed the inhibition of *R. pseudosolanacearum* by three isolates viz. IISRGAB 5, IISRGAB 43 and IISRGAB 146 (Table 5 and Supplementary Fig. 3a). The size of inhibition zone ranged between 3.5- 4.5 mm. However, three isolates viz. IISRGAB 24, IISRGAB 48 and IISRGAB 107 did not show any inhibition.

Among the six isolates IISRGAB 5 was found to be the highest producer of siderophore followed by IISRGAB 24, IISRGAB 48 and IISRGAB 107 based on the radius of the halo formed around the colony, however, none of the short listed isolates produced HCN. Acetoin production by glucose
metabolism was noticed only in IISRGAB 5 and IISRGAB 107 (Table 5 and Supplementary Fig. 3b and c).

All the six isolates are found producing ammonia as well as IAA. The IAA production ranged from 5.37 to 11.83 µg ml⁻¹. IISRGAB 5 and IISRGAB 48 were found to be producing more IAA in comparison with other four bacteria. IISR GAB 5, IISR GAB 24, IISR GAB 107 and IISR GAB 146 could solubilise insoluble phosphate (Table 5 and Supplementary Fig. 4a, b and c).

**Extracellular enzyme production**

Among the six isolates five (IISRGAB 5, IISR GAB 24, IISR GAB 48, IISR GAB 107, IISR GAB 146) could synthesize α-amylase as well as cellulase. But only three isolates (IISRGAB 5, 48, 107) could produce protease (Table 5) while none of them could synthesize lipases.

**Identification of promising apoplastic bacteria**

By 16SrDNA sequencing, 1500 bp amplicons was obtained for all the six isolates (Supplementary Fig. 5). The sequences were assembled and compared with sequences of NCBI by Blastn (http://www.ncbi.nlm.nih.gov/BLAST). IISRGAB 5 showed 99% similarity towards both Bacillus subtilis and B. amyloliquefaciens and IISRGAB 107 showed 99% similarity towards B. licheniformis and B. sonorensis. Based on the salt tolerance test the identity of IISRGAB 5 was confirmed as B. subtilis. Similarly, in tryptic soy broth with NaCl concentrations from 1% to 10%, turbidity was observed in all concentrations within 24 h. of incubation confirming the identity of IISRGAB 107 as B. licheniformis. IISRGAB 43 was identified as B. marisflavi, IISRGAB 24 as Agrobacterium tumefaciens, IISRGAB 48 as Micrococcus luteus and IISRGAB 146 as Staphylococcus haemolyticus. The identity and GenBank accession numbers are given in Table 6.

**Pot culture evaluation**

There was significant reduction in bacterial wilt with apoplastic bacterial treatment, individually and in combination, when compared with pathogen control as well as chemical control. After 10 days of pathogen inoculation, 33-59% disease reduction was observed with apoplastic bacteria over pathogen control while it was only 12.28% and 21.39% respectively with streptomycin sulphate and copper oxychloride, the recommended chemicals against bacterial wilt. After 20 days of pathogen inoculation there was significant difference in the disease incidence by different treatments and the disease reduction over control by different apoplastic bacteria showed wide difference ranging from 9 to 79%. The treatments T15 (IISRGAB5+ IISRGAB 43+ IISRGAB 48+ IISRGAB 107) and T4 (IISRGAB 107) showed a disease reduction of 78.82% and 72.1% respectively over pathogen control and were at par with absolute control (79.94%). The same trend was noticed even after 30 days of inoculation. But the situation was changed after 30 days where most of the apoplastic bacterial treatments, infection was found gradually increased and reached the level as in pathogen control. After 40 dai, only IISRGAB 107 treated plants showed significant reduction (66.42%) in disease over control (Fig. 4). The periodical disease incidence is given

| Bacteria | Family                  | Population (%) | Isolates                                      |
|----------|-------------------------|----------------|-----------------------------------------------|
| Gram positive rods | Bacilaceae          | 38.66          | IISRGAB 4, 5, 7, 9, 12, 14, 31, 32, 37, 43, 44, 49, 52, 53, 60, 61, 68, 69, 72, 73, 74, 76, 79, 80, 82, 89, 90, 92, 94, 98, 100, 101, 102, 103, 104, 106, 107, 109, 111, 116, 117, 118, 123, 124, 127, 130, 131, 132, 133, 134, 135, 136, 138, 139, 140, 145, 147, 148 |
|          | Lactobacilaceae        | 3.33           | IISRGAB 15, 18, 19, 21, 25                    |
| Gram negative rods | Pseudomonadaceae    | 22.66          | IISRGAB 10, 16, 29, 38, 45, 50, 56, 57, 67, 70, 71, 75, 77, 78, 81, 95, 96, 97, 99, 105, 115, 119, 121, 122, 125, 126, 128, 129, 137, 141, 142, 143, 149, 150 |
|          | Euterobacteriaceae     | 7.33           | IISRGAB 6,13, 22, 36, 42, 40, 51, 108, 110, 113, 120 |
|          | Rhizobiaceae           | 2.66           | IISRGAB 1, 2, 24, 91                          |
|          | Aeromonadaceae         | 3.33           | IISRGAB 23, 30, 84, 86, 87                    |
|          | Moraxellaceae          | 3.33           | IISRGAB 34, 35, 39, 93, 112                   |
| Gram positive cocci | Staphylococccaceae   | 10.0           | IISRGAB 3, 8, 20, 28, 55, 58, 62, 63, 64, 66, 83, 88, 114, 144, 146 |
|          | Micrococccaceae        | 6.0            | 26, 33, 46, 47, 48, 54, 59, 65, 85             |
| Gram negative cocci | Unknown              | 2.66           | IISRGAB 11, 17, 27, 41                        |
During harvest after eight months, the average yield obtained per pot with IISR GAB 107 treated plants was 161.74g, compared to absolute control where the average yield per pot was 179.5g. No yield was obtained from any other treatments. The result indicated the potential of IISR GAB 107 as an antagonist to *R. pseudosolanacearum*.

Soil parameters and population of pathogen and antagonistic bacteria in rhizosphere soil.

Different soil parameters like pH, Electrical Conductivity (EC) and dehydrogenase activity of the IISRGAB 107 treated soil were studied in comparison with absolute control and pathogen control (Table 4). Dehydrogenase activity, which is an indicator of soil microbial activity, was found to be more in IISRGAB 107 treated soil. There is considerable reduction in the population of *R. pseudosolanacearum* in the soil treated with IISRGAB 107 i.e., 1.2×10⁸ CFU g⁻¹ (which is below the infection level) when compared with the pathogen control where the highest population of pathogen was present i.e. 2×10⁹ CFU g⁻¹. In uninoculated control, no *R. pseudosolanacearum* could be detected. Similarly, rhizosphere bacterial population was found to be more in the case of soil treated with IISRGAB 107 when compared with absolute control and pathogen control. In *R. pseudosolanacearum* treated soil there is significant reduction in the other soil bacterial population (Table 8).

**Comparison of endophytic population of ginger roots, rhizomes, pseudostems and leaves**

There was considerable difference in the population and diversity of bacteria in the plants treated with IISRGAB107 when compared to control. In general endophytic bacterial colonization was found higher in ginger roots in both control and IISRGAB 107 treatment, but when TSA amended with NaCl was used there was a 10 fold increase in the bacterial population in IISRGAB 107 treatment (T4) in roots and 100

**Table 5. Characterization of shortlisted isolates for biocontrol and growth promoting traits**

| Isolate     | Inhibition zone by Agar gel diffusion (mm) | Production of Siderophore | Production of HCN | Production of Acetoin | Production of Ammonia (µg ml⁻¹) | Production of IAA (µg ml⁻¹) | Phosphate solubilization | Production of Amylase | Production of Protease | Production of Cellulase | Production of Lipase |
|-------------|--------------------------------------------|---------------------------|-------------------|-----------------------|---------------------------------|-----------------------------|---------------------------|------------------------|-----------------------|-----------------------|-----------------------|
| IISR GAB 5  | 4                                          | +                         | -                 | +                     | +                               | 11.12                       | +                        | +                      | +                    | +                     | +                     |
| IISR GAB 24 | 0                                          | +                         | -                 | -                     | +                               | 6.54                        | +                        | +                      | -                    | +                     | -                     |
| IISR GAB 43 | 3.5                                        | -                         | -                 | -                     | +                               | 5.37                        | -                        | -                      | -                    | +                     | -                     |
| IISR GAB 48 | 0                                          | +                         | -                 | -                     | +                               | 11.83                       | -                        | +                      | +                    | -                     | -                     |
| IISR GAB 107| 0                                          | +                         | -                 | +                     | +                               | 5.45                        | +                        | +                      | +                    | +                     | -                     |
| IISR GAB 146| 4.5                                        | -                         | -                 | -                     | +                               | 6.05                        | +                        | +                      | -                    | -                     | +                     |

+ Denotes presence of trait
- Denotes absence of trait

**Table 6. Identification of shortlisted apoplastic bacteria by 16S rDNA sequencing**

| Sl. No | Isolate name | Identification          | GenBank Acc. |
|--------|--------------|-------------------------|--------------|
| 1      | IISR GAB 5   | *Bacillus subtilis*     | KU196772     |
| 2      | IISR GAB 24  | *Agrobacterium tumefaciens* | KU196773   |
| 3      | IISR GAB 43  | *Bacillus marisflavi*   | KU258013     |
| 4      | IISR GAB 48  | *Micrococcus luteus*    | KU258014     |
| 5      | IISR GAB 107 | *Bacillus licheniformis* | KU258015   |
| 6      | IISR GAB 146 | *Staphylococcus haemolyticus* | KU258016 |

in table 7 and Fig. 5. During harvest after eight months, the average yield obtained per pot with IISR GAB 107 treated plants was 161.74g, compared to absolute control where the average yield per pot was 179.5g. No yield was obtained from any other treatments. The result indicated the potential of IISR GAB 107 as an antagonist to *R. pseudosolanacearum*.

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Apoplastic bacteria against *Ralstonia pseudosolanacearum* race 4

Table 7. Effect of selected apoplastic bacteria and their consortia in bacterial wilt management (in planta effect)

| Treatments          | Disease incidence (%) |
|---------------------|-----------------------|
|                     | 10dai | 20dai | 30dai | 40dai |
| T1 IISRGAB 5        | 19.00 | 64.21<sup>ABC</sup> | 62.41<sup>ABC</sup> | 66.73<sup>A</sup> |
|                     | (9.58) | (79.89) | (83.33) | (83.33) |
| T2 IISRGAB 43       | 17.77 | 45.82<sup>B</sup> | 47.82<sup>DEF</sup> | 77.00<sup>A</sup> |
|                     | (14.58) | (51.40) | (75.22) | (100.00) |
| T3 IISRGAB 48       | 16.97 | 49.00<sup>AB</sup> | 54.74<sup>BCD</sup> | 65.74<sup>A</sup> |
|                     | (6.57) | (54.43) | (64.01) | (82.51) |
| T4 IISRGAB 107      | 15.12 | 21.14<sup>EF</sup> | 30.39<sup>GF</sup> | 26.28<sup>B</sup> |
|                     | (11.69) | (11.69) | (26.77) | (19.40) |
| T5 IISRGAB 5+43     | 22.74 | 66.67<sup>AB</sup> | 66.67<sup>ABCD</sup> | 69.10<sup>A</sup> |
|                     | (18.33) | (88.89) | (88.89) | (88.89) |
| T6 IISRGAB 5+48     | 23.12 | 52.48<sup>BCD</sup> | 53.91<sup>CDEF</sup> | 68.83<sup>A</sup> |
|                     | (17.57) | (79.37) | (77.78) | (79.37) |
| T11 IISRGAB 5+43+48 | 22.61 | 56.97<sup>ABCD</sup> | 62.71<sup>ABCD</sup> | 76.96<sup>A</sup> |
|                     | (17.78) | (83.49) | (81.00) | (100.00) |
| T10 IISRGAB 107     | 35.77 | 50.67<sup>ABCD</sup> | 53.17<sup>DF</sup> | 76.96<sup>A</sup> |
|                     | (35.15) | (51.85) | (70.00) | (100.00) |
| T12 IISRGAB 5+43+48 | 20.03 | 68.88<sup>AB</sup> | 78.24<sup>A</sup> | 78.24<sup>A</sup> |
|                     | (19.80) | (83.33) | (100.00) | (100.00) |
| T13 IISRGAB 43+48+107 | 41.33 | 65.93<sup>AB</sup> | 78.81<sup>A</sup> | 74.78<sup>AB</sup> |
|                     | (40.51) | (71.93) | (100.00) | (100.00) |
| T14 IISRGAB 5+43+107 | 23.35 | 36.53<sup>CDEF</sup> | 77.60<sup>AB</sup> | 77.60<sup>AB</sup> |
|                     | (20.32) | (38.10) | (100.00) | (100.00) |
| T15 IISRGAB 48+107  | 16.05 | 16.05<sup>F</sup> | 62.01<sup>ABC</sup> | 76.91<sup>ABC</sup> |
|                     | (8.33) | (8.33) | (80.00) | (97.10) |
| T16 Copper-oxide chloride (200 ppm) | 27.71 | 68.88<sup>AB</sup> | 76.70<sup>AB</sup> | 76.30<sup>ABC</sup> |
|                     | (21.73) | (88.52) | (97.43) | (95.24) |
| T17 Streptomycin sulphate (200 ppm) | 30.92 | 30.92<sup>DEF</sup> | 77.71<sup>AB</sup> | 77.71<sup>AB</sup> |
|                     | (27.95) | (27.95) | (100.00) | (100.00) |
| T18 Pathogen control | 35.25 | 75.78<sup>A</sup> | 67.37<sup>ABCD</sup> | 78.27<sup>A</sup> |
|                     | (34.45) | (93.97) | (100.00) | (100.00) |
| T19 Absolute control | 13.17 | 15.20<sup>F</sup> | 15.81<sup>G</sup> | 18.24<sup>B</sup> |
|                     | (0.00) | (0.00) | (0.00) | (0.00) |
| General Mean        | 24.49 | 50.14 | 60.68 | 68.87 |
| CV (%)              | 55.13 | 35.15 | 23.38 | 12.71 |
| SE (d)              | 11.022 | 14.391 | 11.585 | 7.145 |
| LSD at 5%           | NS** | 29.134 | 23.452 | 14.465 |

LSD at 5% denotes the Least Significant Difference at P ≤ 0.05
* Figures in parenthesis is original value
** NS denotes non-significant

Since the inoculated antagonist is inhabitant of intercellular spaces (apoplast), the apoplastic colonization was also tested in the leaves and pseudostems of IISRGAB 107 treated plants i.e., when the apoplastic fluid from the pseudostem and leaf were plated in TSA and also in TSA amended with 7% NaCl, a higher number of bacteria were found in the treated plant tissues. The amount of apoplastic bacterial population in leaf and pseudostems of the uninoculated plant (control) remains the same. But there was a 10 fold increase in the population of apoplastic bacteria in the pseudostems and leaves of IISRGAB 107 treated plants and also found that the apoplastic niche was occupied by *B. licheniformis* (Table 8). This indicated the re-colonization of apoplastic bacteria after rhizobacterization.

*Ralstonia pseudosolanacearum*, being a universal pathogen infecting tomato, potato, tobacco, banana, ginger etc., various disease management strategies including cultural, chemical and biological control were attempted, however no complete success was obtained and due to the diverse species ecology, universal control measures are not feasible also (Saddler, 2005). *R. solanacearum* has been known for its multiplicity in the xylem and intercellular sap, where the intercellular space is important in many biological functions like nutrient transport, plant pathogen interaction etc (Bakon and Hinton, 2006). So, in the current study the apoplastic fluid was extracted from the intercellular spaces of leaves and pseudostem of healthy ginger plants collected from various locations and also from germplasm collections. The vacuum infiltration and centrifugation technique was employed for the isolation of bacteria from the apoplastic fluid of ginger. The same technique was employed by Bell et al., 1995 to extract the xylem sap from the roots of grapevine and also by Gardner et al. 1982 from citrus plants. The infiltration–centrifugation technique has been widely used for many plant species for intercellular fluid extraction due to efficiency and simplicity (Klement, 1965; Luwe et al., 1999; Cheng et al., 2007; Nouchi et al., 2012). Dong et al. (1994) used centrifugation procedure for intercellular fluid extraction from sugar cane. The technique involved the selective isolation of bacteria colonized in the intercellular spaces and the xylem. Though the apoplastic fluid was plated in selective fungal and actinomycetes isolation medium, none of the culturable fungi or actinomycetes could be obtained.

In the present study, the population of apoplastic bacteria ranged from $3 \times 10^1$ to $3 \times 10^3$ g$^{-1}$ in the pseudostems and zero to $2 \times 10^1$ g$^{-1}$ in the leaves from ginger plants collected from different ginger growing tracts. Similarly, the population...
of apoplastic bacteria ranged from zero to $3 \times 10^3$ g$^{-1}$ in the pseudostems and zero to $9.9 \times 10^2$ g$^{-1}$ in the leaves from plants collected from different ginger germplasm accessions. Various researchers reported varying population level of endophytic bacteria according to the host plants. For example, alfalfa xylem tissue have $6.0 \times 10^4$ to $4.3 \times 10^5$ g$^{-1}$ (Gagne et al., 1987), cotton xylem tissue have $1 \times 10^2$ to $10^7$ g$^{-1}$ (Jacobs et al., 1985) and potato tubers have $0$ to $1.6 \times 10^4$ g$^{-1}$ (De Boer and Copeman, 1974). Considering the population level of endophytic bacteria in the apoplastic fluid, the number is less than the endophytic population obtained by trituration of homogenized plant tissues as already reported by Gardner et al. (1982) and Hallmann et al. (1997a). Even if some variations observed in the population level, this may be due to the selected solid agar medium and incubation conditions employed in the current study. Also, Bell et al. (1995) observed a lower population of endophytic bacteria isolated in solid medium up on vacuum extraction from grape vine stem when compared with direct staining with acridine orange or tissue maceration and plating on solid media. The difference in the population level of endophytes among different ginger samples may be due to the difference in geographic location and the environmental factors from where the plants were collected. The plants collected from germplasm bank showed difference in the endophytic

![Fig. 4. Pot trial evaluation of apoplastic bacteria against bacterial wilt of ginger. (a) Forty five days old ginger plants in pots before Ralstonia pseudosolanacearum inoculation. (b) Bacterial wilt incidence after 15 days of pathogen inoculation. (c) Uninfected ginger plants treated with IISRGAB 107 in comparison with absolute control.](image)

![Fig 5. Graph showing bacterial wilt reduction by apoplastic bacteria.](image)
Apoplastic bacteria against Ralstonia pseudosolanacearum race 4 colonization, which may be due to the original location from where these accessions were collected. This shows the diversity of indigenous endophytic population within a plant species. Chen et al. (2014) studied the population density and distribution of endophytic bacteria from different plant parts at three growth stages of ginger viz. seedling stage, vigorous growth stage and rhizome enlargement stage. They observed a higher colonization of endophytes at the seedling stage both in number and diversity and it was reduced when plant growth occurred. The prominent genera were Bacillus and Pseudomonas, both of which were found in all growth stages.

Of the 150 bacteria isolated from ginger pseudostems and leaves, 43.3% were gram positive rods and 38% were gram negative rod which is in accordance with the endophytic bacteria isolated from corn roots which consisted of 88% Gram positive bacteria (Lalande et al., 1989). Similar observations were made by Zinniel et al. (2002) from corn stalks and Aravind et al. (2009) from black pepper. However, the population diversity was contradictory to the reported population of endophytic bacteria from different plants like grapevine, citrus, corn, cotton, tomato, chilli, eggplant etc (Bell et al., 1995; Gardner et al., 1982; Hallmann et al., 1997; Amaresan et al., 2012; Achari and Ramesh, 2014; Upreti and Thomas, 2015).

Each bacterium has its own collection of enzymes that enable them to use diverse carbohydrates. This is often exploited in the identification of bacterial species. (www2.muw.edu/~lbrandon/Micro/carbs.doc). Based on the sugar utilization pattern the apoplastic bacteria isolated were grouped into 22 groups which include the families viz. Bacillaceae, Lactobacillaceae, Enterobacteriaceae, Pseudomonadaceae, Aeromonadaceae, Moraxellaceae, Rhizobiaceae, Staphylococcaceae and Micrococcaceae.

The result of both in vitro and in planta screening clearly revealed that all the apoplastic inhabitants are not always antagonistic to the invading pathogens. Most of them may coexist with the pathogen while some of them resist the pathogen by antibiosis or by systemic resistance or competition for space and nutrients as shown by IISRGAB 24, IISRGAB 48 and IISRGAB 107. The lack of inhibition may be due to the inability of the apoplastic resident to grow in pace with the fast multiplication of the pathogen.

When the six bacteria were screened for production of diffusible inhibitory compounds like siderophore and hydrogen cyanide, it was found that most of the isolates are producing siderophores, but none of them are producing HCN. Similar observations were reported in case of endophytes associated with sugar cane by Mendes et al. (2007). Two of the Bacillus isolates IISRGAB 5 and IISRGAB 107 could produce acetoin by glucose metabolism. Acetoin is an important volatile organic compound produced by plant associated bacteria, which has been reported as one of the elicitor of induced systemic resistance in plants up on pathogen inoculation (Kim et al., 2011). The studies conducted by Rudrappa et al. (2010) on Arabidopsis thaliana showed that Bacillus subtilis strain FB17 derived acetoin, triggered induced systemic resistance in plants when infected with Pseudomonas syringae pv. tomato. Though in small quantities, all the six isolates, short listed in our study, could produce ammonia and IAA which has a crucial role in plant growth and its additional supply can support the host in stress conditions like drought and pathogen attack (Belimov et al., 2015).

16S rDNA sequence identification revealed two of the six isolates as A. tumefaciens and S. haemolyticus, which were also isolated from the xylem sap of egg plant and chilli (Achari and Ramesh, 2014). The occurrence of B. marisflavi as an endophyte of plant has been recorded for the first time in ginger. Earlier this bacterium has been isolated from yellow sea in South Korea (Yoon et al., 2003) and from agricultural waste in Tamil Nadu India (Anthony et al., 2014).

The short listed bacteria were used for the control of R. pseudosolanacearum under green house conditions individually and in consortia. In an initial trial (data not presented) there was 100% disease incidence and no yield could be recorded. This may be due to the insufficient colonization of the bacteria in the plant rhizosphere or endophytically. It was already reported in the case of some endophytes that even if the inoculum level is high but that whole population will not colonize endophytically. That means there is always an optimum holding capacity of the plants for endophytes, which may fluctuate according to the age of the plant and environmental factors (Hallmann et al., 1997). In the pot culture evaluation IISRGAB 107 (Bacillus licheniformis) could effectively reduce the wilt incidence by 65% over control. But when IISRGAB 107 was applied in combination with other apoplastic bacteria the concurring disease reduction is not happening. This may be due to the competition between these apoplastic bacteria for the same niche, which may play a role and may contribute to the inferior colonization of IISRGAB 107.

Diverse Bacillus species has been reported as effective biocontrol agents against plant pathogens (Lemessa and Zeller, 2007; Ji et al., 2008; Maketon et al., 2008). Bacillus subtilis has been in use in mulberry and tobacco against R. pseudosolanacearum (Ji, 2008; Lemessa and Zeller, 2007; Maketon et al., 2008) in tomato against Xanthomonas euvesicatoria and Xanthomonas perforans (Roberts et al., 2008) and also against various fungal pathogens and plant.
There are also reports that *B. licheniformis* has been effectively employed for the control of strawberry gray mold and tomato gray mold caused by *Botrytis cinerea* (Kim et al., 2007; Lee et al., 2006). Later Kong et al. (2010) identified two major compounds iturin A and surfactin, from *B. licheniformis* strain N1, showing antifungal activity against many fungal pathogens. Also *B. licheniformis* has been used as a biofungicide against *Colletotrichum graminicola* and *Sclerotinia homeocarpa* in turf farms and arboretum (Cawoy et al., 2011). Amaresan et al. (2014) reported *B. licheniformis* from chilli was effective in reducing the major diseases of chilli. *B. licheniformis* strain GL174 an endophyte from *Vitis vinifera* has been studied extensively for its endophytic nature and biocontrol properties against the major fungal pathogens of host (Nilgris et al., 2018) Hence to conclude, *B. licheniformis* strain IISRGAB 107 identified in this study is promising, possessing major growth promoting traits as well as biocontrol traits, can be effectively used for developing a bactericide for controlling bacterial wilt of ginger.

**Identity confirmation of IISRGAB 5 (*Bacillus subtilis*) and IISRGAB 107 (*Bacillus licheniformis*) using salt tolerance test**

When 16S rDNA sequences were analysed by blasting in NCBI Blastn (http://www.ncbi.nlm.nih.gov/BLAST), programme, IISRGAB 5 showed 99% similarity towards both *B. subtilis* and *B. amyloliquefaciens* and IISRGAB 107 showed 99% similarity towards *B. licheniformis* and *B. sonorensis*. In order to confirm the exact identity of the bacteria tolerance to NaCl was done. According to Welker and Campbell (1967), *B. subtilis* vary from *B. amyloliquefaciens* in salt tolerance i.e. *B. subtilis* cannot grow in a medium containing 10% NaCl but *B. amyloliquefaciens* can grow. So nutrient broth was prepared with differential concentrations of NaCl (0.5%, 2%, 4%, 8%, and 10%) and pure colony of IISRGAB 5 was inoculated in each flask and incubated in an orbital shaker at 280 C at 180 rpm for 3-5 days. Based on the salt tolerance test, it was found that IISRGAB 5 could tolerate only up to 8% NaCl amended nutrient broth. There is no growth in 10% NaCl even after 5 days of incubation (Figure 3-4b). So, this confirmed the identity of IISRGAB 5 as *B. subtilis* Similarly Palmisano et al. (2001) reported that salt tolerance is a method to distinguish *B. sonorensis* from *B. licheniformis*. *B. sonorensis* can tolerate only up to 3% NaCl in the medium, while *B. licheniformis* can tolerate up to 10% NaCl. Accordingly tryptic soy broth was prepared with different concentrations of NaCl ranging from 1% to 10%. IISRGAB 107 was inoculated into each concentration and incubated in an orbital shaker at 28°C at 180 rpm for 7days. Turbidity was observed in all concentrations within 24 hours of incubation. So, it is clear that IISRGAB 107 can tolerate up to 10% NaCl. Hence, the identity of the bacterium was confirmed as *Bacillus licheniformis*.

**CONCLUSION**

Among the 150 bacteria isolates from ginger apoplastic fluid, only one isolate namely GAB107 identified as *Bacillus licheniformis* was found to be a potential candidate against *Ralstonia pseudosolanacearum*. Since bacterial wilt of ginger is a threatening problem and increased and non-judicious and indiscriminate application of plant protection chemicals spoil the nature, it is suggested to scale up the dissemination of this biocontrol agents for saving the nature as well as to save the farmers from bacterial wilt crisis.

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**Supplementary Fig. 2.** Microscopic appearance of Gram stained cells of apoplastic bacteria

**Supplementary Fig. 3.** Characterization of apoplastic bacteria for biocontrol traits a. antibiosis by agar well diffusion method b. siderophore production c. Acetoin production.

**Supplementary Fig. 4.** Characterization of apoplastic bacteria for promoting traits. a. IAA production b. Ammonia production c. Phosphate solubilization.

**Supplementary Fig. 5.** Gel image showing the 1500 bp amplicon of partial 16s rDNA gene of short listed apoplastic bacteria.
Supplementary Table 1. *In vitro, in planta* screening and siderophore production of apoplastic bacteria against *Ralstonia solanacearum* race 4

| Isolate No. | In vitro screening (inhibition zone) | Siderophore | Disease incidence (%) | Days taken for wilting | Isolate No. | In vitro screening | Siderophore | Disease incidence (%) | Days taken for wilting |
|-------------|--------------------------------------|-------------|-----------------------|------------------------|-------------|-------------------|-------------|-----------------------|------------------------|
| IISRGAB 1   | No inhibition +(3)                   | 100         | 10                    | IISRGAB 47             | No inhibition +(4) | 80               | 30                      |
| IISRGAB 2   | No inhibition +(2)                   | 66.66       | 10                    | IISRGAB 48             | No inhibition +(3) | 0                | No symptoms             |
| IISRGAB 3   | No inhibition +(1)                   | 75          | 13                    | IISRGAB 49             | No inhibition +(3) | 100              | 27                      |
| IISRGAB 4   | No inhibition +(1)                   | 75          | 19                    | IISRGAB 50             | No inhibition +(4) | 100              | 10                      |
| IISRGAB 5   | 10.0 +(7)                            | 40          | 30                    | IISRGAB 51             | No inhibition +(2) | 85.7             | 13                      |
| IISRGAB 6   | No inhibition +(2)                   | 100         | 19                    | IISRGAB 52             | No inhibition +(3) | 100              | 23                      |
| IISRGAB 7   | No inhibition -(0)                   | 83.33       | 19                    | IISRGAB 53             | No inhibition +(2) | 100              | 10                      |
| IISRGAB 8   | No inhibition +(3)                   | 80          | 23                    | IISRGAB 54             | No inhibition +(2) | 100              | 10                      |
| IISRGAB 9   | No inhibition +(2)                   | 83.33       | 23                    | IISRGAB 55             | No inhibition +(2) | 100              | 10                      |
| IISRGAB 10  | No inhibition +(2)                   | 100         | 19                    | IISRGAB 56             | No inhibition +(3) | 100              | 23                      |
| IISRGAB 11  | No inhibition -                      | 71.42       | 19                    | IISRGAB 57             | No inhibition +(1) | 100              | 23                      |
| IISRGAB 12  | No inhibition +(3)                   | 75          | 30                    | IISRGAB 58             | No inhibition +(4) | 85.7             | 19                      |
| IISRGAB 13  | No inhibition +(4)                   | 75          | 13                    | IISRGAB 59             | No inhibition +(3) | 100              | 30                      |
| IISRGAB 14  | No inhibition +(1)                   | 66.66       | 10                    | IISRGAB 60             | No inhibition +(1) | 83.3             | 10                      |
| IISRGAB 15  | No inhibition +(2)                   | 71.42       | 10                    | IISRGAB 61             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 16  | No inhibition -                      | 80          | 10                    | IISRGAB 62             | No inhibition +(1) | 100              | 10                      |
| IISRGAB 17  | No inhibition -                      | 80          | 19                    | IISRGAB 63             | No inhibition +(2) | 100              | 13                      |
| IISRGAB 18  | No inhibition +(2)                   | 83.33       | 19                    | IISRGAB 64             | No inhibition +(2) | 100              | 13                      |
| IISRGAB 19  | No inhibition +(2)                   | 100         | 10                    | IISRGAB 65             | No inhibition +(1) | 100              | 27                      |
| IISRGAB 20  | No inhibition +(2)                   | 100         | 10                    | IISRGAB 66             | No inhibition +(1) | 100              | 19                      |
| IISRGAB 21  | No inhibition -                      | 75          | 10                    | IISRGAB 67             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 22  | No inhibition -                      | 100         | 19                    | IISRGAB 68             | No inhibition +(1) | 100              | 19                      |
| IISRGAB 23  | No inhibition +(2)                   | 100         | 13                    | IISRGAB 69             | No inhibition -(0) | 100              | 19                      |
| IISRGAB 24  | No inhibition +(5)                   | 0           | 0                     | IISRGAB 70             | No inhibition +(1) | 87.5             | 19                      |
| IISRGAB 25  | No inhibition +(1)                   | 83.33       | 13                    | IISRGAB 71             | No inhibition +(4) | 85.7             | 13                      |
| IISRGAB 26  | No inhibition -                      | 71.42       | 13                    | IISRGAB 72             | No inhibition +(4) | 100              | 13                      |
| IISRGAB 27  | No inhibition -                      | 80          | 19                    | IISRGAB 73             | No inhibition +(4) | 100              | 13                      |
| IISRGAB 28  | No inhibition +(2)                   | 100         | 23                    | IISRGAB 74             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 29  | No inhibition -                      | 80          | 27                    | IISRGAB 75             | No inhibition +(3) | 83.3             | 23                      |
| IISRGAB 30  | No inhibition +(3)                   | 83.33       | 27                    | IISRGAB 76             | No inhibition +(3) | 100              | 10                      |
| IISRGAB 31  | No inhibition +(1)                   | 100         | 27                    | IISRGAB 77             | No inhibition +(2) | 100              | 10                      |
| IISRGAB 32  | No inhibition +(2)                   | 100         | 13                    | IISRGAB 78             | No inhibition +(2) | 100              | 10                      |
| IISRGAB 33  | 2.0 +(3)                             | 100         | 27                    | IISRGAB 79             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 34  | No inhibition -                      | 80          | 27                    | IISRGAB 80             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 35  | No inhibition +(2)                   | 80          | 19                    | IISRGAB 81             | No inhibition +(1) | 100              | 13                      |
| IISRGAB 36 | No inhibition | + (1) | 100  | 10  | IISRGAB 82 | No inhibition | + (1) | 100  | 13  |
| IISRGAB 37 | No inhibition | + (2) | 100  | 10  | IISRGAB 83 | No inhibition | + (1) | 87.5 | 19  |
| IISRGAB 38 | No inhibition | -     | 100  | 13  | IISRGAB 84 | No inhibition | + (5) | 100  | 10  |
| IISRGAB 39 | No inhibition | + (2) | 80   | 13  | IISRGAB 85 | No inhibition | + (2) | 100  | 19  |
| IISRGAB 40 | No inhibition | + (3) | 100  | 13  | IISRGAB 86 | No inhibition | + (2) | 100  | 19  |
| IISRGAB 41 | No inhibition | -     | 100  | 13  | IISRGAB 87 | No inhibition | + (3) | 100  | 10  |
| IISRGAB 42 | No inhibition | -     | 100  | 19  | IISRGAB 88 | No inhibition | + (2) | 85.7 | 13  |
| IISRGAB 43 | No inhibition | -     | 20   | 23  | IISRGAB 89 | No inhibition | + (1) | 85.7 | 10  |
| IISRGAB 44 | No inhibition | + (1) | 80   | 13  | IISRGAB 90 | No inhibition | + (1) | 100  | 30  |
| IISRGAB 45 | No inhibition | + (1) | 80   | 13  | IISRGAB 91 | No inhibition | + (2) | 100  | 13  |
| IISRGAB 46 | No inhibition | -     | 75   | 13  | IISRGAB 92 | No inhibition | + (2) | 100  | 10  |
| IISRGAB 93 | No inhibition | -     | 100  | 19  | IISRGAB 122 | No inhibition | + (6) | 100  | 27  |
| IISRGAB 94 | No inhibition | + (2) | 85.7 | 30  | IISRGAB 123 | No inhibition | -     | 100  | 19  |
| IISRGAB 95 | No inhibition | + (2) | 100  | 13  | IISRGAB 124 | No inhibition | + (2) | 100  | 19  |
| IISRGAB 96 | No inhibition | + (3) | 87.5 | 10  | IISRGAB 125 | No inhibition | -     | 100  | 23  |
| IISRGAB 97 | No inhibition | + (2) | 100  | 10  | IISRGAB 126 | No inhibition | + (2) | 100  | 10  |
| IISRGAB 98 | No inhibition | -     | 100  | 13  | IISRGAB 127 | No inhibition | -     | 100  | 23  |
| IISRGAB 99 | No inhibition | + (5) | 100  | 13  | IISRGAB 128 | No inhibition | -     | 100  | 13  |
| IISRGAB 100| No inhibition | + (3) | 100  | 13  | IISRGAB 129 | No inhibition | + (2) | 100  | 13  |
| IISRGAB 101| No inhibition | + (2) | 100  | 30  | IISRGAB 130 | No inhibition | -     | 83.3 | 23  |
| IISRGAB 102| No inhibition | -     | 100  | 10  | IISRGAB 131 | No inhibition | -     | 100  | 10  |
| IISRGAB 103| No inhibition | -     | 100  | 30  | IISRGAB 132 | No inhibition | + (2) | 100  | 19  |
| IISRGAB 104| No inhibition | -     | 100  | 13  | IISRGAB 133 | No inhibition | -     | 100  | 13  |
| IISRGAB 105| No inhibition | -     | 100  | 19  | IISRGAB 134 | No inhibition | -     | 100  | 13  |
| IISRGAB 106| No inhibition | -     | 100  | 13  | IISRGAB 135 | No inhibition | -     | 100  | 19  |
| IISRGAB 107| No inhibition | + (3) | 0    | 0    | IISRGAB 136 | No inhibition | + (2) | 100  | 13  |
| IISRGAB 108| No inhibition | + (6) | 100  | 13  | IISRGAB 137 | No inhibition | + (4) | 100  | 19  |
| IISRGAB 109| No inhibition | + (2) | 100  | 23  | IISRGAB 138 | No inhibition | + (3) | 100  | 13  |
| IISRGAB 110| No inhibition | -     | 100  | 13  | IISRGAB 139 | No inhibition | + (5) | 100  | 10  |
| IISRGAB 111| No inhibition | + (4) | 100  | 23  | IISRGAB 140 | No inhibition | + (4) | 83.3 | 23  |
| IISRGAB 112| No inhibition | -     | 100  | 10  | IISRGAB 141 | No inhibition | + (2) | 75   | 13  |
| IISRGAB 113| No inhibition | + (6) | 100  | 19  | IISRGAB 142 | No inhibition | + (1) | 80   | 10  |
| IISRGAB 114| No inhibition | -     | 100  | 23  | IISRGAB 143 | No inhibition | + (3) | 100  | 10  |
| IISRGAB 115| No inhibition | -     | 100  | 10  | IISRGAB 144 | No inhibition | + (4) | 100  | 13  |
| IISRGAB 116| No inhibition | + (2) | 100  | 19  | IISRGAB 145 | No inhibition | + (4) | 80   | 13  |
| IISRGAB 117| No inhibition | + (2) | 100  | 10  | IISRGAB 146 | No inhibition | 5.0   | 50   | 30  |
| IISRGAB 118| No inhibition | -     | 100  | 23  | IISRGAB 147 | No inhibition | + (3) | 100  | 30  |
| IISRGAB 119| No inhibition | -     | 100  | 10  | IISRGAB 148 | No inhibition | + (3) | 100  | 23  |
| IISRGAB 120| No inhibition | + (3) | 100  | 10  | IISRGAB 149 | No inhibition | + (4) | 100  | 13  |
| IISRGAB 121| No inhibition | -     | 88.88| 10  | IISRGAB 150 | No inhibition | + (3) | 100  | 19  |

| Absolute | Control | 0  | 0  | Pathogen | control | -     | 100  | 10  |

* _ not present
+ present (nos. in brackets represents zone diameter in cm)
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