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Research Article

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 phytosanitory 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% H_2O_2 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-301centrifuge. 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 10^{-6} 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 ^{0}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% H_2O_2 (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 28ºC 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 28ºC 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 apoplstic bacterial inoculum at a concentration of 1×10^9CFU 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 28ºC 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 (Aneja, 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' AGAGTTTGATCCTGGCTCAG 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 Sequence Assembler 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 10^9 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 (~ 10^8 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 3×10^1 to 3×10^3 in the pseudostems and 2×10^2 to 2×10^3 in the leaves from plants collected from different ginger growing tracts, whereas the yield ranged from 3×10^1 to 3×10^3 in and 1×10^1 to 9.9×10^2 respectively in the rhizomes and in the leaves of plants collected from different ginger germplasm accessions. It is interested 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.

Gram staining of these 150 isolates clearly indicated that 41.99% are of gram positive rods belonging to families *Bacillaceae* (38.66%) and *Lactobacillaceae* (3.33%) and 37.31% are of gram negative rods of families *viz*. *Pseudomonadaceae* (22.66%). *Enterobacteriacea* (7.33%)

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	2.55× 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)

Table 2. Details of apoplastic bacteria isolated from ginger germplasm accessions

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× 102	No bacteria	2 (IISRGAB 9& 10)
2	CLT-G-0098	Cultivated	Khasi Hills, Meghalaya	2.1× 102	4.6× 102	2 (IISRGAB 102 & 103)
3	CLT-G-0103	Cultivated	Karbhi Anglong, Assam	2.1 × 102	2.7× 102	4 (IISRGAB 104, 105, 106 & 107)
4	CLT-G-0127	Cultivated	Mudigere, Karnataka	3 × 101	7× 101	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× 102	No bacteria	2 (IISRGAB 11 & 12)
7	CLT-G-0160	Velliyur	Kozhikode, Kerala	1.04 × 103	1.9× 102	5 (IISRGAB 13, 14, 72,73 & 74)
8	CLT-G-0165	Cultivated	Wayanad, Kerala	1.0× 102	1× 101	3 (IISRGAB 15, 75 & 76)
9	CLT-G-0171	Cultivated	Wayanad, Kerala	4.5× 102	3.8× 102	6 (IISRGAB 77, 78,79,80,81&82)
10	CLT-G-0187	Cultivated	Assam	1.2× 102	No bacteria	1 (IISRGAB 15)
11	CLT-G-0201	Cultivated	Angamali market, Eranakulam	3× 101	No bacteria	1 (IISRGAB 16)
12	CLT-G-0203	Cultivated	Kottayam, Kerala	1.7× 102	9.9× 102	3 (IISRGAB 83,84&85)
13	CLT-G-0204	Cultivated	Thodupuzha, Kerala	2.3× 102	No bacteria	3 (IISRGAB 86,87&88)
14	CLT-G-0211	Cultivated	Chengannur, Alappuzha, Kerala	1.2× 102	1.2× 102	2 (IISRGAB 89 & 90)
15	CLT-G-0219	Cultivated	Nagerkoil, Tamil Nadu	5× 101	3× 102	5 (IISRGAB 91, 92,93,94&95)
16	CLT-G-0224	Cultivated	Sooranad, Kollam, Kerala	9× 101	No bacteria	1 (IISRGAB 17)
17	CLT-G-0227	Cultivated	Angamali, Eranakulam, Kerala	1.0× 102	1.4× 102	3 (IISRGAB 96, 97 & 98)
18	CLT-G-0238	Cultivated	Pottangi, Orissa	3.22× 103	7.6× 102	2 (IISRGAB 99, 100 & 101)
19	IISR-240	wild	Silent Valley, Palakkad, Kerala	1.3× 102	1.7× 102	2 (IISRGAB 117 & 118)
20	IISR-246	wild	Sabarimala, Kerala	2× 101	1.6××102	4 (IISRGAB 126,127,128 & 129)
21	CLT-G-0249	Cultivated	Jorhat, Assam	1.1× 102	No bacteria	1 (IISRGAB 18)
22	CLT-G-0253	Cultivated	Shillong, Meghalaya	1.5× 102	1.9× 102	1 (IISRGAB 119)
23	CLT-G-0254	Cultivated	Howrah, West Bengal	2.2× 102	3.5× 102	1 (IISRGAB 120)
24	CLT-G-0255	Cultivated	Agarthala, Tripura	3.2× 102	4.6× 102	4 (IISRGAB 130, 131, 132 & 133)
25	CLT-G-0261	Cultivated	ICAR Complex, Shillong, Meghalaya	1.8× 102	1.2× 102	1 (IISRGAB 121)
26	CLT-G-0276	Cultivated	Agarthala, Tripura	3× 101	No bacteria	2 (IISRGAB 19 & 20)
27	CLT-G-0296	Cultivated	Pathanamthitta, Kerala	1.20× 103	2.9× 102	2 (IISRGAB 108 & 109)
28	CLT-G-0413	Cultivated	Visakhapatnam	No bacteria	No bacteria	No colonies
29	CLT-G-0428	Cultivated	Jamaica	2.0× 102	1.8× 102	4 (IISRGAB 110,111,112 & 113)
30	CLT-G-0441	Cultivated	Jamaica	6.4× 102	2.3× 102	1 (IISRGAB 122)

31	CLT-G-0442	Cultivated	Jamaica	3.12× 103	2× 102	3 (IISRGAB 114,115 & 116)
32	CLT-G-0443	Cultivated	Jamaica	2.3× 102	4.2× 102	2 (IISRGAB 123 & 124)
33	CLT-G-0449	Cultivated	Siddapur, Karnataka	1.8× 102	3.4× 102	2 (IISRGAB 134 & 135)
34	CLT-G-0464	Cultivated	Hajipur, Bihar	5× 101	2.4× 102	2 (IISRGAB 136 & 137)
35	CLT-G-0465	Cultivated	Patna, Bihar	2.52× 102	3.4× 102	1 (IISRGAB 125)
36	CLT-G-0469	Cultivated	Wadakkanchery, Palakkad, Kerala	2.8× 102	1.3× 102	4 (IISRGAB 138,139,140 & 141)
37	CLT-G-0485	Cultivated	Parakkode, Kollam, Kerala	3.0× 102	1.2× 102	4 (IISRGAB 142,143,144 & 145)
38	CLT-G-0519	Cultivated	Munnar, Idukki, Kerala	6.2× 102	1.8× 102	5 (IISRGAB 146,147,148,149& 150)

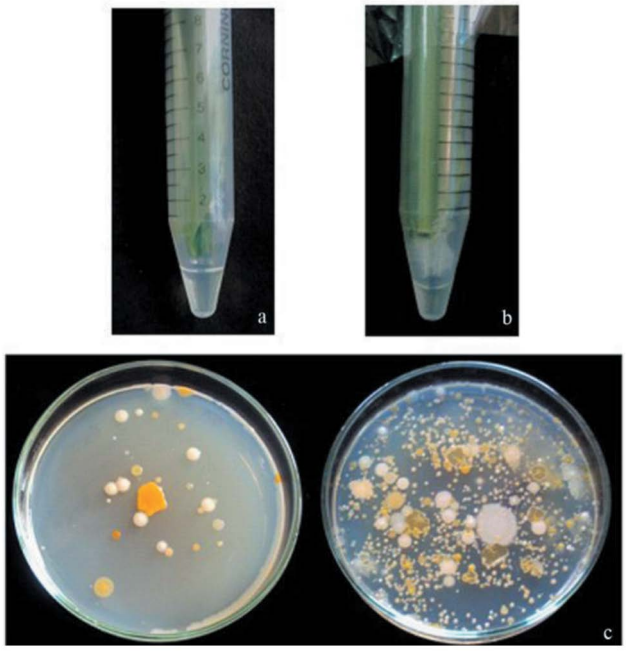

Fig. 1. (a) Apoplastic fluid extracted from ginger leaf. (b) apoplastioc fluid extracted from ginger pseudostem. (c) Bacterial colonies on tryptic soy agar.

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

Biochemical diversity

More than 88.67% of ginger apoplastic bacteria produced the enzyme catalase which is indicated by the bubble formation immediately on adding H_2O_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, where as it was found that seven isolates can utilize glucose alone, five isolates sucrose alone and two isolates sorbitol alone (Table 3).

In vitro and *in planta* Screening

Among the 150 bacteria isolated from apoplastic

Table 3. Carbohydrate utilization of apoplastic bacteria

Sl. No.	Sugar utilization pattern	Apoplastic bacterial isolates (IISR GAB)	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, 51,60,62,65,66,67,69,70,7 1,72,73,74,75,76,77,78,79 ,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	29,57,59,90,97,101,109,1 11,112,116,117,119,121, 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%)

17	Glucose, sucrose, lactose & mannitol	25,49,83,98	4 (2.67%)
18	Glucose, sucrose, lactose & sorbitol	20	1 (0.67%)
19	Glucose, lactose, mannitol & sorbitol	34,35	2 (1.33%)
20	Glucose, sucrose, mannitol & sorbitol	122,150	2 (1.33%)
21	Lactose, Mannitol, sorbitol	18	1 (0.67%)
22	Sucrose, mannitol, sorbitol	38	1 (0.67%)

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 *inplanta* for disease

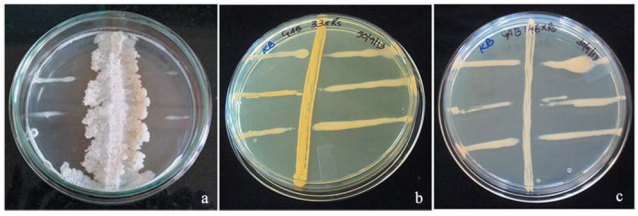

Fig. 2. Bacterial isolates showing antagonism by *in vitro* cross streak method (a) IISRGAB 5. (b) IISRGAB 33. (c) IISRGAB 146.

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.

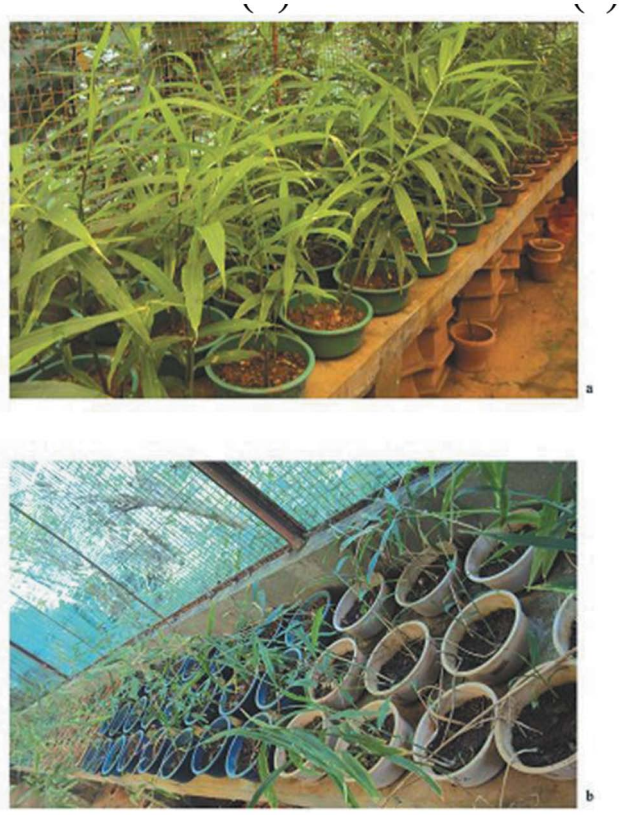

Fig. 3. *In planta* evaluation of apoplastic bacteria a Ginger plants before *Ralstonia pseudosolanacearum* inoculation b Survived plants with potential bacterial isolates.

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

Table 4. Diversity of bacterial families in apoplastic fluid of ginger

Bacteria	Family	Population (%)	Isolates
Gram positive rods	Bacilacae	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
	Eterobacteriaceae	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	IISR GAB 34, 35, 39, 93, 112
Gram positive cocci	Staphylococcaceae	10.0	IISRGAB 3, 8, 20, 28, 55, 58, 62, 63, 64, 66, 83, 88, 114, 144, 146
	Micrococcaceae	6.0	26, 33, 46, 47, 48, 54, 59, 65, 85
Gram negative cocci	Unknown	2.66	IISRGAB 11, 17, 27, 41

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	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.*

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., 1.2×10^3 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. 2×10^9 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 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 (9.58)	64.21^{ABC} (79.89)	62.41^{ABCD} (83.33)	66.73^{A} (83.33)
T2 IISRGAB 43	17.77 (14.58)	45.82^{BCDE} (51.40)	47.82^{DEF} (75.22)	77.00^{A} (100.00)
T3 IISRGAB 48	16.97 (6.57)	49.00$^{AB\ CDE}$ (54.43)	54.74^{BCDE} (64.01)	65.74^{A} (82.51)
T4 IISRGAB 107	15.12 (11.69)	21.14^{EF} (11.69)	30.39^{FG} (26.77)	26.28^{B} (19.40)
T5 IISRGAB 5+43	22.74 (18.33)	66.67^{AB} (88.89)	66.67^{ABCD} (88.89)	69.10^{A} (88.89)
T6 IISRGAB 5+48	23.12 (17.57)	52.48^{ABCD} (79.37)	53.91^{CDE} (77.78)	68.83^{A} (79.37)
T7 IISRGAB 5+107	21.13 (13.66)	59.75^{ABCD} (76.67)	68.78^{ABCD} (81.67)	71.42^{A} (86.67)
T8 IISRGAB 43+48	17.58 (9.92)	41.40$^{BC\ DEF}$ (45.91)	38.91^{EFG} (40.45)	71.69^{A} (86.11)
T9 IISRGAB 43+107	45.65 (45.40)	66.40^{AB} (72.22)	79.13^{A} (100.00)	79.13^{A} (100.00)
T10 IISRGAB 48+107	35.77 (35.15)	50.67^{ABCD} (51.85)	53.17^{DE}F (100.00)	76.96^{A} (100.00)
T11 IISRGAB 5+43+48	22.61 (17.78)	56.97^{ABCD} (83.49)	62.71^{ABCD} (100.00)	77.64^{A} (100.00)
T12 IISRGAB 5+48+107	20.03 (19.80)	68.88^{AB} (83.33)	78.24^{A} (100.00)	78.24^{A} (100.00)
T13 IISRGAB 43+48+107	41.33 (40.51)	65.93^{AB} (71.93)	78.81^{A} (100.00)	74.78^{A} (100.00)
T14 IISRGAB 5+43+107	23.35 (20.32)	36.53^{CDEF} (38.10)	77.60^{AB} (100.00)	77.60^{A} (100.00)
T15 IISRGAB 5+43+48+107	16.05 (8.33)	16.05^{F} (8.33)	62.01$^{AB\ CDE}$ (80.00)	76.91^{A} (97.10)
T16 Copper-oxy chloride (0.25%)	27.71 (21.73)	68.88^{AB} (88.52)	76.70^{ABC} (97.43)	76.30^{A} (95.24)
T17 Strepto mycin sulphate (200 ppm)	30.92 (27.95)	30.92^{DEF} (27.95)	77.71^{AB} (100.00)	77.71^{A} (100.00)
T18 Pathogen control	35.25 (34.45)	75.78^{A} (93.97)	67.37^{ABCD} (100.00)	78.27^{A} (100.00)
T19 Absolute control	13.17 (0.00)	15.20^{F} (0.00)	15.81^{G} (0.00)	18.24^{B} (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 $P \leq 0.05$
* Figures in parenthesis is original value
** NS denotes non-significant

fold increase in rhizomes when compared to control (Table 8) that showed the endophytic colonization of introduced antagonist.

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 multiplication 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.,* 1993; Lyons *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 3×10^{1} to 3×10^{3} g^{-1} in the pseudostems and zero to 2×10^{3} g^{-1} in the leaves from ginger plants collected from different ginger growing tracts. Similarly, the population

Table 8. Soil parameters and population of pathogen and antagonistic bacteria in rhizosphere soil, different plant tissues and apoplastic fluid in the pot experiment

Treatments	pH	EC (µS)	Dehydrogenase activity (µg TPF g-1 soil/ hour)	Rhizosphere bacteria other than R. pseudosolanacearum (CFU g-1)	R. pseudosolanacearum (CFU g-1)	Root (CFU g-1)		Rhizome (CFU ml-1)		Pseudostem Apoplastic fluid (CFU ml-1)		Leaf Apoplastic fluid (CFU ml-1)	
						TSA	TSA + NaCl	TSA	TSA + NaCl	TSA	TSA + NaCl	TSA	TSA + NaCl
Pathogen control**	6.75	258	0.1211	9 × 105	2 × 109	ND	ND	ND	ND	ND	ND	ND	ND
Absolute control	6.39	122.8	0.08075	1.6 × 106	0	2.56 × 106	7 × 104	5.67 × 104	4 × 104	6 × 102	3 × 102	6 × 102	4 × 102
IISR GAB 107	6.7	176.5	0.3733	3.2 × 106	1.2 × 103	2 × 106	3.7 × 105	4.15 × 106	2.5 × 105	2.5 × 103	1.5 × 103	2.7 × 103	1.6 × 103

*ND-not done as there were no plants left in pathogen control at the end of trial
** R. solanacearum inoculated control

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.

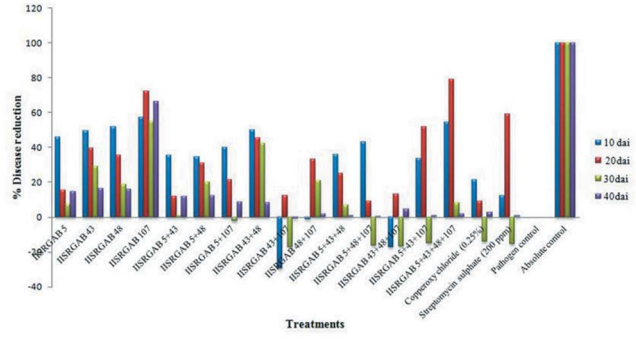

Fig 5. Graph showing bacterial wilt reduction by apoplastic bacteria.

of apoplastic bacteria ranged from zero to 3×10^3 g^{-1} in the pseudostems and zero to 9.9×10^2 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 6.0×10^3 to 4.3×10^4 g^{-1} (Gagne *et al*., 1987), cotton xylem tissue have 1×10^2 to 11×10^3 g^{-1} (Misaghi and Donndelinger, 1990), sugar beet tissue have 3.3×10^3 to 7.0×10^5 g^{-1} (Jacobs *et al*., 1985) and potato tubers have 0 to 1.6×10^4 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

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

pathogenic nematodes (Cawoy *et al*., 2011). 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. amyloliquifaciens* 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 at180 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 psuedosolanacearum*. Since bacterial wilt of ginger is a threatening problem and increased and non-judicoius and indiscrimnate 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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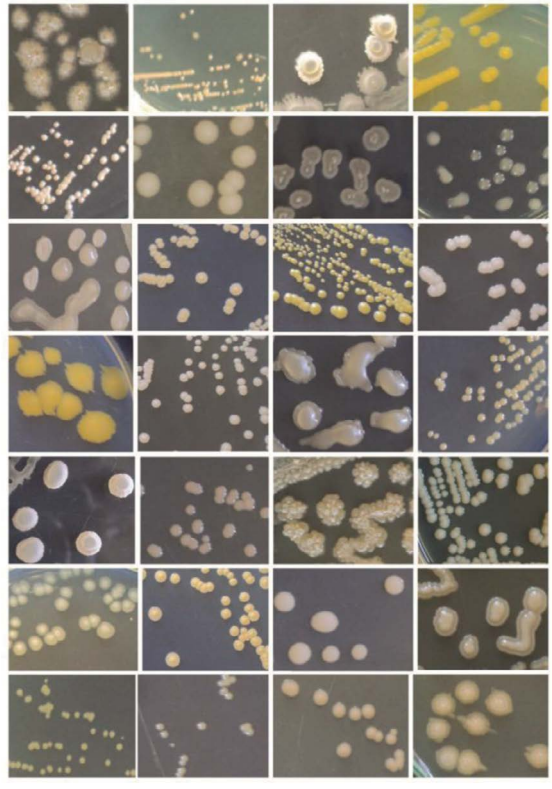
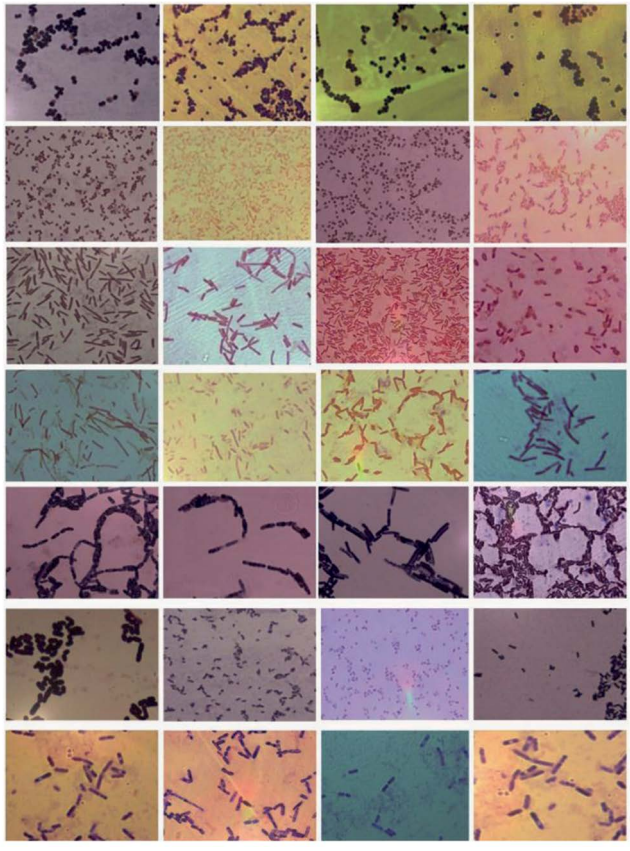
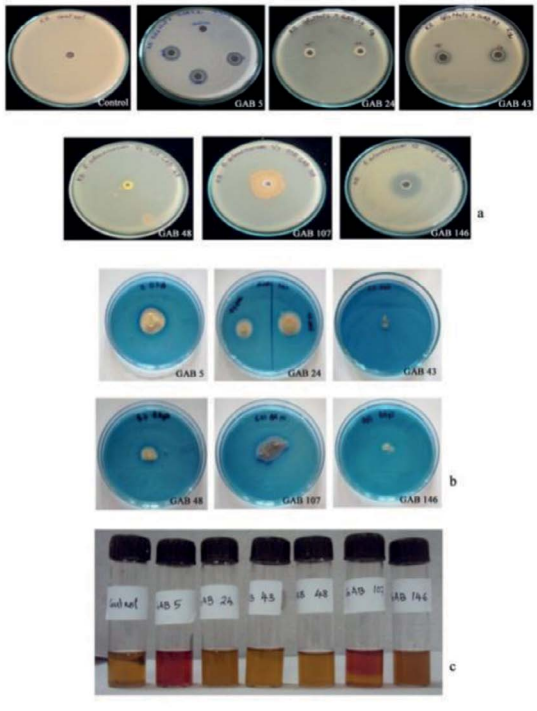


Supplementary Fig. 1. Colony morphology of ginger apoplastic bacteria on tryptic soy agar.

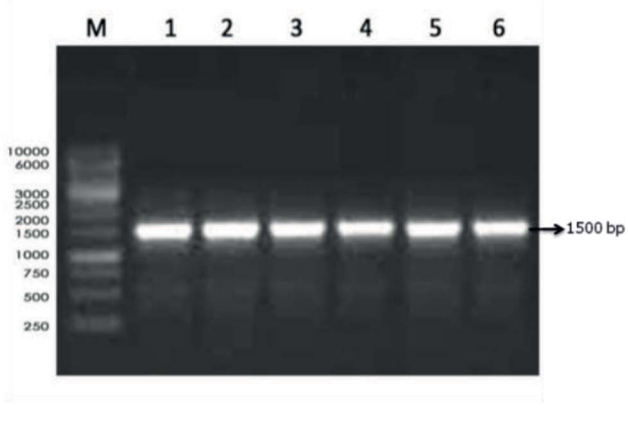


Supplementary Fig. 2. Microscopic appearance of Gram stained cells of apoplastic bacteria



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 with 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)	Sidero-phore	In planta screening	Isolate No.	In vitro screening	Siderophore	In planta screen-ing		
			Disease incidence (%)	Days taken for wilting				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	+	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	-	100	10
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
IISRG									
AB 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	-	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	-	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)	85.71	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	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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