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Isolation, Characterization and Evaluation of Native Antagonistic Rhizobacteria Against *Pythium* Rhizome Rot Disease in Ginger

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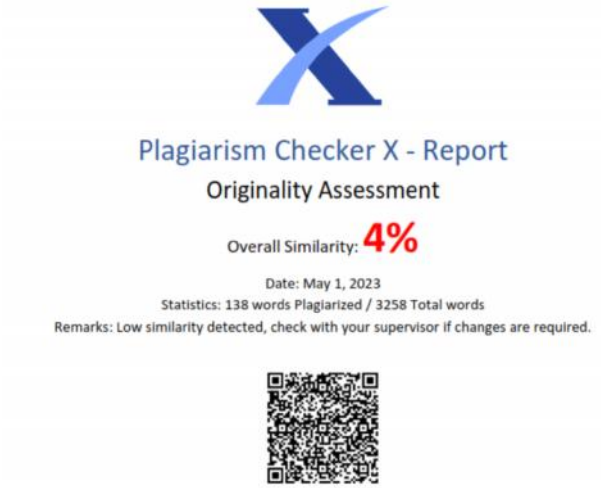


ABSTRACT

The antagonistic potential of rhizobacterial isolates collected from ginger (Zingiber officinale Rosc) cultivated soils and forest soils against the ginger rhizome rot pathogen Pythium myriotylum was assessed. 24 rhizosphere soil samples (16 from ginger growing tracts and 8 from forest area) were randomly collected from different locations of Wayanad, Kozhikode and Pathanamthitta districts, Kerala, India were screened. Soils samples from Muthanga and Ranny forest areas showed significantly higher CFU count and a total of twenty eight bacterial isolates were obtained from this soil samples. They were subjected to in vitro morphological and biochemical studies and generic level identification was performed. These bacteria belongs to 9 genera such as, Bacillus (7 nos.), Pseudomonas (6 nos.), Serratia (3 nos.), Citrobactor (3 nos.), Burkholderia (2 nos.), Klebsiella (2 nos.), Enterobacter (2 nos.), Arthrobacter (2 nos.) and Micrococcus (1 no.). Isolates were screened in vitro for inhibition against Pythium myriotylum. Results revealed that eight isolates showed >60% suppression against pathogen. They were further characterized by molecular traits in vitro and identified that higher inhibition zone exhibited bacterial isolate BA-51 as Bacillus subtilis and another isolate BA-276 as Bacillus amyloliquefaciens. Mechanism traits towards biocontrol/growth promoting ability of these eight bacterial isolates exhibited that, Bacillus subtilis (BA-51) and Bacillus amyloliquefaciens (BA-276) expressed higher production of salicylic acid and siderophore and in other tests viz., the production of HCN, IAA,

ammonia, volatile organic compound, nitrogen fixation and phosphate solubilisation showed positive trends. Pot culture experiment to assess the disease suppressing and growth promoting effect of these two promising antagonistic rhizobacterial isolates and reference culture, revealed that BA-51 with pathogen (T4) and BA-276 with pathogen (T6) registered markedly higher tiller production (14.3 nos. and 14.1 nos. respectively), lower disease incidence (32.69% and 33.19% respectively) and greater rhizome yield (19.79g pot-1 and 19.29g pot-1, respectively), while absolute control and control registered the lower tiller production (8.4 nos. and zero respectively), maximum rhizome rot incidence (44.78% and 100% respectively) and lower rhizome yield (16.49g pot-1and 0.0g pot-1, respectively). Plant growth promoting rhizobacteria (PGPR), Bacillus subtilis (BA-51) and Bacillus amyloliquefaciens (BA-276) could be good alternative for growth promotion and management of rhizome rot disease in ginger.

KEY WORDS

Zingiber officinale Rosc, Plant growth promoting rhizobacteria, Pythium myriotylum, Rhizome rot, Bacillus subtilis, Bacillus amyloliquefaciens.

INTRODUCTION

Ginger (*Zingiber officinale Rosc*. Family: *Zingiberaceae*) is a herbaceous perennial monocotyledonous tropical spice crop, the rhizomes (underground stem) can be used as both spicy vegetable and medicine. The key components of ginger includes gingerols, shogaols, essential oils, oleoresin, carbohydrates (12.3%), fibre (2.4%), proteins (2-3%), fats (0.9%) and is good source of vitamins, minerals and trace elements, these gives its distinct flavour, aroma, pungency and medicinal value (Mahender *et al*., 2015; Zhang *et al*., 2023).

The ginger plants are delicate succulent herb and succumb easily to different diseases *viz*., rhizome rot, leaf spot, bacterial wilt, fusarium wilt, stem rot, mosaic and storage rot (Le *et al*., 2016; Zhao *et al*., 2023). Among these, rhizome rot (soft rot) caused mainly by *Pythium myriotylum* Drechsler is the most destructive in various stages of its growth and most damaging diseases of ginger globally, causing crop losses of 50-100% in the majority of ginger producing nations (Varghese and Thomas, 2023). The disease losses can be limited to a certain extent by applying antibiotics and copper-based chemical fungicides. But excessive and improper uses of these inorganic inputs have caused in deleterious ecological and health problems (Randeep *et al*., 2019). Soil microbial diversity is intimately connected to the health and productivity of plant crops including ginger (Liu *et al*., 2017). In biological plant disease control packages, by using this soil microbial diversity can reduce the detrimental impacts of chemical pesticides in the ecosystem. As a result, a group of beneficial bacteria called as 'plant growth promoting rhizobacteria' (PGPR) are being studied as potential agents for the biological control of plant diseases and plant development without endangering mother nature. They are known to reduce disease through a variety of mechanisms (Karthika *et al*., 2020; Riaz *et al*., 2021) and also act as biostimulants to increase growth of different plant parts (Hamid *et al*., 2021). Studies have shown that plant growth bacteria had positive results on fruits production (Kavino *et al*., 2010), vegetables production (Kurabachew and Wydra, 2013) and significantly decreased the severity of pepper root rot disease (Dinesh *et al*., 2014). It is a well-known fact that native strains of bio control agents are more effective and have potential to control the plant disease than the introduced strains. Hence, the objective of the present study was to screen native rhizobacterial isolates for developing an eco-friendly efficient rhizome rot disease management strategy for ginger cultivation.

Materials and Methods

A total of 24 rhizosphere soil samples were randomly collected for this study, in which 16 soil samples were collected from the rhizosphere of healthy ginger plants from major ginger growing tracts of Wyanad (Meenangadi panchayath), Kozhikkodu (Balussery and Changaroth panchayaths) and Pathanamthitta

(Naranganam panchayath) districts of Kerala, India and four samples each from the rhizosphere soils of other members belonging to family *Zingiberaceae* were collected from Muthanga forest area of Wayanad district and Ranny forest area of Pathanamthitta district. The enumeration of bacterial colonies from collected rhizosphere soil sample were carried out by using serial dilution pour plate method (Johnson and Curl, 1972). The bacterial colonies developed on the nutrient agar and King's B agar plates were counted and calculated using the following formula:

$$\text{No. of colonies(cfu/g of soil)} = \frac{\text{No. of colonies developed on agar plates} \times \text{dilution factor}}{\text{Wt. of sample}}$$

Plates with higher colony forming units (CFU) values were subcultured and purified for bacterial isolates. The isolates were identified by morphological and biochemical features and was confirmed by as per the procedures suggested in Bergy's Manual of Systematic Bacteriology and 28 distinct bacterial isolates were selected for the study.

The *in vitro* antagonistic effect of these isolates against *Pythium myriotylum* (pure culture slant was purchased from ICAR - IISR, Kozhikkodu), was tested by dual culture method (Johnson and Curl, 1972). From this preliminary screening, fifteen bacterial isolates showed antagonistic activity in varied ranges and were tested again individually by dual plate method. For this the bacterial isolates were streaked evenly over petri plate media (containing equal quantity of PDA+NA/KB) at a distance of 30 mm from the border of 90 mm petri dish. By using sterile Cork borer a 5 mm disc of actively growing *P. myriotylum* pure culture was placed on the opposite side of the bacterial streak 30 mm away from the edge of the petri dish. Three replications were maintained for each isolates and plates with pathogen alone was served as the control. Plates were incubated at room temperature, until the leading edge of fungus in the control plate reached the edge of the plate. A reference culture of *Pseudomonas fluorescens* (PGPR II) from Kerala Agricultural University was also examined for its ability to have an antagonistic impact on the pathogen. Fungal mycelium radial growth was evaluated, and the percentage of inhibition was compared to the control using the formula $PI = [C - T/C] \times 100$ Where, PI = Percentage inhibition, C = Radial growth of the pathogen in control plate (mm) and T= Radial growth of the pathogen in dual culture (mm).

Molecular identification on the basis of gene sequence analysis of eight bacterial isolates with antagonistic activity (>60%) *in vitro*, were done for further confirmation. Bacterial isolates were identified by 16S rDNA cataloguing using universal primers and phylogenetic tree was constructed using Basic Local Alignment Search Tool (BLAST). To study the mechanism of biocontrol/growth promoting ability of the isolates following tests were carried out *viz*., salicylic acid production, siderophore production, HCN production, production of indole acetic acid, production of ammonium, nitrogen fixation, volatile organic compound production and phosphate solubilisation (Wahyudi *et al*., 2011).

Two potential bacterial isolates *viz.*, BA-51 and BA-276 were selected for further evaluation based on its mechanism of action and antagonism against the ginger rhizome rot pathogen *Pythium myriotylum*. A pot culture experiment in completely randomized design with 10 replications was laid out, to assess the efficiency in reducing disease and enhancing plant growth of two selected antagonistic rhizobacteria in comparison with reference culture of *Pseudomonas fluorescens* (from Kerala Agricultural University) at ICAR –Krishi Vigyan Kendra, CARD, Pathanamthitta, Kerala (Latitude 9.355922^0 and Longitude 76.675045^0). The eight experiment treatments included T1 - Absolute Control (without any treatment), T2 – Control (pathogen alone), T3 - BA-51 (ST+DS 5times), T4 - BA-51 (ST+DS 5times) +Pathogen, T5 - BA-276 (ST+DS 5times), T6 - BA-276 (ST+DS 5times) +Pathogen, T7 - *P.fl* (ST+DS 5times), T8 - *P.fl* (ST+DS 5times) +Pathogen, where BA- Bacterial isolates, *P.fl- Pseudomonas fluorescens*, ST- Seed treatment, DS-Drenching and spraying, Path.-Pathogen. Each cement pots of capacity 18kg were filled with sieved (<2 mm) and sterilized (by formaldehyde fumigation) 15kg potting mixture containing soil, sand and farm yard manure in the ratio 1:1:1.

Single ginger seed rhizome, var. *Mahima* (25g) with at least two sprouted buds soaked in respective talc based 2% rhizobacterial suspensions ($\times 10^7$cfu ml^{-1}) for 1 hours and later shade dried for one day was used for planting pot^{-1}. Booster doses of each 2% rhizobacteria (x10^{7}cfu ml^{-1}) was given as soil drench and foliar application at 20, 40, 60, 80 and 100 days after planting (DAP) @ 1 lit. pot^{-1}. On 75 DAP, the soft rot causing pathogen (*P. myriotylum*) multiplied in rice bran sand mixture was challenge inoculated at the root zone of the ginger plants @5g kg^{-1} soil of respective pots. All the cultural and agronomical operations except fungicidal application were carried out at appropriate times as per Package of Practices Recommendations: Crops 15'th (Ed.) of Kerala Agricultural University (KAU, 2016). Observations on number of tillers (at 2 MAP, 4 MAP and 6 MAP), rhizome rot incidence (at 150 DAP and 180 DAP) and rhizome yield pot^{-1} were recorded. Data was analysed with ANOVA using OPSTAT.

Results

Soil samples from forest areas showed significantly higher bacterial load (Muthanga forest area showed higher CFU than Ranny forest) than non forest soil. The soil samples with highest bacterial load of forest area were subcultured and twenty eight rhizobacterial colonies were isolated. These isolates were subjected to morphological and biochemical analyses and generic level identification was performed. The rhizosphere bacteria found to belong 9 genera *viz*., *Bacillus* (7 nos.), *Pseudomonas* (6 nos.), *Serratia* (3 nos.), *Citrobactor* (3 nos.), *Burkholderia* (2 nos.), *Klebsiella* (2 nos.), *Enterobacter* (2 nos.), *Arthrobacter* (2 nos.) and *Micrococcus* (1 no.) (Fig. 1). The antagonistic capacity of the 28 isolates along with reference culture were tested and sixteen isolates showed zone of inhibition (including reference culture) towards rhizome rot pathogen *Pythium myriotylum*. Among these, 8 isolated bacterial species exhibited > 60% of antagonistic effect. In which *Bacillus* sp. BA-51 shows maximum antagonism (79.3%) followed by BA-276 (78.87%) (Table 1).

Eight rhizosphere bacteria with strong antifungal property were selected for molecular identification by 16S rDNA sequencing method. The sequence data were subjected to BLAST analysis, in which BA-51 showed 99% identity to *Bacillus subtilis* (Accession Number MH794233.1) and BA-276 showed 99% identity to *Bacillus amyloliquefaciens* (Accession Number KY777346.1) (Table 2). The different mechanism traits towards biocontrol/growth promoting ability of bacterial isolates indicates that, among eight isolates, *Bacillus subtilis* (BA-51) and *Bacillus amyloliquefaciens* (BA-276) showed higher production of salicylic acid and siderophore and also exhibited positive trends to production of HCN, IAA, ammonia, volatile organic compound, nitrogen fixation and phosphate solubilisation (Table 3).

The pot culture study revealed that highest tiller production was exhibited by T_7 (*P. fluorescens*) and maximum at 6 MAP (Table 4). After four and six months of planting, same trend in tiller production was observed *i.e.*, maximum in T_7 (*P. fluorescens*) followed by T_3 (*B. subtilis* - BA 51), T_8 (*P. fluorescens* + Pathogen), T_5 (*B. amyloliquefaciens* - BA 276), T_4 (*B. subtilis* - BA 51 + Pathogen) and T_6 (*B. amyloliquefaciens* - BA 276 + Pathogen). At 6 MAP, control plants (T_2) were found to be completely destructed due to rhizome rot. In all growth periods all the antagonistic treatments recorded increase in tiller production over absolute control (Fig. 2).

For the evaluation of disease suppression effect, rhizome rot disease incidence was recorded. At 150 and 180 days after planting (DAP) a similar trend of infection was recorded and with least disease incidence in T_7 (*P. fluorescens*). The treatments T_3, T_5 and T_7 *ie.*, pathogen untreated plants also showed disease incidence (Table 5). All the bacterial antagonistic treatments exhibited a decreasing disease incidence tendency in respect of growth periods. But T_1 (Absolute control) and T_2 (Control) expressed increasing tendency. At 150 and 180 DAP, T_3 and T_5 were *at par* and T_4 and T_6 also were *at par*. Due to rhizome rot attack 100% disease incidence were noticed in all control pots. Disease incidence percentage over control in pathogen inoculated treatments *ie.*, T_8, T_6 and T_4 at harvest stage were -73.1%, -66.81% and -67.31% respectively (Fig. 3).

Treatments T_7 (*P. fluorescens*), T_3 (*B. subtilis* - BA 51), T_5 (*B. amyloliquefaciens* - BA 276) and T_8 (*P. fluorescens* + Pathogen) were *at par* regarding rhizome yield (231.11 g plant^{-1}, 229.81g plant^{-1}, 224.11g plant^{-1} and 219.51 g plant^{-1} respectively). In control pots all the rhizomes were demolished by rhizome rot (Table 6). The selected bacterial antagonists along with pathogen treatments *ie.*, T_4 and T_6 also exhibited good yield potential.

Discussion

Native bacterial isolates are found to be effective in preventing ginger rhizome rot, leading to enhanced growth attributes. 28 bacterial isolates were obtained from 24 rhizosphere soil samples and majority belongs to *Bacillus* genera and 8 of them exhibited > 60% *in vitro* antagonistic effect against ginger rhizome rot pathogen *Pythium myriotylum*. This inhibition effect may be due to the production of a variety of antimicrobial compounds that cause cytolysis, potassium ion leakage, membrane disruption, inhibition of mycelial growth, inhibition of spore germination, and protein biosynthesis (Quan *et al.*, 2010; Yuan *et al.*, 2012; Dinesh *et al.*, 2015).

Ginger plants treated with the three bacterial isolates *viz.*, BA-51, BA-276 and *Pseudomonas fluorescens* produced significantly more number of tillers than the controls. The influence was more profound during the later stages of growth. The better growth attained by the treated plants can be attributed to nitrogen fixation, phosphate solubilisation, release of organic nutrients and plant growth regulators like IAA by these bacterial isolates (Islam, 2018). Similar other results on growth promotion by PGPR in crops such as ground nut (Goswami *et al.*, 2014), Bt-cotton (Kumar and Gera 2014) and ginger (Dinesh *et al.*, 2015) were reported.

Incidence of varying degrees of rhizome rot pathogen infection was noted in all the treatments of the present study. Compared to control pots, treatment with these bacterial isolates, BA-51 and BA-276 exercised higher degree of disease suppression towards harvesting stage of the crop. The rhizobacterial isolates' inhibition can be related to the formation of secondary metabolites such as HCN and NH_3, that control phytopathogens or by competing for colonization sites, nutrients, antibiotics, salicylic acid and siderophores (Bhattacharyya and Jha, 2012). In previous studies, *Bacillus* spp. has been shown the ability to prevent banana and cucumber *fusarium* wilt (Yuan *et al.*, 2013; Xu *et al.*, 2014).

The fresh ginger yield was highest in pots treated with *P. fluorescens* followed by BA-51 and BA-276.This can be attributed to the ability of *Bacillus* spp. to promote growth by enhancing the bioavailability of nutrients like phosphorus and zinc, fixing atmospheric nitrogen, sequestration of iron through siderophores, ACC deaminase and production of phytohormones (Barea and Richardson, 2015, Sansinenea, 2019). Studies have shown that PGPR had positive results on vegetables production (Zaidi *et al.*, 2015) ginger production (Dinesh *et al.*, 2015) and flowers production (Lyu *et al.*, 2022).

CONCLUSION

The native bacterial isolates, *Bacillus subtilis* (BA-51) and *Bacillus amyloliquefaciens* (BA-276) showed significant inhibition effect against *Pythium myriotylum* and can be used as a prophylactic agent for ginger cultivation. Both the selected isolates have significant PGPR effect too, which is well evident with the decrease rate of disease incidence, increased tiller production and yield. Hence *Bacillus subtilis* (BA-51) and *Bacillus amyloliquefaciens* (BA-276) are recommended for wider studies and experimental field usage in order to get better yield and prevent ginger rhizome rot.

Table 1: Details of soil samples and % of inhibition of selected bacterial isolates (n=72)

District	Field	Name of Bacterial Isolates	% of Inhibition of Bacterial Isolates
Wayanad	Field:-1 Muthanga Forest Area	BA-51	79.300±0.536
	Field:-2 Meenangadi	BA-97	62.467±0.669
Kozhikode	Field:-1 Balussery	BA-121	68.200±0.059
		BA-147	67.200±1.457
	Field:-2 Changaroth	BA-154	68.400±0.361
Pathanamthitta	Field:-1 Naranganam	BA-222	64.800±0.473
		BA-251	65.267±1.212
	Field:-2 Ranny Forest Area	BA-276	78.870±0.493
BA - Bacterial Antagonist, KAU - Kerala Agricultural University		Reference Culture of KAU (*P.fl*)	83.033±0.177
		CD SE(m)	2.341 ±0.833

Table 2: Summary of identification of rhizosphiric isolates obtained in the study by 16s rDNA sequence based on blast analysis

Sl.no.	Code of the rhizosphiric isolates	Closest NCBI match with accession number	Percentage of identity	Isolates identified
1	BA-7	MH760804.1	99	*Pseudomonas aeruginosa*
2	BA-51	MH794233.1	99	*Bacillus subtilis*
3	BA-92	MH746068.1	99	*Pseudomonas aeruginosa*
4	BA-222	KU937375.1	96	*Citrobacter freundii*
5	BA-227	KC465728.1	98	*Bacillus subtilis*
6	BA-240	MF682011.1	99	*Pseudomonas azotoformans*
7	BA-251	MH767054.1	99	*Klebsiella variicola*
8	BA-276	KY777346.1	99	*Bacillus amyloliquefaciens*

Table 3: Mechanism of biocontrol of native bacterial strains

Rhizobacterial isolates	Salicylic acid production	Siderophore production	HCN production	IAA production	Ammonia production	Nitrogen fixation	Volatile organic compound production	Phosphate solubilisation
	OD AT 527 nm	OD AT 440 nm			Intensity of ammonia production			
Pseudomonas aeruginosa (BA-7)	0.24	1.362	-	+	++	-	+	-
Bacillus subtilis (BA-51)	0.762	3	+	++	+++	+	+	++
Pseudomonas aeruginosa(BA-92)	0.656	2.735	+	+	++	-	+	-
Citrobacter freundii (BA-222)	0.538	2.641	-	+	++	-	+	-
Bacillus subtilis (BA-227)	0.712	2.874	+	++	++	-	+	++
Pseudomonas azotoformans (BA-240)	0.732	2.987	+	+	+++	-	+	-
Klebsiella variicola (BA-251)	0.58	2.664	-	+	+	-	+	-
Bacillus amyloliquefaciens (BA-276)	0.746	3	+	++	+++	+	+	++

BA - Bacterial Antagonist HCN - Hydrogen cyanide IAA - Indole acetic acid nm – Nanometer
+++ High intensity (3) ++ Moderate intensity (2) + Less intensity (1) - Nil intensity (0)

Table 4: Effect of selected rhizobacterial antagonists on number of tillers/plant of ginger plants in pot culture study

Treatments	2 MAP*	4 MAP*	6 MAP*
T_1	1.300±0.153^{d}	5.500±0.224^{g}	8.400±0.452^{e}
T_2	1.200±0.133^{d}	1.500±0.153^{h}	0.000±0.000^{f}
T_3	2.000±0.167^{b}	9.500±0.342^{ab}	14.600±0.371^{ab}
T_4	2.00±0.167^{b}	8.100±0.348^{cde}	14.300±0.423^{bc}
T_5	1.800±0.249^{c}	8.500±0.342^{bcd}	14.300±0.423^{bc}
T_6	1.800±0.249^{c}	7.600±0.427^{def}	14.100±0.547^{cd}
T_7	2.300±0.260^{a}	9.800±0.490^{a}	14.900±0.482^{a}
T_8	2.200±0.260^{a}	8.700±0.335^{bc}	14.600±0.452^{ab}
C.D.	0.638	1.017	1.292
SE(m)±	0.227	0.362	0.460

MAP: Month after planting. *Mean of ten independent observations. Figures in a column followed by the same alphabet does not differ significantly (Pd-0.05).

Table 5: Effect of selected rhizobacterial antagonists on disease incidence (%) on ginger plants in pot culture study

Treatments	150 DAP*	180 DAP*
T_1	44.682±0.964^{b}	44.781±0.966^{b}
T_2	100.000±0.000^{a}	100.000±0.000^{a}
T_3	25.780 ± 0.580^{ef}	24.890±0.537^{e}
T_4	33.187±0.716^{c}	32.687±0.705^{c}
T_5	26.881±0.556^{de}	25.191±0.544^{e}
T_6	35.086±0.757^{c}	33.187±0.716^{c}
T_7	25.589±0.552^{f}	22.690±0.489^{f}
T_8	27.689±0.597^{d}	26.890±0.580^{d}
C.D.	2.081	0.701
SE(m)±	0.741	0.249

DAP: Days after planting. *Mean of ten independent observations. Figures in a column followed by the same alphabet does not differ significantly (P-0.05).

Table 6: Effect of selected rhizobacterial antagonists on yield of ginger plants in pot culture study

Treatments	Yield (g/plant)*
T_1	170.528±3.895^{e}
T_2	0.000±0.000^{f}
T_3	229.810±4.958^{a}
T_4	212.514±4.585^{bc}
T_5	224.111±4.836^{ab}
T_6	205.320±4.430^{cd}
T_7	231.111±4.986^{a}
T_8	219.513±4.736^{ab}
C.D.	12.070
SE(m)±	4.300

*Mean of ten independent observations. Figures in a column followed by the same alphabet does not differ significantly (P-0.05).

FIG. 1 MAJOR GENERA OF RHIZOBACTERIA ISOLATED FROM RHIZOSPHERE SOIL

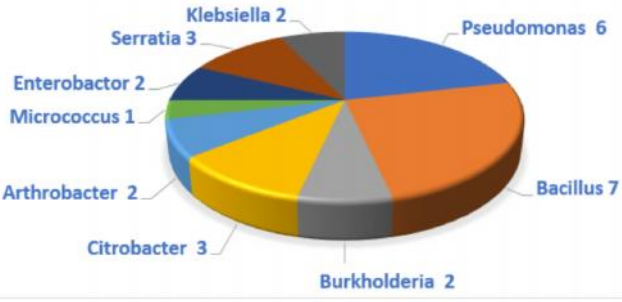
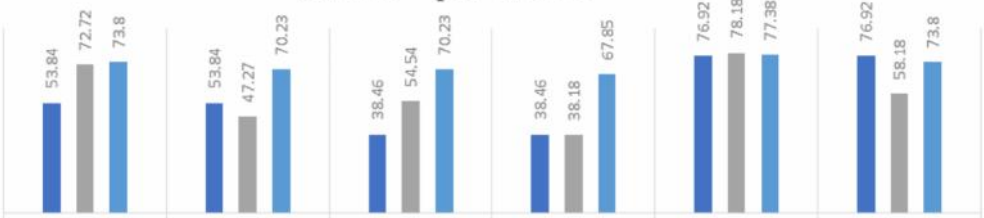
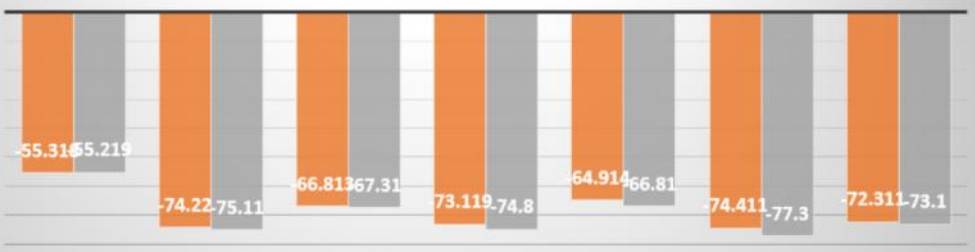


Fig. 2 Number of tillers/plant percent over absolute control - pot culture



	T3 B. subtilis	T4 B. subtilis+Pathogen	T5 B. amyloliquefaciens	T6 B. amyloliquefaciens +Pathogen	T7 P. fluorescens	T8 P. fluorescens +Pathogen
2 MAP	53.84	53.84	38.46	38.46	76.92	76.92
4 MAP	72.72	47.27	54.54	38.18	78.18	58.18
6 MAP	73.8	70.23	70.23	67.85	77.38	73.8

Fig. 3 Disease incidence percent over control - Pot culture



	T1 Absolute control	T3 B. subtilis	T4 B. subtilis+Pathogen	T5 B. amyloliquefaciens	T6 B. amyloliquefaciens +Pathogen	T7 P. fluorescens	T8 P. fluorescens +Pathogen
150 DAP	-55.318	-74.22	-66.813	-73.119	-64.914	-74.411	-72.311
180 DAP	-55.219	-75.11	-67.31	-74.8	-66.81	-77.3	-73.1

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