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Field application of *Bacillus subtilis* isolates for controlling late blight disease of potato caused by *Phytophthora infestans*

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ABSTRACT

Field studies on the biocontrol activity of four *B. subtilis* isolates viz., *B. subtilis* MTCC-2422 (T-3), *B. subtilis* KU936344 (T-4), *B. subtilis* KU936345(T-5) and *B. subtilis* KU936341 (T-6), against late blight disease of potato caused by fungus like-organism *Phytophthora infestans* were investigated. These isolates produce mycotoxins against the causal organism. The fungicide (Mancozeb) M 45 (CURZATE®) was used as positive control. Potato (*Solanum tuberosum*) cultivar, Kufri Jyoti was used. The treatments were utilized as soil drenching as well as foliar spray. Results reveal that, bacterial treatments significantly reduced disease incidence of late blight compared with the control. Bacterial treatments increased the plant vegetative parameters like plant height, sprouting, number of leaves, fresh weight and dry weight of plants. In addition, treatments also showed the clear difference between commercial and non-commercial tuber yield/hectare. In this experiment, two observations displayed the reduction of the disease severity. There was 32.73% increase in the control plot and 14.3% in chemical fungicide, while in bacterial treatments there was 12.75%, 14.09%, 4.7% and 4.13% increase in the T3, T4, T5 and T6, respectively. Soil drenching of bacterial culture gave the high yield in commercial potato tubers, even when compared with the chemical fungicide. The total yield of control block was 166.04 quintals/hectare but non-commercial yield was 101.07 quintals/hectare. In chemical fungicide, total yield was 188.8 quintals/hectare and non-commercial tubers was 69.66 quintals. The bacterial treatments T4, T5, T6, showed less non-commercial potato yield 48.29, 22.60 and 24.67 quintals/hectare in the total yield of 177.02, 212.89 and 190.737 quintals/hectare in respective treatments. So this study uncovers that *B. subtilis* treatments are effective under field condition for the control of potato late blight.

1. Introduction

Late blight of potato, caused by the fungal like-organism, *Phytophthora infestans* Mont. (de Bary), first occurred in Europe in the 1840s when it led to the devastating Great Irish Potato Famine (Bourke, 1991). It can infect and destroy the leaves, stems, fruits, and tubers of potato and tomato plants. Late blight has been a challenge for potato growers worldwide. In recent years, change in the characters of late blight was observed, including changes in aggressiveness to potato and tomato plants. Some literatures have reported more aggressive strains which are resistance to fungicide (Goodwin et al., 1994). Fungicides cannot be employed solely for effective control of late blight, but could be incorporated in integrated management strategy. If the disease is well established on the crops, the use of old or new fungicides is not worthy as the fungus will become resistant. Plant pathogenic fungi and oomycetes are major threats for crops and plant production. Therefore, the control of fungal diseases by bacilli represents another interesting opportunity for agricultural biotechnology (Fry and Goodwin, 1997).

The bacterium *B. subtilis* is a potential biocontrol agent, for which many studies have reported its broad antagonistic activity against various plant diseases across the globe (Cavaglieri et al., 2005; Boland, 1997; Tu, 1997). *B. subtilis* is common in nature, nontoxic and harmless

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to humans and other animals and non-pathogenic to plants (Acea et al., 1988). The bacterium produces antimicrobial compounds *in vitro*, including the antibiotics zwittermicin-A and kanosamine (Leifert et al., 1995), lipopeptides (Ahimou et al., 2000; Bais et al., 2004; Raaijmakers et al., 2002) and antifungal protein bacisubin (Liu et al., 2007). As a group, Bacillus species also offer several advantages over other bacteria for protection against root pathogens. These advantages include the broad-spectrum activity of their antibiotics and ability to form endospores, which facilitates long-term storage and commercialization.

It is known that Bacillus species protect plants against phytopathogenic bacteria and micromycetes through a number of mechanisms, in particular, through the synthesis of different cyclic lipopeptides with inhibitory activity against phytopathogens (Hossain et al., 2015). The genes responsible for biosynthesis of antimicrobial peptides were identified in different species of Bacillus, and mostly in *B. subtilis* and *B. amyloliquefaciens* strains (Hossain et al., 2015). Majority of strains carry srfA, bacA, bmyB, fenD genes (Mora et al., 2015).

For protecting the plants from pathogens, biological control is an environmentally-friendly alternative to chemical pesticides and it is an attractive method, because the wide usage of chemicals has a negative impact on the environment and human health. Many biocontrol agents were isolated by screening of the large number of soil or plant-associated microorganisms for antagonism against phytopathogens *in vitro* or in Planta (Berg et al., 2001; Cazorla et al., 2007). Rhizosphere of different plants having high diversity of bacterial species with antagonistic activity, among those, bacilli and pseudomonads are the most common isolates (Berg et al., 2001; Cazorla et al., 2007; Anelise Beneduzi et al., 2012; Kumar et al., 2012). It is known that various species of the Bacillus genus are able to stimulate the plant growth (Choudhary and Johri, 2009). Bacteria can promote plant growth in various ways, such as improvement of plant nutrition; induction of systemic resistance; toxicity to pests and antagonism pathogens (Choudhary and Johri, 2009; Pinchuk et al., 2002; Wang et al., 2009). The antagonistic activity of Bacillus is associated with the synthesis of various antimicrobial peptides (Kim et al., 2010; Falardeau et al., 2013), secreted enzymes (Baysal et al., 2013), proteins (Tan et al., 2013) and volatile organic compounds (VOCs) (Choudhary and Johri, 2009; Baysal et al., 2013). Many *Bacillus* isolates were shown to have antifungal activity against phytopathogenic fungi, making them potential biocontrol candidates (Li et al., 2014; Baffoni et al., 2015; Shternshis et al., 2015). The strains of the species *B. subtilis* can vary considerably both phenotypically and genetically, ultimately affecting their antagonistic potential. Comparative proteome analysis of two *B. subtilis* strains possessing antagonistic potential revealed notable differences in the composition of intracellular as well as extracellular proteins (Tan et al., 2013; Zhang et al., 2009), some of which can be associated with antimicrobial properties. Owing to their fast growth, ability to effectively grow in low cost media and to sporulate under undesirable conditions, Bacillus isolates are the attractive candidates for application as the biocontrol agents.

The objective of this research was to evaluate the potential effect of using *B. subtilis*, as biological control agents to control naturally infested late blight of potato under field conditions with respect to chemical fungicide and proper control.

2. Materials and methods

2.1. Source of biocontrol agent cultures

In total, seventy soil samples were screened from different locations for the isolation of *B. subtilis* strains. Both morphological and molecular identification were explained. Sample showing positive result towards 16s rRNA sequencing, with NCBI accessions KU936341, KU936344 and KU936345 were used for this study with proper fungicide control and *B. subtilis* reference strain MTCC-2422 procured from The Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Treatments were listed in Table 1.

2.2. Phylogenetic analysis

For the construction of phylogenetic tree Phylogey. fr (http://www.phylogeny.fr/simple_phylogeny.cgi) program was used to perform a multiple sequence alignment with default parameters (Dereeper et al., 2008).

2.3. Preparation of biocontrol agent inoculums and chemical fungicide

In vitro studies were taken for all bacterial strains (data not shown) *B. subtilis* KU936341, KU936344, KU936345 and MTCC-2422 were inoculated in Luria broth (Casein enzymic hydrolysate 10 gm, Yeast extract 5 gm, Sodium chloride 10 gm add H_2O to 1000 ml, Final pH 7.5 ± 0.2). The bacterial cultures were added to 500 mL flasks containing 200 mL Luria broth and grown aerobically in a rotating shaker at 180 rpm and 30 °C for 16–18 h. The bacterial cultures were suspended in water and adjusted to final concentration 2×10^6 CFU/ml for use in the following experiment. Fungicide M 45 (Mancozeb) - 2 g/l and CURZATE® - 3 g/l concentrations were used for field experiment.

2.4. Application of biocontrol agents

Before the sowing of potato seeds, all the blocks (T3, T4, T5 & T6) were drenched with different bacterial cultures at the concentration 2×10^6 CFU/ml per block, with the exception of chemical fungicide and control blocks. For the fungicide block, pieces of potato seed tubers were treated with 0.2–0.3% of M 45 (Mancozeb) fungicide before ten days of planting. The grown culture of different bacterial strains were sprayed on the plants (foliar spray) in field with a hand-pump sprayer at a rate 2 L/block and for fungicide block 1.5 L/block.

2.5. Plant materials

Potato tubers (*Solanum tuberosum* L.) cultivar, Kufri Jyoti was used in this experiment. The seed tubers were obtained from local dealer.

2.6. Field experiment

This study was conducted in College of Agriculture, Hassan, India (12° 58′ 16N and 76° 15′ 46E) during 2016 season. It was aimed to determine the effect of *B. subtilis* treatments on the management of late blight of potato with chemical fungicide and proper control, in a randomized complete block design. This experiment involved four *B. subtilis* treatments, with chemical fungicide and control. Each treatment consisted of four replications with a total 324 m^2 plot area. Each replicate contains four lines with total of 72 potato pieces.

2.7. Vegetative growth parameters

At 65th day of planting potato plants were randomly chosen from each replicate, within each treatment as well as control. The effect of the treatments on plants were recorded, growth parameters including plant height (inches), number of sprouting, number of branches, number of leaves and weight of the plant (fresh and dried) of potato plants were

Table 1
All the four *Bacillus subtilis* treatments and treatment names, used in the experiments with chemical fungicide and proper control.

Sl. No.	Treatments	Treatment name
1	Control	CONT
2	Fungicide control M 45 (Mancozeb) & CURZATE®	FUNGICIDE
3	*Bacillus subtilis* MTCC-2422	Treatment 3 (T-3)
4	*Bacillus subtilis* (KU936344)	Treatment 4 (T-4)
5	*Bacillus subtilis* (KU936345)	Treatment 5 (T-5)
6	*Bacillus subtilis* (KU936341)	Treatment 6 (T-6)

measured.

2.8. Potato tuber yield

After harvesting of potato all the individual treatment blocks were combined, commercial and non-commercial size potatoes were separated by grading. All treatments were determined in field experiment.

2.9. Disease incidence

Incidence is a measure of disease that allows us to determine the occurrence of disease during a given period of time. It is observed day by day for individual plants from each block. Presence of the infection of late blight was recorded. Final disease occurrence was calculated when control block reached 100% incidence. Disease incidence was calculated using the following formula.

Disease incidence (%) = Number of diseased plants / Total number of plants × 100

2.10. Late blight of potato disease severity

Disease severity was calculated using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison posttest. Differences between groups were considered to be significant at a P value of 0.05. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

2.11. Peroxidase assay

According to Hammerschmidt and Kuc (1982) peroxidase activity was recorded at 470 nm as the oxidation of guaiacol using hydrogen peroxide as substrate. Activity was expressed as the increase in absorbance at 470 nm/min/g of fresh tissue. The assay was measured on 65th day of planting.

2.12. Statistical analysis

One-way analysis of variance (ANOVA) was carried out to compare the differences of plant morphological parameters and late blight severity.

3. Results

3.1. Phylogenetic analysis

To determine the evolutionary relationship, phylogeny analysis was carried out using phylogeny. fr program with nucleotide sequences. The three *B. subtilis* strains KU936341, KU936344 and KU936345 branched out differently in three leaves, KU936341 is highly similar with GQ360038, EU931563 and AB701293. On the other hand, KU936344 was found to be closely related with KU936333 and MF614911. Finally, NCBI accessions EU257453, FJ527656 and EU257442 were found to be comparable with KU936945 (Fig. 1). Among the three NCBI accessions, KU936341 and KU936345 are unique ancestor, while KU936344, an outward group, branched out separately.

3.2. Disease incidence rate in two intervals

Two observations were taken for the disease incidence and each of the treatments were compared with control (Table 2). Final observation was made when control plot reached 100% severity. On the 60th day, the disease occurrence in control block was 67.27% and fungicide control exhibited 49.08%, but other treatments showed between 53 and

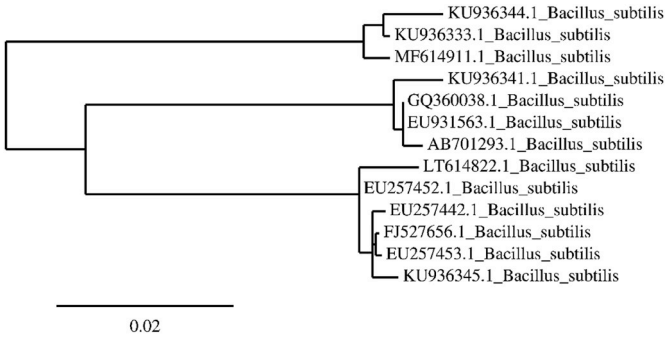

Fig. 1. Phylogenetic analysis of bacterial strains sequences used in the field study. Tree was constructed using Phylogey. fr (http://www.phylogeny.fr/simple_phylogeny.cgi) program with default parameters. All the sequences were used belongs to *Bacillus subtilis.* The sequences were obtained from the databases of the National Centre for Biotechnology Information.

Table 2
Disease incidence percentage of individual treatments on 60th day and 80th day. Readings explains the increase in the disease incidence percentage.

Treatments	Disease incidence percentage in different intervals	
	60th day	80th day
Control	67.27	100
Fungicide	49.08	63.38
T3	57.83	70.58
T4	59.04	73.13
T5	55.88	60.6
T6	53.84	57.97

59%. Interval application of the *B. subtilis* on 80th day even on the fungicide block also increased to 14.3%, but in treatments 5 and 6 there were slight increases of 4.72% and 4.13%, respectively (Fig. 2). This observation is evident that treatment of *B. subtilis* also gave the best protection against potato plants infected with late blight.

3.3. Late blight of potato disease severity

ANOVA results showed the late blight severity between the treatments. There was only a significance between control and fungicide treatment. Notably, other treatments did not show significant difference (Fig. 3). Control exhibited 81.25% of severity, while the lowest severity 65%, was found in fungicide. Apart from these, all the *B. subtilis* severity

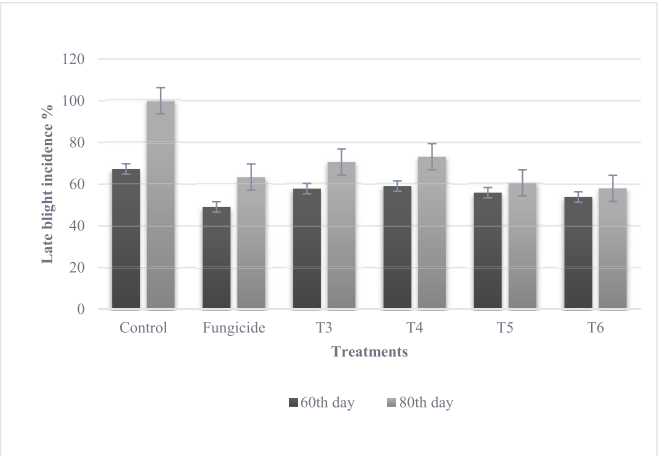

Fig. 2. Difference of disease incidence percentage between the individual treatments, 60th and 80th days. Final observation was taken on 80th day when the control block reached the 100% of disease incidence.

percentages are found between 73 and 79% (Table 4). These observations highlight there were slight differences between the *B. subtilis* treatments.

3.4. Vegetative growth parameters

The efficacy of the tested bacterial treatments demonstrated improvement in some vegetative growth and yield parameters, and protection of the potato plants against late blight. Table 3 explains the difference in the biomass of all the treatments before and after the attack of late blight. These observations depict the difference of biomass of all the treatments in field conditions.

One-way analysis of variance (ANOVA) was carried out to compare the difference between plant height before and after late blight (Table 3; Fig. 4) other morphological characters *viz,* sprouting, number of branches and number of leaves present in the potato plants in different treatments were recorded (Table 5). All the parameters were compared between control, Fungicide and *B. subtilis* strains. ANOVA results for plant height before the attack of late blight indicated there was no significant difference between the treatments, but after disease attack, significant difference was observed under control with fungicide, Treatment 4 and Treatment 5 ($P < 0.05$) (Fig. 4). There was a great difference of plant height in control, reducing to 5.17 inches. In Treatment 4, there was no difference between plant heights. Other treatments displayed slight height reduction after the bacterial culture application (Table 6), fungicide (1.59 inches), Treatment 3 (2.75 inches), Treatment 5 (2.5 inches) and Treatment 6 (4 inches).

3.5. Potato tuber yield

This result indicates that there was a requirement of biocontrol agents for the disease management in field crops. These observations found that without any treatment (control) the non-commercial potato yield was very high out of 166.04 quintals per hectare there was only 65.025 quintals of potato are commercial uses, the rest of 101.01 quintals of potato are non-commercial. Bacterial treatment elevated the total commercial potato tuber weight. Even in fungicide treatment, 69.66 quintals of non-commercial yield was recorded. Bacterial strains T4, T5 and T6 recorded less non-commercial potato yield 48.29, 22.60 and 24.67 quintals, respectively. Late blight of potato symptoms compared with the control. *B. subtilis* treatments gave a high level of protection to tubers against the infection. Fig. 5 explains the difference between commercial and non-commercial yield of potato in the field condition. These results suggested that the soil application and foliar spray of bacterial cultures were suitable to protect against late blight of potato in the field condition, which increases the commercial potato tuber yield.

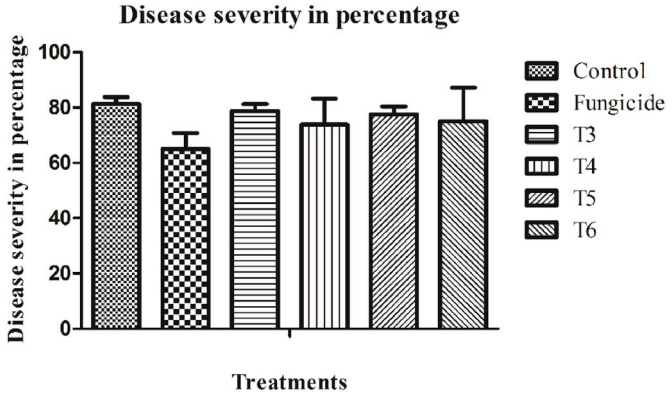

Fig. 3. Effect of late blight disease severity on different treatments. Values are mean ± SD (mean of data with replications). T3, T4, T5 and T6 indicates the different *B. subtilis* treatments.

Table 3
Vegetative growth parameters (fresh weight and dry weight) of each treatment before the disease incidence and after the disease incidence (Data are mean of three replications).

	Weight of the plants before disease incidence.		Weight of the plants after disease incidence.	
	Fresh weight in grams	Dry weight in grams	Fresh weight in grams	Dry weight in grams
Control	808	132	149.5	41.5
Fungicide	1083	185	302	96
T3	899.5	173	316	92.5
T4	997	165.5	388	127
T5	1152	190.5	317	94
T6	901.5	141.5	331.5	107

Table 4
ANOVA results showed the late blight severity between the treatments. Differences between groups were considered to be significant at a P value of 0.05.

Treatments	Severity %
Control	81.25
Fungicide	65
T3	78.75
T4	73.75
T5	77.5
T6	75

3.6. Peroxidase assay

This assay was carried out to check the activity of the *B. subtilis* on late blight of potato caused by fungus, *Phytophthora infestans*. Peroxidase activity showed that there was increase in the absorbance of the *Phytophthora infestans* infected samples as compared with the uninfected samples. This absorbance was carried out with the control and absolute control treatment (Fig. 6).

4. Discussion

Plant fungal diseases reduce yield and productivity of several economically important crops all over the world. Resistant plant cultivars, cultural practices and chemical applications are routinely used as intervention to provide disease control. In this study, the biocontrol agent of *B. subtilis* treatments reduced late blight of potato plants (Kufri Jothi, cultivar) under field applications. It demonstrated that bacterial cultures are promising antagonistic property towards controlling the effect of *Phytophthora infestans*, causative agent of potato late blight. All the factors of plant vegetative growth like height, sprouting, branches and number of leaves observations were taken before and after the disease incidence, under field conditions. They gave impressive differences as compared to chemical fungicide and control treatments. Improved growth and health of the potato plants were found effective by the plant growth promoting substances, which were produced by biocontrol agents (Marlin et al., 2001).

Our findings highlight the effectiveness of *B. subtilis* is one of the suitable biocontrol against late blight (Fig. 2), the results are consistent with the different worker. According to Essghaier et al. (2012) *B. subtilis* J9 shown the inhibition on the growth of various phytopathogenic fungi including: *Sclerotinia, Phytophthora, Penicillium* and *Alternaria,* with high growth mycelial inhibition (superior to 95.3%), *Fusarium graminearum* (63.1%) and slightly growth inhibition of about 35% had been reported for *Fusarium avenaceum* and *Fusarium oxysporum*. This halotolerant strain J9 of *B. subtilis* which failed to inhibit the pathogen, *Botrytis cinerea,* in *in vitro* condition. The biocontrol activity of halotolerant strain J9 strain suppressed the growth of pathogen by releasing metabolites

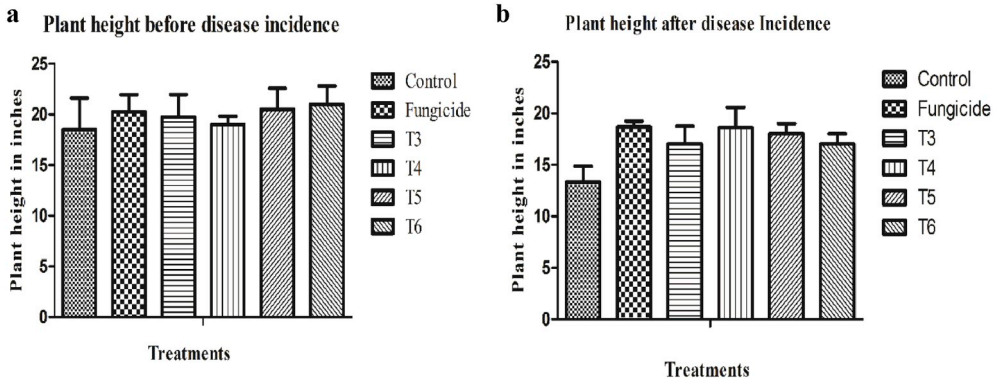

Fig. 4. **a** Plant height before disease incidence. **b** Plant height after disease incidence. Values are mean ± SD (mean of data with replications). T3, T4, T5 and T6 indicates the different *B. subtilis* treatments.

Table 5
Vegetative parameters of potato plants before late blight incidence under field condition (Data are mean of three replications).

	Plant height (inches)	Sprouting/ plant	Branches/ plant	Leaves/ plant
Control	18.5	2.5	4.25	41.75
Fungicide	20.5	1.75	8.25	67.5
T3	19.75	3	6.25	50.75
T4	19	3	7.5	69.5
T5	20.5	3.25	7.25	71
T6	21	2.75	5.75	58.5

Table 6
Plant vegetative parameters (Height and Sprouting) after the disease incidence under field condition (Data are mean of three replications).

	Height (inches)	Sprouting/plant
Control	13.33	4
Fungicide	18.66	6
T3	17	6
T4	19	9
T5	18	6
T6	17	8

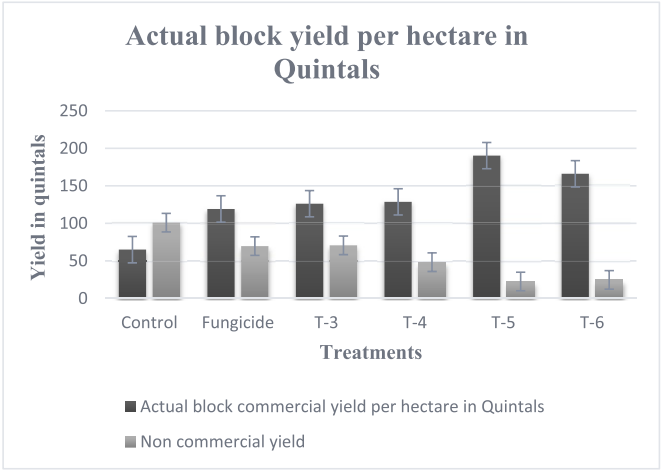

Fig. 5. The total yield of potato in quintals/hectare in different treatments, including actual commercial yield and non-commercial yield.

into proper culture medium in *in vitro*.

Hundreds of combinations have been tested for antagonism against plant pathogens using *B. subtilis* and *B. cereus* strains and proved to be capable of suppressing broad-spectrum diseases and promoting plant growth. The effects of the tested mixture of *B. subtilis* strain SM21 and *Bacillus cereus* strain AR156 on a range of biological parameters of plant growth including biomass, yield, chlorophyll content and fruit nutrients of pepper, as well as, its efficacy in controlling bacterial wilt caused by *Ralstonia solanacearum* under field conditions in 2009 and 2010 by a variety of inoculation methods, at 1.0×10^6 CFU/mL concentration were found to be encouraging (Zhou et al., 2014).

B. subtilis treatments gave a high level of protection to tubers against the infection. Fig. 5 explains the difference between commercial and non-commercial yield of potato in the field condition. Vegetative parameters and weight of tuber were recorded, this observation presented the difference among the treatments. Comparison of treatments with proper control with regard to the grading of potato tubers data showed a remarkable difference. These results assert that the soil application and foliar spray of bacterial treatments were effective to protect the potato tubers against late blight of potato disease. The application of bacterial treatments hold abilities as biocontrol agents with antifungal effect against late blight. The success of Bacillus species as biocontrol agent could be ascribed to a wide array of peptide antibiotics produced such as iturin A, Mycobacillin, subtilin and bacilysin as well as 25 different basic chemical structures with proven antifungal secondary metabolites (Yoshida et al., 2000; Mutaz and Hasnain, 2006). Within the *B. subtilis,* there is a large variation in the amount and variety of antifungal substances (Pinchuk et al., 2002).

According to Welinder (1992) peroxidase assay is useful marker for plant development, physiology, infection and stress. Interestingly, this result indicated that there was a rise of peroxidase activity in *Phytophthora infestans* infected samples compared with the uninfected samples. Therefore, the induction of peroxidase by *B. subtilis* on late blight of potato can be considered as one of the marker of plant pathogenesis.

To improve the stability of a biocontrol agent, it is important to discern their mode of action and subsequently improve the mechanism. In addition, enhancing the conditions where biocontrol agents are predicted to be more successful can easily be established to promote the growth of biocontrol agents. The mechanisms of biocontrol have been studied extensively and reviewed in several investigations (Adams, 1990; Cook and Baker, 1983; Fravel, 1988; Ghisalberti and Sivasithamparam, 1991; Kessmann et al., 1994; Lam and Gaffney, 1993). Such mechanisms of action that have been implicated in enhancing colonization of plant pathogens include antibiosis, cell wall degrading enzymes, mycoparasitism and induced resistance (Lo, 1997; Lo et al., 1998).

The findings in this study involves the value of discovering the suitable biocontrol agents and its inoculation methods, which can give the appreciable enhancement of plant growth and most importantly supress plant diseases. Although biological control approach is suitable, the major impediment is slow in action, as it takes considerably longer to

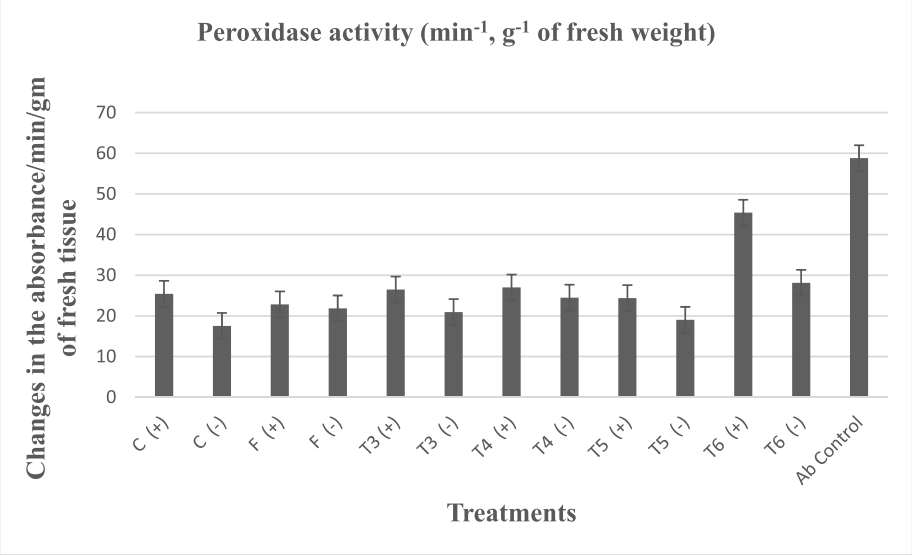

Fig. 6. Peroxidase activity of potato plant induced with *Bacillus subtilis* isolates, Standard deviation at the rate of 0.5. (Legends: C- Control, F- Fungicide control, T3- Treatment 3, T4- Treatment 4, T5- Treatment 5 and T6- Treatment 6; [+] indicates sample infected with *Phytophthora infestans* and [−] sample without infection of *Phytophthora infestans*).

show the activity against plant diseases. Further, the activity of the biological agents are dependable on variation in climatic conditions. For future consideration, new approaches like CRISPR/Cas9 mediated genome editing technology for the control of plant disease management will be convenient.

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Declaration of competing interest

None.

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