SCREENING AND APPLICATION OF RHIZOBACTERIAL ISOLATES AS BIOLOGICAL CONTROL AGENT AGAINST CHARCOAL ROT OF CHILLIES

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ABSTRACT: *This research work was conducted in order to evaluate the potential of rhizobacterial isolates to minimize the application of chemical pesticides against charcoal rot of chilies. Charcoal rot is one of the most devastating fungal diseases in chilli and causes significant losses to chilli crop in Pakistan. A detailed survey was conducted to different vegetable farms, Agricultural research farms and farmer fields in order to collect the diseased specimen and soil samples from infected field of chilli by charcoal rot. Pathogenic fungus (Macrophomina phaseolina) causing charcoal rot was isolated and identified. Forty eight ectophytic and endophytic rhizobacterial species were also isolated and identified by means of their biochemical tests. Rhizobacterial isolates were screened for their antagonistic potential. Antifungal potential of different rhizobacterial species was evaluated in vitro as well as in field. Fresh biomass, dry biomass, root length, shoot length, disease severity, disease incidence and percentage mortality were the parameters that were used to evaluate the potential of rhizobacterial species. Finally it was concluded that among the rhizobacterial isolates only ChE1 (Bacillus subtilis) and UCaE4 (Bacilis lichenifromis) have increased fresh and dry biomass, root and shoot length increased; disease incidence and percentage mortality was reduced. It was concluded that the species of Bacillus were the best biological control agents against Macrophomina phaseolina.*

Key words: Charcoal rot, chilli, rhizobacteria, disease incidence, disease severity, biological control.

lNTRODUCTION

Chilli *(Capsicum annuum* L.*)* belonging to family *Solanaceae* and cultivated about in 130 countries of the world. The largest producer is India in the world followed by China and Pakistan. World production was 2.098 million tonnes in 2007-2008. Pakistan's share is 6% in global production [1]. The chillies growing areas in Pakistan are decreasing gradually from 64.6 to 63.6 thousand hectares but per hectare yield is increasing from 1.9 to 2.7 tonnes per hectare and total production is also increasing from 122.9 to 171.8 thousand tonnes during 2005-2011 [2]. Fungi are the most dangerous pathogen among all type of damaging microorganisms against the production of cultivated plants and wild plants in forests causing extensive damage [3]. The impact of fungi with regards to plant health and food losses is staggering. The world's renowned famines and human suffering blame due to plant pathogenic fungi [4]. Charcoal rot disease is caused by *Macrophomina phaseolina* (Tassi) Goid. It is important soil borne pathogen causes diseases above the 500 plants species [5]. Management of *M. phaseolina* is challenging task because it is a soil borne pathogen and ubiquitous but prevalent in tropical, arid and sub-tropical environment, especially in those areas where high temperature and low rainfall occur [6]. It causes charcoal rot disease in chillies and destroyed whole field. *M. phaseolina* might be present in plants without showing any symptoms of disease [7]. Micro-organism such as bacteria, yeast or saprophytic fungi has significant potential to reduce and manage disease caused by root rotting fungi and induce defence mechanism [8]. There are many methods to control fungal diseases and reduce yield losses but the most commonly used method is chemical fungicides [9]. Extensively use of chemical fungicides developed resistance in plant pathogens [10]. These chemicals adversely effect environment and human health [11]. The organisms that suppress the growth of pathogen are referred as biological control agent [12]. Biocontrol has the potential to control crop diseases while causing no detrimental effect on environment [13]. The aim of present research work was to screen rhizobacterial species as biological control agents for charcoal rot disease of chilies.

MATERIALS AND METHODS

Sample collection and isolation of pathogen

Diseased and healthy plant samples of chillies were collected from different locations of district Lahore. The main areas selected for sampling were Research Farm, Institute of Agricultural Sciences (IAGS), University of the Punjab, Vegetable farm of University of the Punjab, Research Farm of Auriga Chemicals Private Limited, Farmer's fields in Shamki Battian, and Farmer's fields in and around Manga Mandi, Lahore. The pathogen was isolated on Potato Dextrose Agar (PDA) plates. The isolated fungus was identified on the basis of morphology and microscopic characteristics [14].

Isolation of Rhizobacteria

Ten (10 g) of washed roots were chopped and suspended in saline solution which was further diluted to six times and 100 µl were inoculated on fresh Luria Bertani agar (LB agar) plates. Purification of

morphologically different colonies was done on LB plates by following the protocol of [15].

Qualitative and Quantitative Screening of Rhizobacteria for Antifungal Activity

In qualitative screening 1 mm disks were punched from the pure culture of *Macrophomina phaseolina*. These disks were placed in the centre of fresh Potato Dextrose Agar (PDA) plates along with rhizobacterial isolates for the inhibition of pathogenic fungi and incubated at 28 ± 2^{o} C for 7-10 days. For the quantitative screening the percentage growth inhibition was determined according to Anis *et al*. [16].

Properties of hizobacteria

Morphological and physiological properties of 2 different rhizobacterial isolates (ChE1 and UCaE4) were investigated according to the Bergey's Manual of Systematic Bacteriology [17]. These isolates were identified by performing different biochemical tests such as Gram staining, Spore staining, Catalase test, Starch hydrolysis test, Voges proskauser test, Citrate utilization and some biometric measurements.

Field Trial

For field trials the land was prepared with dung manure and urea and sterilized hybrid seeds were sown on raised beds and replicated. The experiment was conducted into Randomized Split Plot design trial with 9 treatments and three replicates.

Inoculum Preparation of Pathogens and Rhizobacterial Isolates

To prepare the inoculum of rhizobacterial isolates ChE1 and UCaE4 (*Bacillus subtilis* and *Bacillus licheniformis*) was inoculated in 4 prepared fresh LB broth bottles [18]. In field trial growth and disease related parameters such as shoot and root length, fresh weight and dry weight of plants, disease incidence, disease severity [19] and mortality [20] were evaluated.

RESULTS

Isolation and Identification of Pathogenic Fungus

Macrophomina phaseolina (Figure 1) was identified on the basis of fumaceous hyphae bearing numerous minute sclerotia. Sclerotia are small, numerous in number, oblong to round or irregular in shape, black color attached with mycelium [21].

Rhizobacteria Isolation

About 48 different rhizobacteria were isolated, purified and preserved for their biocontrol activity against fungal diseases. (Figure: 2).

Properties of Rhizobacteria

The isolated rhizobacteria ChE1 and UCaE4 were found as aerobic, spore forming gram positive and motile bacteria. It was clear from "Bergey's Manual of Systematic Bacteriology 1986" these bacteria belong to the genus *Bacillus*. ChE1 was identified as Bacillus subtilis and UCaE4 as *Bacillus licheniformis* at species level (Table: 1).

Screening of Rhizobacteria for Antifungal Activity

Rhizobacterial isolates (31 isolates) were observed with antagonistic activity against pathogenic fungus *in-vitro*. Out of 30 antifungal rhizobacterial isolates, 13 rhizobacterial isolates were found as promising antifungal rhizobacteria against *Macrophomina phaseolina* (Figure 3).

From these rhizobacteria isolates ChE1 and UCaE4 were selected as highly antagonistic, UPD3 and URE8C were selected as moderately antagonistic, ChE3 and ChD3 were selected as slightly antagonistic and URE3 and UCaD2were selected as non-antagonistic rhizobacterial (Figure 4).

Disease Severity, Incidence and Mortality after Inoculation

Bacillus subtilis had shown better results and significantly reduced the level of disease severity, incidence and mortality against *Macrophomina phaseolina*. These parameters were measured for 30 days at 5 days interval. Treatment showed that *Bacillus subtilis* reduced disease severity up to 38.06%. Maximum disease severity was observed in B_0F_1 plot and minimum in B_1F_0 plot (Figure 5).

Disease incidence reduced best by *Bacillus subtilis* as compare to *Bacillus licheniformis*. Treatment showed that *Bacillus subtilis* reduced disease incidence up to 32.88% while *Bacillus licheniformis* inhibit disease incidence up to 14.48%. Maximum disease incidence was observed in B_0F_1 plot and minimum in B_1F_0 (Figure 6).

It was observed that *Bacillus subtilis* have ability to reduce mortality rate among chilli plants. Treatment showed that *Bacillus subtilis* reduced mortality rate up to 21.13% and *Bacillus licheniformis* also inhibited disease mortality upto 18.30%. Maximum disease mortality was observed in B_0F_1 plot and minimum in B_1F_0. The data were subjected to Analysis of Variance (ANOVA) and significance differences among the comparisons were taken by LSD test at $P \leq 0.05$. According to statistical analysis a significant difference was observed in the performances of both rhizobacterial isolates (Figure 7).

Effect on Fresh and Dry Weight of Chilli seedlings after inoculation

Fresh and dry weight of chilli seedlings were increased when inoculated with *Bacillus subtilis* and *Bacillus licheniformis*. The fresh and dry weight of plants was measured for 30 days at 5 days interval (15 days, 20 days, 25 days and 30 days). Treatment exhibited that *Bacillus subtilis* increased fresh weight 9.00% in treated plots while *Bacillus licheniformis* increased up to 7.13%. Maximum fresh weight was observed in B_1F_0

plot (where distilled water with *Bacillus subtilis* and distilled water were inoculated) and minimum in B_0F_1 where distilled water with *M. phaseolina* was inoculated) (Figure 8). Dry weight of plants was increased when inoculated with *Bacillus subtilis* and *Bacillus licheniformis*. Non-significantly, *Bacillus subtilis* was shown better results as compared to *Bacillus licheniformis*. Comparison of treatment showed that *Bacillus subtilis* increased dry weight up to 17.76% and *Bacillus licheniformis* increase dry weight up to 7.94% in treated plots. Maximum dry weight of plant was observed in B_1F_2 plot (where *Bacillus subtilis* with *Macrophomina phaseolina* were inoculated) and minimum in B_0F_1 (where distilled water with *M. phaseolina* was inoculated) (Figure 9).

Effect on Root and Shoot Length of Plants

Increase in shoot and root length of the infected chilli plants were measured after inoculation with antagonistic *Bacillus subtilis* and *Bacillus licheniformis*. The shoot and root length (cm) of plants were measured for 30 days at 5 days intervals (15 days, 20 days, 25 days and 30 days). Treatment showed that *Bacillus subtilis* increased shoot length up tp 6.04% while *Bacillus licheniformis* increased shoot length up to 3.43%. Maximum shoot length was observed in B_1F_0 plot (where distilled water with *Bacillus subtilis* was inoculated) and minimum in B_0F_1 where distilled water with *M. phaseolina* was inoculated) (Figure 10). *Bacillus subtilis* had significantly increased length of roots when plants were inoculated with *Bacillus subtilis* and *Bacillus licheniformis*. Comparison of treatment shows that *Bacillus subtilis* when inoculated with *Macrophomina phaseolina*, it increased root length up to 12.74% while *Bacillus licheniformis* increased root length up to 6.70%. Maximum root length was observed in B_1F_0 (plot where distilled water with *Bacillus subtilis* was inoculated) and minimum in B_0F_1 (where distilled water with *M. phaseolina* were inoculated). All the data was subjected to Analysis of Variance (ANOVA) and significance differences among the comparisons were taken by LSD test at $P \leq 0.05$. According to statistical analysis a significant difference was observed in the performances of both rhizobacterial isolates (Figure 11).

DISCUSSION

Macrophomina phaseolina recognized as pathogenic organism responsible for Charcoal rot of chillies. It was identified on the basis of fumaceous hyphae bearing numerous minute sclerotia. Sclerotia are small, numerous in number, oblong to round or irregular in shape, black color attached with mycelium (Figure 1). Kamalakannan [21] also concluded that *Macrophomina phaseolina* have fumaceous hyphae bearing numerous minute sclerotia which were oblong to round or irregular in shape and in black colour attached with mycelium. The isolated rhizobacteria ChE1 and UCaE4 were found as aerobic, spore forming gram positive, motile bacteria with ability to hydrolyse catalase and starch (Table: 1). The present results were in line with the findings of Ann [22] *who concluded that Bacillus species have ability to produced antibiotics (iturin, mycosubtilin and baculysin), spore are resistant to UV light induced defence response and growth. Moreover,* Akgul and Mirik [23] also Isolated 16 rhizobacteria were having ability to produce indole acetic acid belonging to genus *Bacillus* and *Pseudomonas*.

ChE1 and UCaE4 isolates were belonging to genus Bacillus and identified as *Bacillus subtilis* and *Bacillus licheniformis* according to "Bergey's Manual of Systematic Bacteriology 1986". These isolates were found as most promising biocontrol agent during recent studies. Thirty one rhizobacterial isolates were found with antagonistic activity against pathogenic fungi *in-vitro*. Out of thirty antifungal rhizobacterial isolates, thirteen rhizobacterial isolates were found as promising antifungal rhizobacteria against *Macrophomina phaseolina*. In present work *Bacillus subtilis* (ChE1) and *Bacillus licheniformis* (UCaE4) found as excellent antagonist against *Macrophomina phaseolina in vitro* conditions (Figure 3 and 4). Same results were found by Samrah [24] while working with the *Psudomonas* species and concluded that the efficacy of *Pseudomonas* sp. against root rotting fungi *in vitro* was excellent. The results showed that Bacillus subtilis is very important rhizobacteria for their bio control activity it reduced diseased severity, mortality and incidence also increase fresh and dry weight of plant, shoot and root length and statistical analysis showed that it has significant effect than *Bacillus licheniformis* but meanwhile *Bacillus licheniformis* also have good bio control activity against fungal diseases. The *M. phaseolina* induced disease severity up to 35.33% and 27.33% respectively. *B. subtilis* reduced disease severity of *M. phaseolina* up to 38.06%. *B. licheniformis reduced disease severity up to* 10.97% against *M. phaseolina* (Figure 5). The disease incidence was observed 25.33% in case of *M. phaseolina* but it reduced up to 25.28% and 32.88% by *B. subtilis and B. licheniformis respectively (Figure 6).* Richards and Peter [25] also concluded while working with same disease that plant disease losses can be minimizing after the correct diagnosis of causal agent first and then accurate estimation of the disease severity can be helpful to seek remedies to manage such diseases. Other parameters such as fresh and dry weight of chili seedlings were also amplified when vaccinated with *Bacillus subtilis* and *Bacillus licheniformis*. *Bacillus subtilis* increased fresh weight 9.00% while *Bacillus licheniformis*

Figure 1: Pure Culture of *Macrophomina phaseolina*

increased up to 7.13% (Figure 8). *Bacillus subtilis* improved dry weight up to 17.76% and *Bacillus licheniformis* increase dry weight up to 7.94% in treated plots (Figure 9). *Similar results were reported by* Karimi [26], they enlightened the ability and performance of *Bacillus subtilis* in *in vitro* and in field, shows it have significant results for controlling soil borne pathogens as well as have ability to increase plants growth.

Increase in shoot and root length of the infected chili plants was also observed after inoculation with antagonistic *Bacillus subtilis* and *Bacillus licheniformis*. *Bacillus subtilis* increased shoot length up tp 6.04% while *Bacillus licheniformis* increased shoot length up to 3.43% (Figure 10). In case of root

Table 1: Identification Tests for Most Promising Antifungal Rhizobacteria

Test Name	ChE1	UCaE4
Gram stain	Positive	Positive
Sporulation	Positive	Positive
Catalase test	Positive	Positive
Starch hydrolysis	Positive	Positive
Voges-proskauer Test	Positive	Positive
Citrate utilization test	Positive	Positive
Growth 6% NaCl solution	Positive	Positive

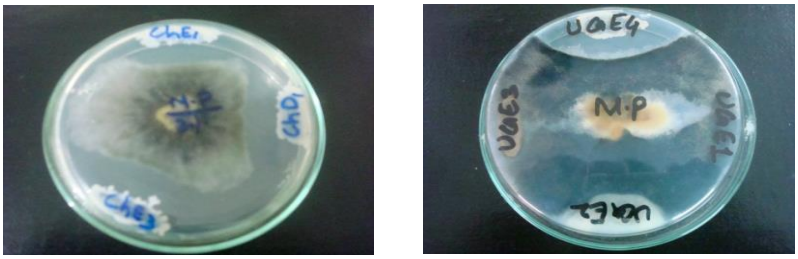

Figure 2: Antifungal Activity of Rhizobacterial Isolates

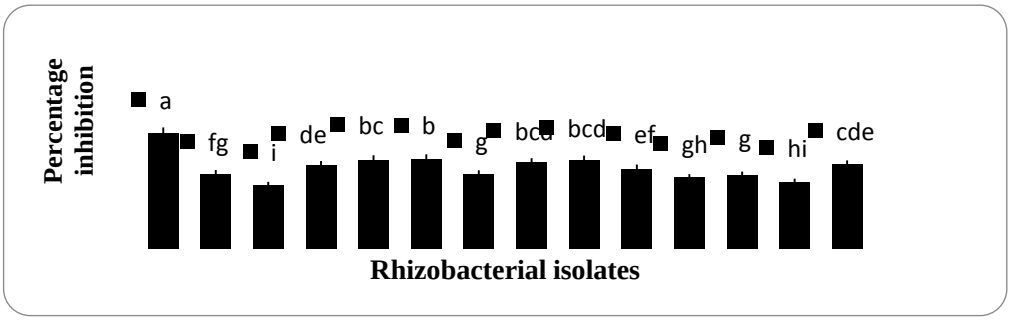

Figure 3: Quantitative Analysis of Rhizobacteria against *Macrophomina phaseolina*

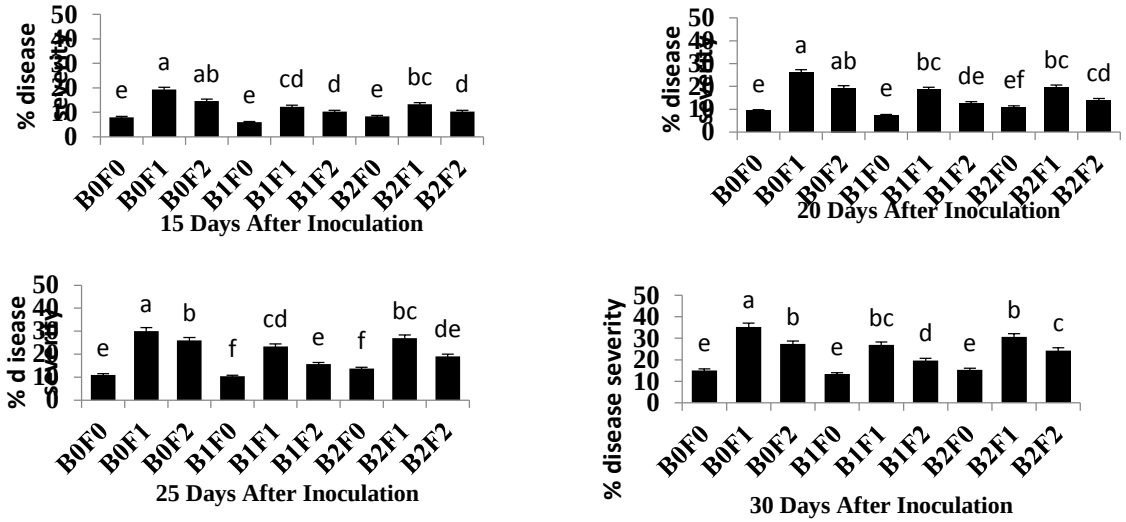

Figure 4: Percentage disease severity 15, 20, 25 and 30 Days after Inoculation

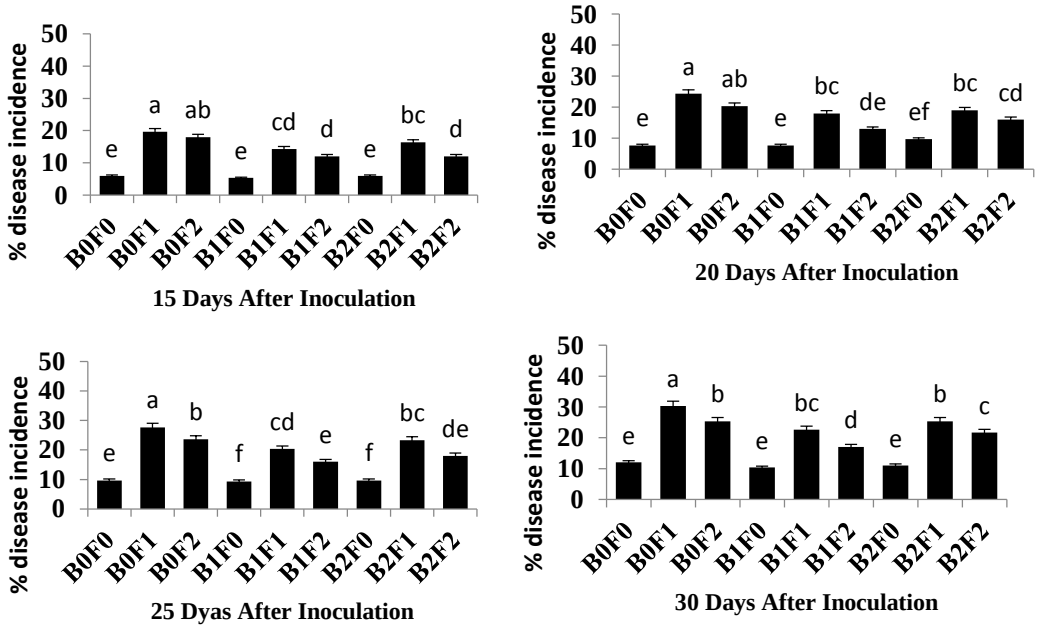

Figure 5: Percentage disease incidence 15, 20, 25 and 30 Days after Inoculation

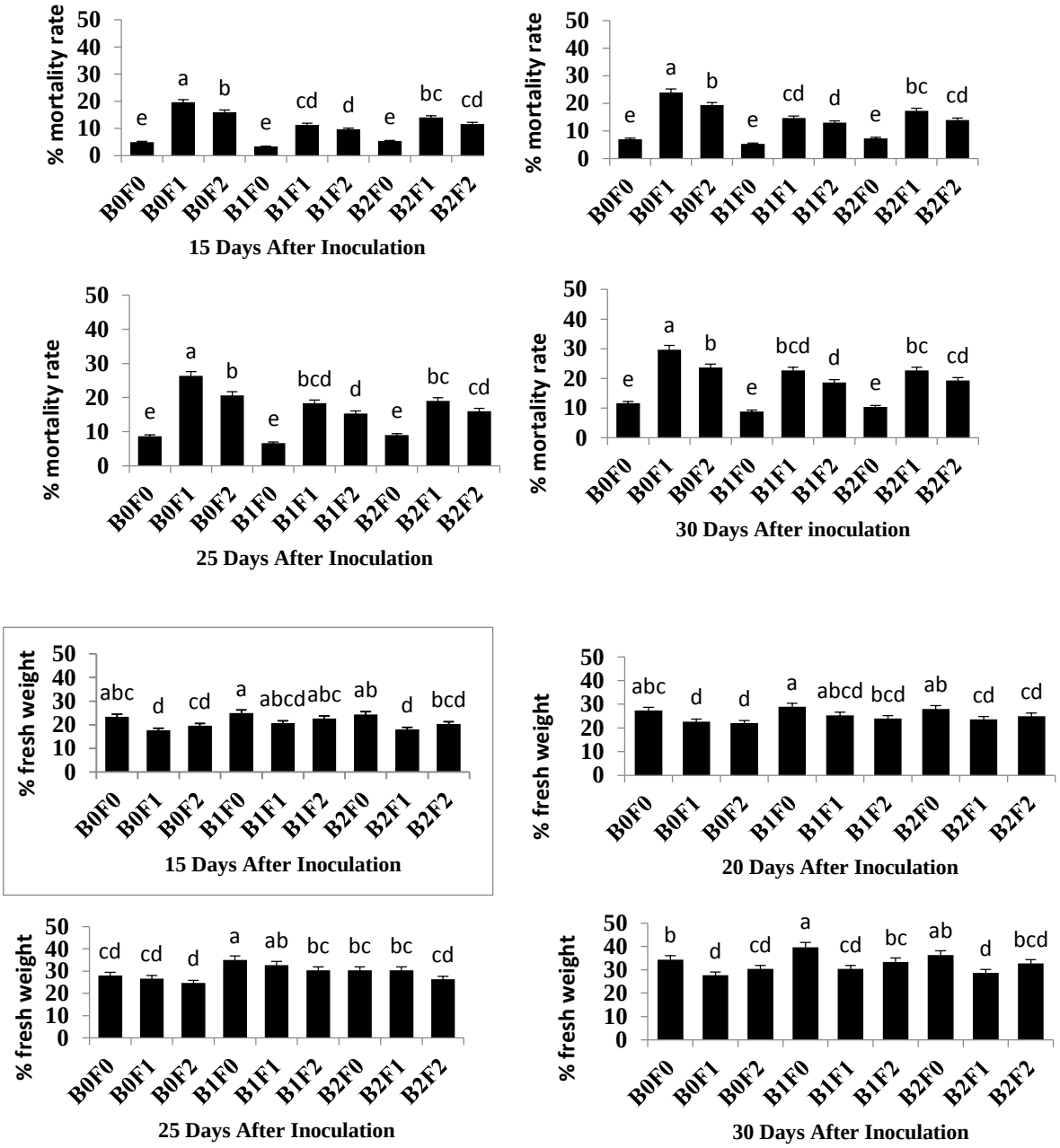

Figure 6: Fresh weight of Plants 15, 20, 25 and 30 Days after Inoculation

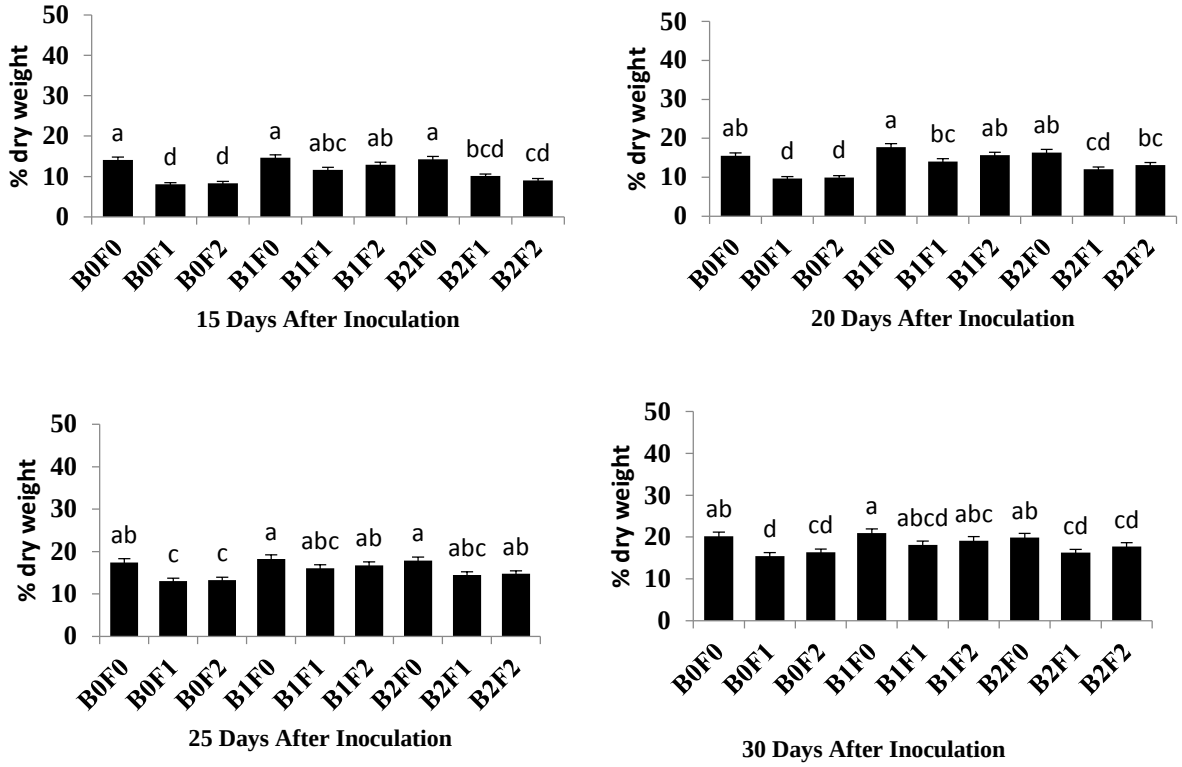

Figure 7: Percentage dry weight 15, 20, 25 and 30 Days after Inoculation

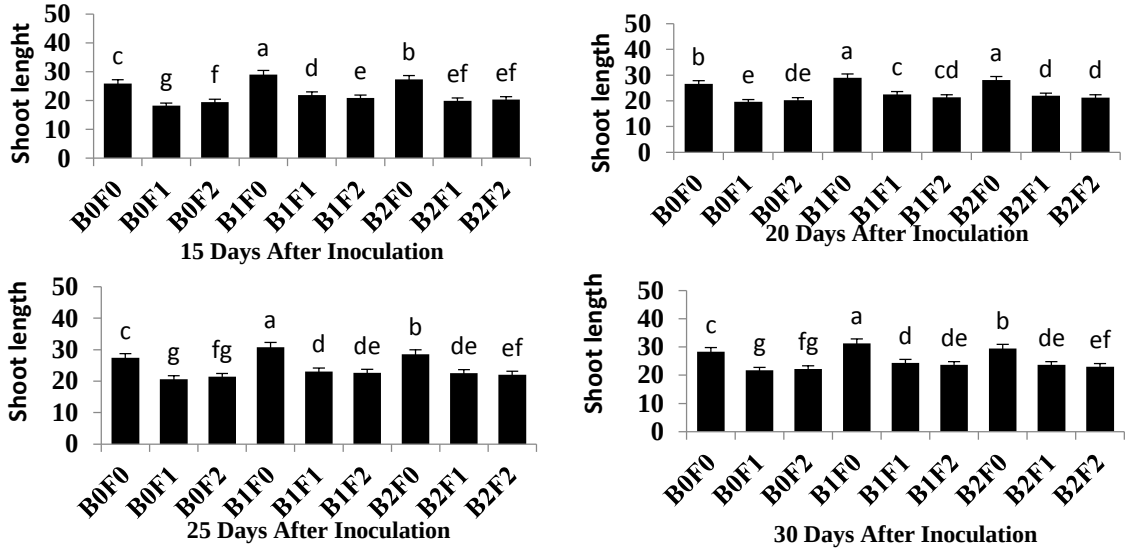

Figure 8: Shoot length of plants 15, 20, 25 and 30 Days after Inoculation

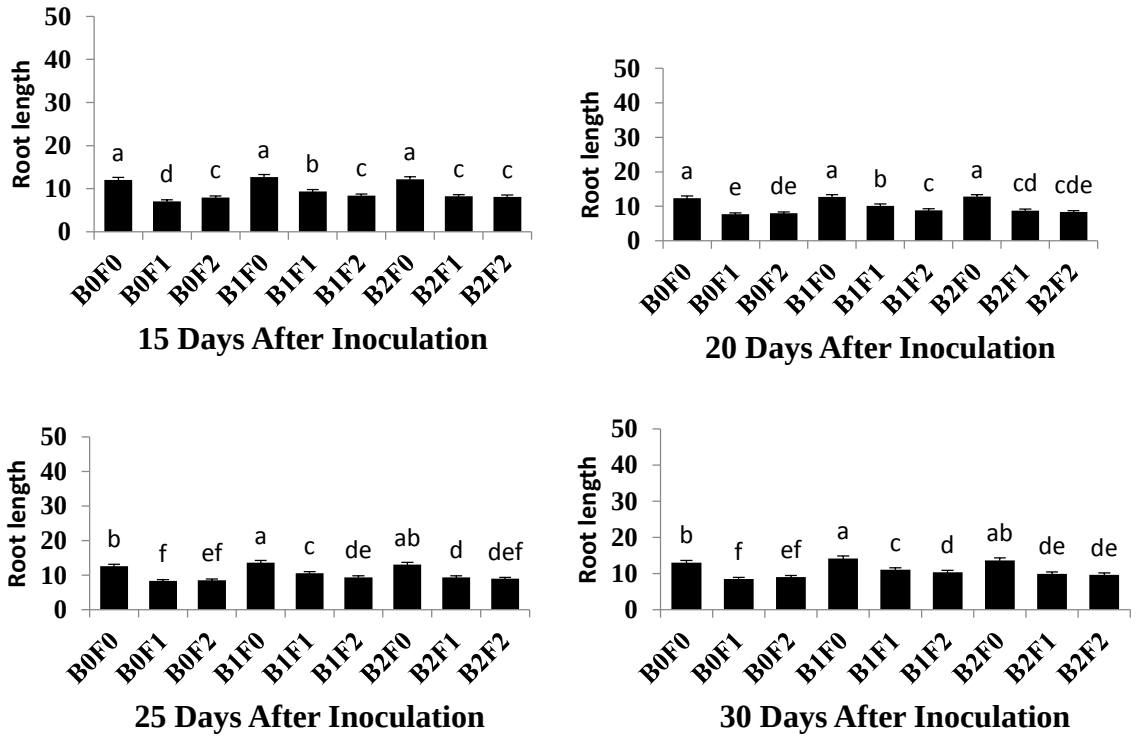

Figure 9: Root length of plants 15, 20, 25 and 30 Days after Inoculation

length *Bacillus subtilis* had significantly increased length of roots up to 12.74% while *Bacillus licheniformis* increased root length up to 6.70%. Similar findings were discussed in the work of Hassni [8]; Kulkarni [27] who reported the use of micro-organism such as bacteria, yeast or saprophytic fungi have significant potential to reduce and manage disease caused by root rotting fungi by acting as antagonists and biological control method is best and potential method to overcome disease and environmental pollution related problems. Benizri [28] also studied on metabolites production of antagonistic bacteria, used for bio control of plant diseases.

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