AJAB

Asian J Agric & Biol. 2019;7(3):320-333.

Original Article

Biocontrol potential of *Bacillus gibsonii* and *Brevibacterium frigoritolerans* in suppression of *Fusarium* stalk rot of maize: a sustainable approach

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Received:
March, 12, 2019

Accepted:
June 30, 2019

Published:
September 30, 2019

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Abstract

Natural interactions between plant and associated microbes have vital importance in plant growth and vigor. Plant growth promoting rhizobacteria (PGPR) modulates growth promotion and suppression of plant diseases. Maize (*Zea mays* L.) being an important cereal crop faces loss in annual yield due to stalk rot caused by fungal pathogen *Fusarium moniliforme*. Native bacteria can be used to reduce fungal disease and could provide a sustainable solution to reduce yield loss by pathogen attack. Two antagonistic PGPR, *Bacillus gibsonii* and *Brevibacterium frigoritolerans* were investigated for their potential to enhance growth and ameliorate the negative effects of *F. moniliforme* on both diseased effected and normal plants. Two maize varieties TP-1217 (Variety A) and TP-1221 (Variety B) were subjected to different treatments under greenhouse conditions by using a completely randomized design. Analysis of plant growth parameters, chlorophyll and proline contents, electrolyte leakage, antioxidant enzyme activities, and disease index assessment was done to examine the induced tolerance and plant growth promotion by applied PGPR. Results indicated potential antifungal activity of bacterial strains. Inoculation of bacterial strains to plants reduced disease and enhanced plant growth parameters. Disease suppression was influenced by 67% and plant growth was enhanced significantly. Relative electrolyte leakage reduced by 52 -55% and more than 80% disease control in both varieties of the plant was observed. Application of bacteria as biocontrol agents in combination with current disease protection strategies could aid in global food security.

Keywords: Biocontrol, Phytopathogens, Antagonistic activity, PGPB, Stalk rot disease, *Fusarium moniliforme*

How to cite this:

Batool R, Rehman SU, Rafique M, Amna, Ali J, Mukhtar T, Mahmood S, Sultan T, Munis FH and Chaudhary HJ, 2019. Biocontrol potential of *Bacillus gibsonii* and *Brevibacterium frigoritolerans* in suppression of *Fusarium* stalk rot of maize: a sustainable approach. Asian J. Agric. Biol. 7(3):320-333.

Introduction

Maize (*Zea mays* L.) being an important cereal crop (Figueroa-López et al., 2016), also known as the queen of cereals, cultivated throughout the world. It has an important position in cropping system of Pakistan after wheat and rice (Naseem and Bano 2014). In Pakistan, maize constitutes 4.8% of total cropping area and 3.5% of total agronomic production. *Fusarium moniliforme* (also known as *Fusarium verticillioides*) is the most common soil-borne fungal pathogen infecting maize crop. It causes stalk, ear and root rot (SERR) diseases in maize, and is responsible for significant economic losses globally (Kenganal et al., 2017). Around 35% loss in total yield has been reported due to *Fusarium* stalk rot (AICRP, 2014). This soil-borne pathogen (*F. moniliforme)* leads to breakage of the stalk, rotting, lodging and premature death of the infested plants. The plants express symptoms of drying from leaf margins extending towards midrib covering entire leaf lamina, the subsequent death of all leaves lead to drying of the whole plant before seed set (Kenganal et al., 2017).

Increasing demand for the supply of steady food to growing world population required controlling of plant diseases that significantly reduce the crop yield. Current practices for controlling crop disease are largely based on the development of resistant varieties, use of synthetic pesticides, crop alteration, solarization and chemical control (Ge et al., 2004). As *F. moniliforme* is a soil-borne pathogen and chemical application is not feasible as it directly effects the beneficial soil microflora. Moreover, their constant use results in the development of chemical resistance in target pathogens. Considering that, there is a need for alternative control (Kenganal et al., 2017). Bio control of fungal diseases by using plant growth promoting bacteria (PGPB) is a better option due to its low cost and environment friendly approach and its dual effects results in its successful application on a commercial scale (Souza et al., 2015). Generally, PGPB help in plant growth promotion directly by facilitating in the acquisition of nutrients such as phosphorus, nitrogen and essential minerals; modulating hormone levels of plant, or indirectly as a bio-control agent by decreasing the drastic effect of various plant pathogens on the growth and the development of plant (Glick, 2012). Documented mechanisms for biocontrol of plant diseases mediated by PGPB includes struggle to survive for an ecological niche, synthesis of inhibitory metabolites, and induction of systemic resistance (ISR) in host plants to a broad spectrum pathogens(Rojas-Solís et al.,, 2018). *Bacillus* genera offer several advantages over rest of bacteria for protection against root pathogens because of their ability to form endospores and the broad-spectrum activity of antibiotics. There are numerous reports of *Bacillus* spp. which repress pathogen (Bacon et al., 2001; de Jensen et al., 2002).The *Bacillus* genus is able to produce many secondary metabolites with antifungal effects on diverse plant pathogens (Raaijmakers and Mazzola 2012).

The objective of this study was to determine the ability of *B. gibsonii* and *B. frigoritolerans* (1) to enhance the maize plant growth and chlorophyll contents (2) *in vitro* and *in vivo* inhibition of *F. moniliforme* growth (3) controlling *Fusarium* stalk rot disease in maize and (4) substituting the use of agrochemicals with biofertilizers (PGPB) to control pest and pathogen attack on maize crop.

Material and Methods

In vitro antagonistic activity of bacterial strains against *F. moniliforme*

The bacterial strains used in this study were obtained from Plant Microbe Interactions Laboratory, Quaid-i-Azam University Islamabad, which were previously isolated from rhizosphere of sugarcane plant; collected from Punjab, Pakistan. The isolates were identified as *Bacillus gibsonii* and *Brevibacterium frigoritolerans*. The antagonistic activity of *Bacillus gibsonii* and *B. frigoritolerans* against the fungal pathogen *F. moniliforme* (obtained from Plant Microbe Interactions Laboratory, Quaid-i-Azam University Islamabad, Pakistan)was evaluated by following the dual culture technique as described by Cray et al. (2015). Bacteria and fungus, both were inoculated in the same plate containing dual culture of LB and PDA (1:1). An agar plug of fungus taken from the fresh fungal culture with the help of borer was placed on a plate containing culture media and, a thin smear of bacterial cells was streaked on other sides about 2 cm away from fungal disc (Gupta et al., 2016). For control, culture media were inoculated with fungus only. The test was applied in 3 replicates for each bacterial strain. After being sealed with parafilm, plates were incubated at 27°C. The percentage inhibition zone compared to control was computed.

Exopolysaccharide production

EPS-producing ability of bacteria was qualitatively

analyzed in optimized mineral salt medium. Bacteria having the ability to produce EPS will form thick mucoid colonies on this media(Bramhachari and Dubey, 2006).

In vivo antagonistic assay

The pot experiment was performed in green house at National Agriculture Research Centre (NARC) in complete randomized design (CRD) with factorial arrangements in three replicates. The treatments applied were C (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *B. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritolerans* and *F. moniliforme*) T5 (*F. moniliforme* infected plants).

Seed sterilization

The seeds of two varieties TP-1217 (Variety A) and TP-1221 (Variety B) obtained from NARC, Crop Research Institute and were surface sterilized by dipping in 95% ethanol for few moments and in 0.2% $HgCl_2$ solution for 3 min, and then washed carefully with distilled water (Naseem and Bano 2014).

Inocula preparation and application

Both bacterial isolates (*B. gibsonii* and *B. frigoritolerans*) were inoculated in 100 ml of LB broth separately and incubated at 30°C for 48 h. at 120 rpm. After 48 h. broth culture was centrifuged for 10 min. at 3000 rpm and pallets were collected. Pallets were then re-suspended in distilled water to make optical density equivalent to 1 at 600 nm (Naseem and Bano 2014). Surface sterilized seeds were soaked in this suspension prior to sowing for 3 to 4 hours and seeds for control treatment were soaked in distilled water only. In total five seeds were sown in each pots of 23 cm in diameter and 19 cm in length, filled with 5 kg of autoclaved soil: sand: vermicompost (1:1:1) at pH 7.76 and EC 380 µs/cm. There were three replicates of each treatment in making total of eighteen pots. Plants were irrigated one time daily with distilled water. After 45 Days of germination, thedisease was applied to the plants of relevant treatments (T3, T4, and T5) by soil drench method. Six days before disease application, 25ml of 48 h old bacterial culture adjusted to 10^8 cells was applied to plant by soil drench method around the stem of each plant and exactly after six days of bacterial application, 25 ml of conidial suspension of *F. moniliforme* (10^6 conidia/mL) was applied by soil drench method in each pot of T3, T4 and T5 (Abdallah et al., 2016). Plants were harvested after 15 days of disease application.

Disease index assessment

After harvest, the stalks were cut longitudinally and rated the disease severity. Discoloration and rotting on the inoculated stalks were rated from 1 to 5, where 1; 0–25%, 2; ≥ 25–50%, 3; ≥ 50–75%, 4; ≥ 75 < 100% of the inoculated internodes, and 5; 100% with infection extending into the adjacent internodes. The formula for obtaining the disease index is as follows:

$$Disease\ Index\ (\%) = \frac{0A + 1B + 2C + 3D + 4E + 5F}{5T}\ x100$$

Where A, B, C, D, E, and F are the total number of maize stalks with an index of 0, 1, 2, 3, 4, and 5, respectively, and 5T is the total number of stalks multiplied by the maximum disease rating (Hooker 1956).

Physiological and biochemical analysis of plant

Shoot and root length of freshly harvested plants was taken in cm-scale by using a measuring tape(Bano and Muqarab 2017). Fresh weight of plants from each pot was taken by using an electrical balance then these plants were dried at 80°C in a hot air oven for 24 h, after that their dry weight was measured with electrical balance (Reetha et al., 2014).

Root parameters

Roots were scanned for plant root imaging. Image analysis of the roots was done by using high throughput computing platform called GiA Roots (software for the high throughput analysis of plant root system architecture). Root length, maximum number of roots, average root width, network area and network depth was analyzed (Singh et al., 2015)

Leaf area

Leaf area of freshly harvested plants was measured manually by using following formula (McKee, 1964):

$$L.A = (Length\ x\ Width)\ x\ 0.74$$

Relative water content

The relative water content of leaf was determined by the method of Ahmed et al. (2016) with slight modification. A 0.5 Gram fresh weight of the leaf was taken and kept in distilled water overnightin the dark then turgor weight of these leaves was measured by

using weighing balance. After that, these leaves were oven dried at 70°C in dry air oven for one day, and their dry weight was measured. RWC was calculated by using following formula:

$$RWC\ (\%) = \frac{(Fresh\ Weight - Dry\ Weight)}{(Turgor\ Weight\ -\ Dry\ Weight)}\ x\ 100$$

Relative electrolyte leakage

Electrolyte leakage was measured by using EC meter. 1 g of the leaf was cut into small pieces and was immersed in 20 mL of distilled water at 25°C for 24 h. After the completion of incubation period initial EC (value A) was recorded. Samples were then autoclaved for 20 min. at 120°C to disrupt the leaf tissues completely and final EC (Value B) was recorded after cooling the samples to room temperature (Jiang et al., 2014) Percentage electrolyte leakage was measured by the following formula:

$$EL\ (\%) = \frac{(Value\ A)}{Value\ B}\ x\ 100$$

Estimation of antioxidant enzymes and Proline content of leaves

Peroxidase estimation of plants was performed by the method of Reddy et al. (1985) with slight modification.1 gram of fresh plant material was ground in 10 mL phosphate buffer and centrifuged for 10 minutes. Clear supernatant was collected. Spectrophotometer was adjusted to read zero at 430 nm and absorbance was taken for 3 minutes by adding 0.5 mL of 1% H_2O_2 in test cuvette along with plant extract.

Superoxide dismutase activity was performed by the method of Beauchamp and Fridovich (1971). Superoxide dismutase activity was performed by the method of (Beauchamp and Fridovich, 1971). 0.2 g of plant material was ground in 4mL phosphate buffer (pH 7.8), containing 1 g of polyvinyl pyrrolidone (PVP) and 0.0278 g of Na_2EDTA and then centrifuged at 4°C for 10 minutes. Supernatant was collected and its volume was raised up to 8 mL with phosphate buffer of pH 7. 1 mL of reaction mixture containing 0.0278 g of Na_2EDTA, 1.5 g Methionine and 0.04 g of Nitro blue tetrazolium chloride (NBT) in 100 mL phosphate buffer (pH 7.8) and 0.5 mL of reaction mixture containing 0.00113 g Riboflavin in 100 mL phosphate buffer (pH 7.8) is mixed with 0.5 mL of enzyme extract. One sample was kept in the light to initiate the reaction at 30 °C for 1 h, while an identical sample was kept in the dark. Absorbance was recorded at 560 nm.

Catalase activity of plants was determined by the method of Luck et al. (1974). 0.5 g of plant material was homogenized in 8 mL of phosphate buffer (pH 7) and centrifuged for 10 minutes to collect supernatant. Now 3 mL of H2O2 (2 mM) was mixed with 40 µl of supernatant. By using spectrophotometer reduction in absorbance by 0.05 units at 240 nm was recorded.

Proline content of leaves was determined by following the method of Li et al. (2010). 0.5 g of leaf sample was homogenized in 4 mL of 3% sulfosalicylic acid. The samples were centrifuged for 10 minutes to collect clear supernatant. 2 mL of supernatant was mixed with 2 mL of glacial acetic acid and 3 mL of acetic ninhydrin. Acetic ninhydrin reagent was prepared by heating 1.25 g of ninhydrin in a mixture of 30 mL glacial acetic acid and 20 mL of 6M phosphoric acid, with agitation, until dissolved. Then the samples were heated in hot water bath for 1 hour at 100°C. The samples were then allowed to cool and mixture was extracted by adding 5 mL of toluene. The absorbance was measured at 520 nm using spectrophotometer and toluene was taken as a blank. The calculation was made by following formula:

$$Proline\ (\mu g/g) = \frac{k\ value\ x\ dilution\ factor\ x\ absorbance}{Sample\ weight}$$

$$Where, k\ value = 17.52\ and\ dilution\ factor = 2$$

Photosynthetic pigments

Photosynthetic pigments (chlorophyll *a*, *b* and carotenoid) were determined according to Saeidi and Zabihi-e-Mahmoodabad (2009). 0.1g of the fresh leaf was homogenized in 6 mL of 80% acetone, and the extract was centrifuged for 10 minutes at 6000 rpm. The supernatant was used to record the absorbance at 645, 663 and 470 nm. 80% acetone was taken as a blank.

Statistical analysis

Data collected from all experiments were analyzed using standard analysis of variance (two way ANOVA) with factorial treatment structure and interactions using Statistix 8.1 software. The significance of treatments means at $p \leq 0.05$ was tested by using Tukey test.

Results

In vitro antagonism activity of PGPR against F. moniliforme

Both bacterial strains (*B. gibsonii and B. frigoritolerans*) strongly inhibited the growth of *F. moniliforme*. Bacterial isolate *B. gibsonii* found to cause 67.8% inhibition and *B. frigoritolerans* inhibited 67.4% as compared to control (Table 1) (Plate 1)

Table 1: Percentage growth inhibition of *F. moniliforme* by bacterial strains

S. No	Bacterial strains	*F. moniliforme* Inhibition (%)
1.	*Bacillus gibsonii*	67.8 ± 0.06
2.	*B. frigoritolerans*	67.4 ± 0.07

Plate 1: In vitro antifungal activity of bacterial isolates against *F. moniliforme*

Exopolysaccharide production

Both of the bacterial strains showed mucoid growth on optimized mineral salt medium, which indicates that they have exopolysaccharide producing ability.

In vivo effect of bacteria for growth promotion and disease suppression

The fresh and dry weights of plants

Both fresh and dry weights significantly increased after *B. gibsonii and B. frigoritolerans* inoculation. The percentage increase of fresh and dry weight was much higher (32.7% and 56% respectively) in *B. gibsonii* inoculated plants in variety A than in *B. frigoritolerans* inoculated plants. Disease occurrence decreased fresh and dry weights by 76.8% and 73% respectively in variety A and 78% and 61% respectively in variety B, as compared to non-infected un-inoculated control. Inoculated plants significantly overcame the disease induced reduction in fresh and dry weights over diseased controls. *B. gibsonii* increased fresh and dry weights by 74% and 79%, respectively, and *B. frigoritolerans* induced 81% and 82% increase in fresh and dry weights respectively under disease stress condition over disease control (Fig. 1).

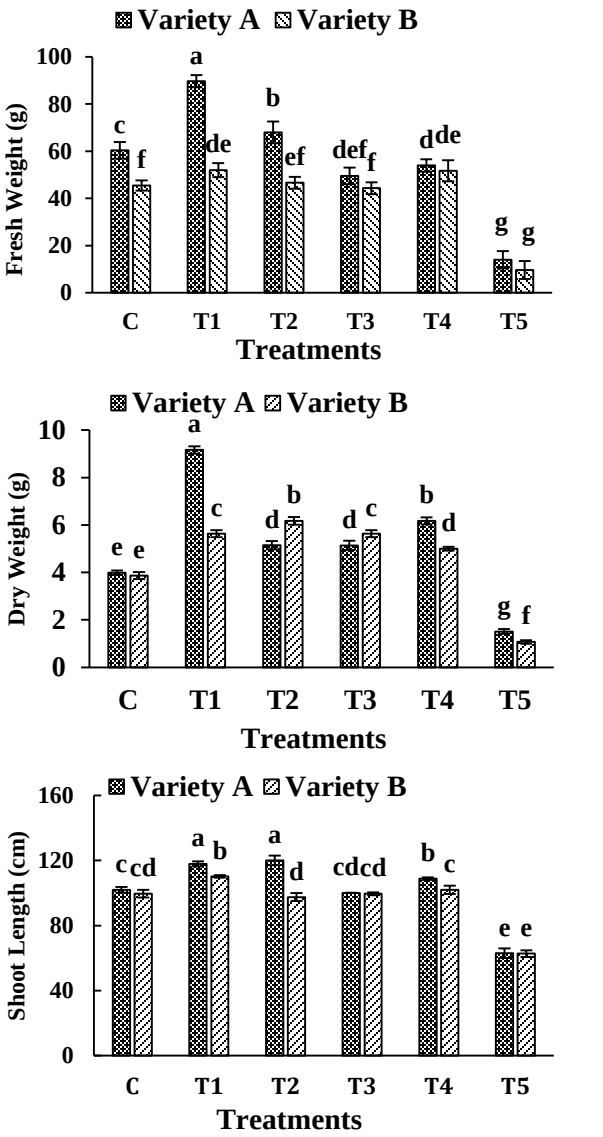


Fig. 1: Effect of PGPB strains on morphological parameters of two maize varieties under disease stress and non-stress condition.

Letters indicate significant differences *(p<0.05)*. Error bars indicate standard deviation. C (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *B. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritolerans* and *F. moniliforme*), T5 (*F. moniliforme* infected control).

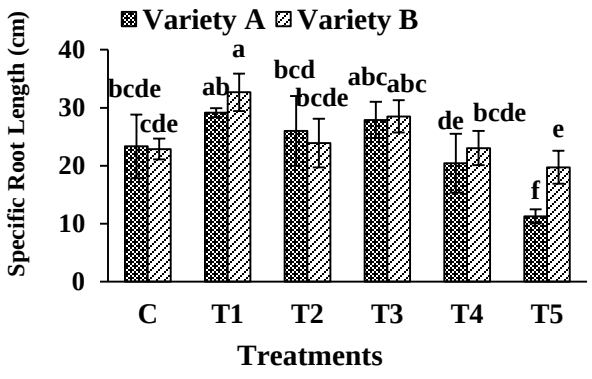
Shoot length of plants

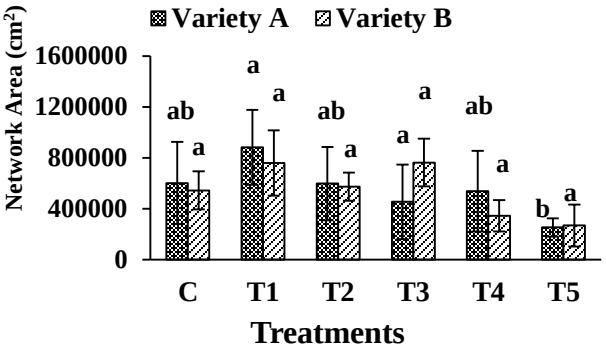
Interestingly, both *B. gibsonii* and *B. frigoritolerans* increased a higher rate of shoot/growth (13.5 and 15% respectively), as compared to the non-infected un-inoculated control. Plants inoculated with *B.*

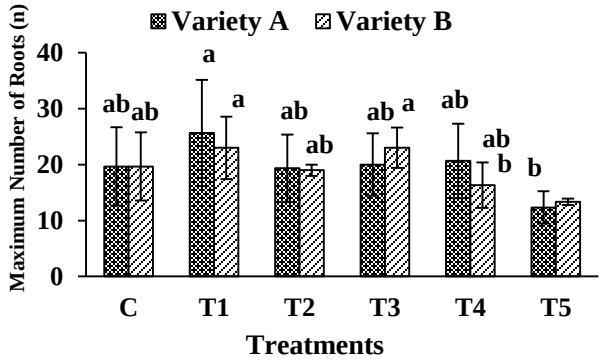
gibsonii,and *B. frigoritolerans* increased 37% and 42% of shoot length respectively in variety A and 37% and 38.5% respectively in variety B. Fungal application in disease treatment decreased shoot length by 38% and 37% in variety A and B respectively (Fig. 1).

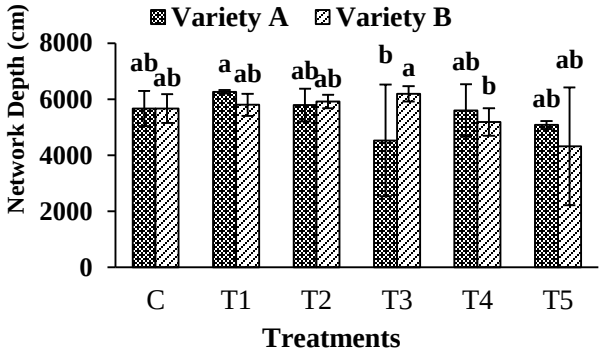
Root parameters

A large number of roots was analyzed using GiA root software, and our results showed a significant increase in length, network area, number of roots and network depth of roots treated with bacteria *B. gibsonii and B. frigoritolerans*. A maximum number of roots about 23.4% in variety A and 14.5% in variety B was observed in plants treated with *B. gibsonii* under nonstress conditions. Disease stress decreased the number of roots up to 37.5% and 32.5% in variety A and B respectively non-infected un-inoculated control. Similarly, bacteria *B. gibsonii* and *B. frigoritolerans* increased root length up to 20% and 10.4% respectively in variety A and 30% and 4.2% respectively in variety B. root length was decreased due to disease stress up to 52% and 14% in variety A and B respectively. Network area of root was significantly increased by treating plants with *B. gibsonii* and up to 32% and 28.3% in variety A and B respectively. Whereas, *B. frigoritolerans* increased 5.4% root network area in variety B. 58% and 50% disease induced decrease in root network area was observed in variety A and B respectively as compared to non-infected un-inoculated control. Similarly, network depth was increased by bacterial treatment and decreased up to 10% and 24% in variety A and B respectively. Average root width was increased by 4% and 3% in plants infected with disease stress (*F. moniliforme*) as compared to non-infected un-inoculated control. All other treatments do not show any noticeable difference in root width as compared to control.









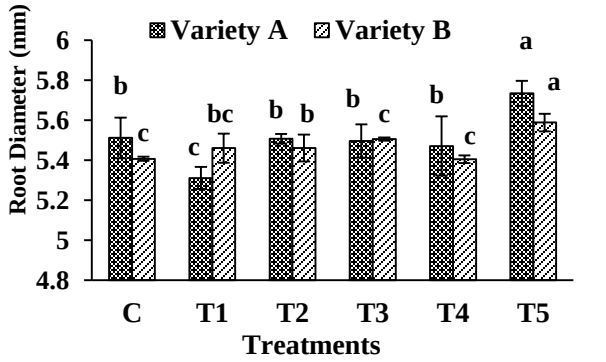


Fig. 2: Effect of PGPB strains on root parameters of two maize varieties under disease stress and non-stress condition.

Whereas application of bacteria *B. gibsonii* and *B.*

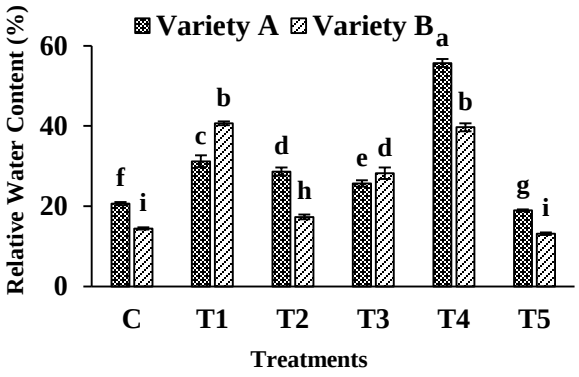
frigoritolerans under disease stress condition, by reducing the negative effect of fungi, increased the network area by 44% and 53%, root length by 59% and 44.6%, number of roots by 38% and 40% and network depth by 9% respectively in Variety A. In variety B network area increase by 65% and 22%, root length by 31% and 14.5%, number of roots by 41% and 18.4% and network depth by 30% and 17% were observed when inoculated with bacteria *B. gibsonii* and *B. frigoritolerans* respectively under disease stress condition as compared to disease control (Fig. 2).

Letters indicate significant differences ($p<0.05$). Error bars indicate standard deviation. C (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *B. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritolerans* and *F. moniliforme*), T5 (*F. moniliforme*infected control)

Leaf area

Leaf area of plants was found to increase significantly up to 37% and 32% due to *B. gibsonii* and *B. frigoritolerans* inoculation respectively, but in disease stress condition, it is reduced by 19% and 13% in variety A and B respectively. Whereas, bacterial inoculation in disease stressed plants improved the leaf area of plants significantly as compared to diseasedcontrol. The maximum increase of 53% was observed in *B. frigoritolerans* inoculated plants of variety A under disease stress condition (Fig. 3)

Letters indicate significant differences *($p<0.05$)*. Error bars indicate standard deviation. C (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *B. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritolerans* and *F. moniliforme*), T5 (*F. moniliforme* infected plants).



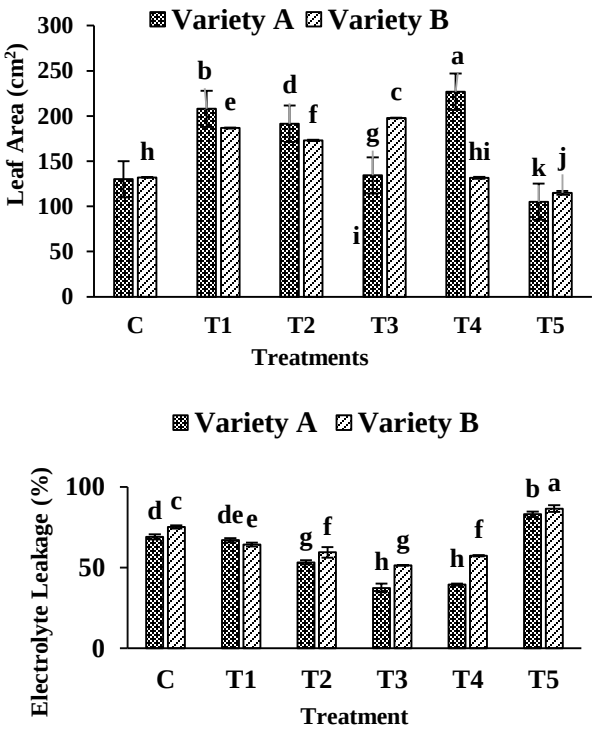


Fig.3: Effect of PGPB strains on relative water content, leaf area and electrolyte leakage of two maize varieties under disease stress and non-stress condition.

Relative water content

Results indicated that relative water content decreased in plants infected with *F. moniliforme* as compared to non-infected and un-inoculated controls. The increase of 34% and 28% were recorded when inoculated with *B. gibsonii* and *B. frigoritolerans*, respectively, in variety A and an increment of 64% and 17% were recorded by inoculation of *B. gibsonii* and *B. frigoritolerans* respectively in variety Bas compared to non-infected un-inoculated. Plants infected with *F. moniliforme* and inoculated with *B. gibsonii* and *B. frigoritolerans* had up to 53% and 67% higher relative water content, over disease control. The maximum increase of 67% was observed in *B. frigoritolerans* inoculated, and disease stressed plants (T4) (Fig. 3).

Relative electrolyte leakage

Electrolyte leakage increased by 17% and 13% in plants infected with *F. moniliforme* in variety A and B respectively, as compared to non-infected and un-inoculated control. Whereas,plants infected with *F.moniliforme* and inoculated with *B. gibsonii* and *B. frigoritolerans* showed a significant decrease in

relative electrolyte leakage up to 55% and 52% respectively, in variety A and 41% and 34% respectively in variety B (Fig. 3).

Disease Index assessment

In vivo results of antagonistic activity against *F. moniliforme* have shown significant effect to control stalk rot disease. The inoculation by *F. moniliforme* (without PGPB) showed that plant was fully infected. Application of PGPB strains with *F. moniliforme* showed that the PGPB controlled the infection/disease at different ranges. *B. gibsonii* controlled 86% and 80% infection in varieties A and B respectively. *B. frigoritolerans* controlled 93% and 86% of the infection in varieties A and B respectively (Table 2).

Table 2: *In vivo* disease index assessment of maize crop

Treatments	*In vivo* Stalk rot Disease Index (%)	
	Variety TP-1217	Variety TP-1221
Control	0.00 ±0.01	0.00 ±0.30
F. moniliforme	100.00 ±0.10	100.00 ±0.02
F. moniliforme + *B. gibsonii*	13.33±0.05	20.00 ±0.05
F. moniliforme +*B. frigoritolerans*	6.67±0.03	13.30 ±0.40

Mean of three replicates and ± values indicate standard deviation

Activity of antioxidant enzymes and Proline content of leaf

SOD activity in maize leaves was enhanced by 13% and 14% over controls under stressed condition in both varieties. Plants inoculated with *B. gibsonii*and *B. frigoritolerans* and infected with *F. moniliforme* had significantly increased SOD activity of 24% and 10% respectively in variety A and 6% and 10% in variety B over non-infected and un-inoculated control. Similar pattern of response to PGPB and disease infection was observed for POD activity. Maximum increase of 43% and 37% in POD was observed in *B. gibsonii* and *B. frigoritolerans* inoculated plants respectively after fungal infection as compared to diseased control. Similarly catalase activity of plants increased by 60% and 70% in variety A and B respectively under disease stress condition. Bacterial inoculation under disease stressed plants further increased the catalase activity over fungal infected plants. Maximum increase was observed by bacteria *B. frigoritolerans* up to 31% and 30% in variety A and B respectively under disease stress condition.

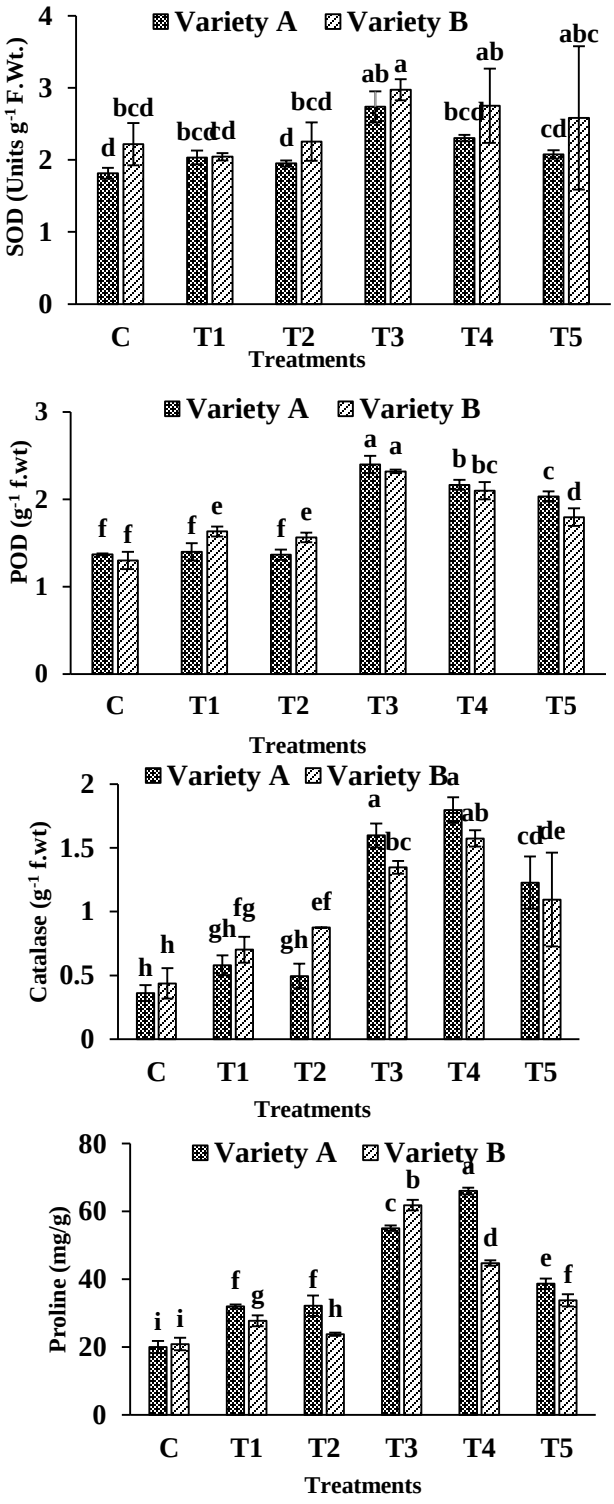


Fig.4: Effect of PGPB strains on antioxidant enzyme activity of two maize varieties under disease stress and non-stress condition.

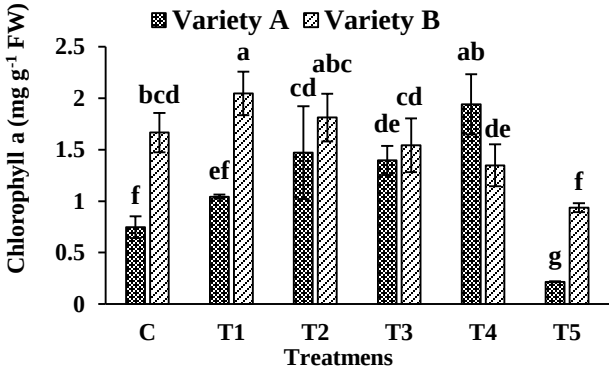
Plants infested with *F. moniliforme* had 48% and 38% higher proline content in variety A and B respectively

than non-infected un-inoculated control. *B. gibsonii*and *B. frigoritolerans* inoculation in plants infected with disease stress (*F. moniliforme*) increased the proline production by 64% to 70% over the plants infected with disease stress only (Fig. 4).

Letters indicate significant differences ($p<0.05$). Error bars indicate standard deviation (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *F. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritolerans* and *F. moniliforme*), T5 (*F. moniliforme* infected plants).

Chlorophyll content

Chlorophyll "*a"* and "*b*", and carotenoid contents of maize plants decreased by 71%, 72% and 58% respectively when compared with non-infected un-inoculated control. Bacterial inoculation significantly improved these attributes under disease stress condition. Inoculation by bacteria *B. gibsonii* under disease stress condition improved chlorophyll "*a*" and "b" content (upto 84% and 86%, respectively) and carotenoids contents (up to 76%) and bacteria *B. frigoritolerans* increased chlorophyll *a, b* upto 89% and 96% and carotenoids upto 84% as compared to disease control. Whereas chlorophyll a/b ratio was found to be increased by 7% under disease stress in both varieties as compared to non-infected un-inoculated control. Decreased pattern of chlorophyll a/b ratio was observed in first treatment (inoculated with *B. gibsonii* only) of variety B and treatment 4 (inoculated with *B. frigoritolerans* under disease stress) of Variety A of about 22% and 26% respectively. All other treatment showed almost same pattern (Fig. 5).



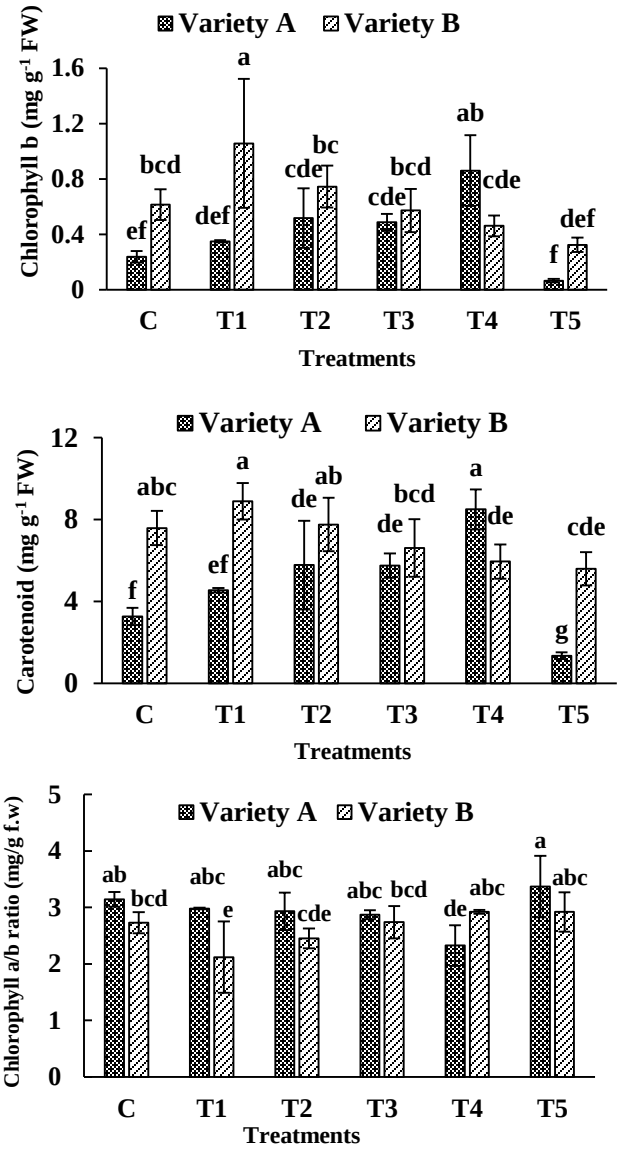


Fig.5. Effect of PGPB strains on chlorophyll content and chlorophyll a/b ratio of two maize varieties under disease stress and non-stress condition.

Letters indicate significant differences ($p<0.05$). Error bars indicate standard deviation. C (untreated, control), T1 (seeds inoculated with *B. gibsonii*), T2 (seeds inoculated with *B. frigoritolerans*), T3 (seeds inoculated with *B. gibsonii* and *F. moniliforme*), T4 (seeds inoculated with *B. frigoritoleran s*and *F. moniliforme*), T5 (*F. moniliforme* infected plants).

Discussion

Plants provide a diverse platform for microorganisms that participate in plant growth promotion by the production of beneficial secondary metabolites and suppression of disease in plants (Gupta et al., 2016). (Figueroa-López et al., 2016). Current study revealed the potential of PGPR in plant growth promotion by increased root and shoot length, fresh dry weight, and enhanced chlorophyll content. Moreover, disease suppression in inoculated plants as compared to uninoculated diseased plants revealed the biocontrol traits of the inoculated PGPR. EPS produced by play a key role in the attachment and colonization of bacteria to surfaces. Various techniques have been adopted for the proper study and understanding of biofilm attachment and its role in biocontrol mechanism. Bacterial biofilms established on plant roots could protect the colonization sites and act as a sink for the nutrients in the rhizosphere, hence reducing the availability of root exudate nutritional elements for pathogen stimulation or subsequent colonization on the root (Weller and Thomashow, 1994). Root-associated *Pseudomonads* have been studied extensively, these not only promote plant growth of host plants but also are used as biocontrol agents (Lugtenberg et al., 2001). *Pseudomonas putida* can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and starting steady biofilms network (Espinosa-Urgel et al., 2002). Haggag and Salme Timmusk (2008) studied the role of biofilm-forming bacteria, *Paenibacillus polymyxa* strains in controlling crown root rot disease and also emphasized importance of biofilms in biocontrol beginning. To make biocontrol mechanisms effective, successful colonization with the biocontrol agent must be ensured. In the present study, *in vitro* prescreening of two isolates *B. gibsonii* and *B. frigoritolerans* against *F. moniliforme* with a variable range of percentage inhibition by dual-culture technique revealed noticeable antagonistic activity. The *Bacillus* genus is able to produce many secondary metabolites with antifungal compounds against various plant pathogens Plant growth promotion is a key attribute of PGPB derived by PGP hormones and other associated traits (Glick 2005). Both bacterial isolates were biochemically positive for protease, pectinase, amylase, catalase, oxidase, cellulase and chitinase (Fig. 6).These antifungal proteins contribute in inhibition against diverse pathogens including *F. moniliforme* (Fig.7), and other fungi including *F. oxysporum, F. solani, P. ultimum* and *Rhizoctonia solani* (Chang et al., 2009).

Seed priming with both bacterial strains significantly reduces the disease index in comparison to uninoculated diseased plants. It was reported that *Bacillus* reduces the endorhizosphere colonization of *F. moniliforme* (Cavaglieri et al., 2005).

Both of the bacterial isolates (*B. gibsonii* and *B. frigoritolerans*) showed a significant increase in growth parameters of the plant in comparison with un-inoculated control. Shoot length, root length, root network area, number of roots, and average root width of plants increased by EPS-producing bacteria in both varieties over disease control as well as un-inoculated control due to IAA producing ability (Fig.6. (f)) of bacterial isolates as IAA is an important phytohormone which aids the plant in development of organized root system enabling the plant to uptake essential nutrients more efficiently (Tsavkelova et al., 2007). (Farooq and Bano 2013). All growth parameters were found to decrease in *F. moniliforme* treated plants due to disease occurrence whereas bacterial application minimized the effect of disease and increased the plant growth. As *F. moniliforme* is the soil-borne fungal pathogen (Kenganal et al., 2017) and can penetrate through roots and produces its micro conidia inside the roots (Rodriguez-Galvez et al., 1995). Voorhees et al. (1934) reported that when new roots grow out from cortex, they rupture the cell and provide easy channel for *F. moniliforme* hyphae to penetrate into the roots and wall of cell lining the area of rupture, appear start becoming thicker and apparently become more or less suberized, due to which cell size increases and root width also increases. Similarly, a noticeable increase in fresh and dry weight was observed due to the bacterial application which has EPS-producing ability under stress condition (Yaish et al., 2015). Remarkable increase in seedling strength, it's development, plant height, shoot and root length, fresh, and dry weight were observed in plants inoculated with bacteria. It has also been reported that PGPB isolates are more efficiently confer plant growth stimulation under stress condition than in normal environment (Rubin et al., 2017). Leaf area content is a measure of plant water status and it has a vital role in plant growth as well as in photosynthesis (Gou et al., 2015). Relative water content was increased in all treatments inoculated with EPS-producing bacteria but *B. frigoritolerans* showed maximum increase under disease stress condition. Bacterial EPS have an ability of water holding due to which EPS-producing

bacteria assisted in maintaining the moisture content of soil and flow of water across the plant roots due to the formation of soil aggregates (Roberson and Firestone, 1992).

Relative electrolyte leakage aggravated severe negative effect due to stress in both varieties that could be attributed to the enhanced POD and catalase activity indicating that membrane damage is caused by oxidative stress. Bacterial inoculation in this regard reduced the adversity of stress in both varieties. The findings are in accordance with Vardharajula et al. (2011) who reported that inoculation of *Bacillus* sp. in maize seedlings under stress condition decreased electrolyte leakage thus imparting membrane stability. Disease stress creates an osmotic imbalance and induces oxidative stress in plants. Proline serves as an energy source and hydroxyl radical scavenger (Munns and Tester, 2008). Our results indicated that plants adapted to osmotic stress increased the level of proline content but proline content were further increased in plants under diseased condition inoculated with PGPB which are similar to findings observed in Bano and Muqarab (2017). Increased level of proline via up-regulation of proline biosynthesis pathway keep plants safe from stress by membrane protection and maintaining cell water content (Sandhya et al., 2010).

The enhanced activities of defense-related enzymes contributed to bio protection of plants against pathogens and insects. Resistance to stress is strongly correlated to antioxidant enzyme activity (Bano and Muqarab, 2017). Production and scavenging of reactive oxygen species (ROS) are balanced by different antioxidant enzymes like SOD, POD, CAT etc. The SOD is first enzyme in the series which scavenge ROS induced during biotic and abiotic stresses. Current study showed an increased level of SOD, POD and CAT in disease stressed plants which is in accordance with Kużniak and Skłodowska (2005) who stated that tomato plant infected with *Botrytis cinerea* resulted in increment of SOD level in leaves and increase in POD level under disease stress is reported by Anjum et al. (2017).Moreover, PGPB inoculation under disease stress further enhanced the activities of ROS scavenging enzymes as compared to control and *F. moniliforme* control. Similar results are supported by Bano and Muqarab (2017)where antioxidant enzymes production was significantly stimulated in insect-infested–PGPR inoculated plants. Gururani et al. (2013) also reported elevation of similar activities in PGPR inoculated plants under stress than non-inoculated plants. In another study, elevation in enzymatic activities was observed in okra plants under stress condition, when inoculated with PGPB (Habib et al., 2016). Chlorophyll content is an indicator of stability under stress. In current study, disease stress significantly reduced chlorophyll content of plants. Reduction in Chlorophyll content (*a*, *b*) is an indication of photo-oxidation and has also been reported in bean and *Paulownia imparialis* (Rahdari et al., 2012). Bacterial inoculation improved chlorophyll content (*a*, *b*) under disease stress as well as in non-stressed condition. Chlorophyll a/b ratio was found to increase in stress condition. Vejan et al. (2016) also reported an increase in chlorophyll content of plants by PGPB application under disease stress. Carotenoids are non-enzymatic scavengers of reactive oxygen species present in substantial amounts in plants (Jung et al., 2000). In our study, carotenoid content was noticeably increased due to bacterial application in both varieties under stress and non-stressed plants. Chandrasekar et al. (2000) attributed high carotenoid content to genotype tolerance, since they are responsible for breakdown of singlet oxygen. Most root and foot rots of economic importance are caused by fungi that infect roots and cause progressive rotting of the root system. They can also infect the basal part of the stem (foot rot). Infected plants cannot absorb sufficient water and nutrients for their needs so the shoot system becomes stunted and the leaves turn yellow and wilt. Affected plants can eventually collapse and die. However, the severity of symptoms and the speed with which they appear depend on the rate of development of the root rot as well as the ability of the host to produce.

Conclusion

This study concludes that both the strains *B. gibsonii* and *B. frigoritolerans* have potential to control stalk rot disease by inhibiting *F. moniliforme* poliferation and can improve plant growth. This PGPB-induced disease resistance helps plants to cope the biotic stress and its negative effects on plant growth and yield. It is an environment-friendly strategy and its use as antagonistic bio fertilizer helped to cut off the heavy input of chemical fertilizers and pesticides in crop fields. Thus, considered as strong candidates for a novel bio-control agent against stalk rot disease.

Human and animal rights

This research does not include any animal and/or

human trials.

Contribution of Authors

Batool R: Data collection and manuscript writing
Rehman SU: Statistical analysis
Rafique M: Data interpretation
Amna: Manuscript Writing
Ali J: Designed research methodology
Mukhtar T: Literature search
Mahmood S: Statistical analysis
Sultan T: Manuscript final reading & approval
Munis MFH: Data interpretation
Chaudhary HJ: Conceived idea and manuscript final approval

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: None.

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