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Identification of New Biocontrol Agent against Charcoal Rot Disease Caused by *Macrophomina phaseolina* in Soybean (*Glycine max* L.)

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Abstract: Controlling agricultural pests using suitable biocontrol agents has been considered the best strategy for sustainable agriculture. Charcoal rot caused by a necrotrophic fungus *Macrophomina phaseolina* is responsible for a 30–50% annual reduction in soybean yield worldwide. Little is known about the role of *Bacillus clausii* in reducing charcoal rot disease severity in the soybean crop. In this study, we investigated plant growth promoting and antagonistic potential of *Pseudomonas putida* (MT604992) and *Bacillus clausii* (MT604989) against charcoal rot disease incidence in soybean. Among twenty bacteria isolated from soil and water samples of two different hot springs of Gilgit-Baltistan, Pakistan, 80% were siderophore positive; 65% were hydrogen cyanide (HCN) positive; 55%, 30%, and 75% were phosphate, potassium, and zinc solubilizers, respectively. Based on higher antagonistic activities and plant growth promoting traits five strains were selected for in vitro screening. Out of all tested strains, *Pseudomonas putida* and *Bacillus clausii* showed a significant increase in germination, growth, and disease suppression in soybean. These strains produced a pronounced increase in relative water content, photosynthetic pigments, membrane stability, proline, antioxidant enzymes status, phytohormones content (Salicylic acid, and Jasmonic acid), and disease suppression in comparison to control plants. *Bacillus clausii* mitigated the disease by 97% with a marked increase in the proline content (73% and 89%), superoxide dismutase (356% and 208%), peroxidase (439% and 138.6%), catalase (255.8% and 80.8%), and ascorbate peroxidase (228% and 90%) activities in shoots and roots, respectively. Infected plants showed an increase in salicylic acid and jasmonic acid content which was further increased with the application of the selected strains to increase resistance against pathogens. To our knowledge, this is the first study showing a rise in salicylic acid and jasmonic acid in *Macrophomina phaseolina* infected plants. These two strains are suggested as a cost-effective, eco-friendly, and sustainable alternative to chemical fungicides. However, there is a need to explore the field testing and molecular mechanisms leading to disease suppression by these strains.

Keywords: PGPB; charcoal rot; *Macrophomina phaseolina*; biocontrol; salicylic acid; jasmonic acid

1. Introduction

Soybean is a nitrogen fixing, eco-friendly annual oilseed crop with 18–23% oil content [1]. About 98% of soybean meal is used for making aquaculture and livestock feeds due to higher protein contents (38–44%). Industrial uses of soybean oil include the manufacturing of printing inks, soaps, insecticides, paints, linoleum, and disinfectants [2].

One of the primary causes of soybean yield loss is fungal diseases which damage >125 million tons of important crops annually [3]. *Macrophomina phaseolina* is an economically important necrotrophic fungus that causes soil- and seed-borne disease in soybean called “charcoal rot” or “dry root rot”. Charcoal rot causes approximately 50% annual yield loss in soybean [4]. This fungus causes disease in ~500 plant species, particularly in arid regions [5]. Remnants of infected plants in the soil incubate the pathogen *M. phaseolina* which subsequently infects the seeds and roots of next crop. It penetrates the surface of roots through germination tubes formed from sclerotia [6]. In the early stages of infection, from 7–42 days, mycelia of fungus perforate in the epidermis of soybean. The name “charcoal” is given to this infection because it blackens the infected tissues with numerous black microsclerotia [4]. Elevated soil temperatures and low moisture content of soil increase the growth of *M. phaseolina* [7]. Early morphological indication of charcoal rot is rolling and wilting of leaves along with their reduced size [6].

To remove or lessen the effect of resistant spores of *M. phaseolina*, farmers apply synthetic fungicides, soil fumigators, and some traditional agricultural processes like sterilizing the plowing machinery. Synthetic fungicides are costly and harmful for the environment, induce resistance in target organisms and bio-accumulate in the food chain, while traditional methods are hard to put in practice. The practice of using growth-promoting bacteria (PGPB) as biocontrol agents against disease causing fungi is a cheaper, eco-friendly, efficacious way to boost plant defense responses and sustainable strategy for agricultural production. Biological control of fungal pathogens by using PGPB has various associated benefits i.e., target specificity, negligible antagonism against beneficial microflora, lower chances of resistance in target organisms, and no bioaccumulation in the environment [8]. These PGPB can play a vital role in supporting the sustainability of agriculture by reducing the yield losses of crops.

Plant growth-promoting bacteria (PGPB) exert their beneficial effects on plants via two different strategies, direct and indirect. Direct strategies include the production of phytohormones, mineralization of organic compounds, mineral nutrients solubilization, and nitrogen fixation. Indirect strategies neutralize or completely prevent the damaging effects of plant pathogens by phytochemicals which increase the natural resistance of plants [9]. For instance, production of antibiotic compounds, the formation of siderophores, production of lytic enzymes, i.e., proteases, phosphatases, chitinases, lipases, etc. [8]. It is also reported that the inoculation of plant roots with PGPB enhances the immune functions of plants before disease onset. This strategy of physiological priming produces local and systemic resistance in combination with faster and strong defense responses [10].

A plant starts to respond to fungal spores in a very short duration of time, i.e., within 30 min after contact. After the recognition of spores, a cascade of defense reactions is activated inside the plant. Resistance gene-dependent responses of plants to fungal pathogens includes a hypersensitive response, formation of reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide radicals, fortification of a plant cell wall, accumulation of salicylic acid and benzoic acid, pathogenesis-related (PR) and other defense-related proteins and secondary metabolites such as phytoalexins [11].

PGPB activates the ROS scavenging system of plants to remove destructing ROS species via an internal enzyme-catalyzed cleaning system. Other responses of plants provoked by PGPB are cell wall composition changes, cell death, and synthesis of antimicrobial compounds [12]. Plants respond to biological agents through induced systemic resistance (ISR). PGPB provokes ISR in plants by efficient roots colonization, increasing accumulation of pathogenesis-related (PR) and other defense-related proteins, secondary metabolites, plant hormones, and antioxidant enzymes against ROS produced by pathogens.

The consistent performance of biocontrol agents is affected by changing climatic conditions. For agricultural sustainability it is essential to discover new microbial strains from a different habitat with the potential to adapt to the changing environment. Therefore, we aimed to explore the biocontrol activity of PGPB strains from hot springs and screened *P. putida* and *B. clasuii* for their biocontrol potential. To the best of our knowledge, the biocontrol potential of *B. clasuii* against charcoal rot disease in soybean (*Glycine max* L.) was unknown so far. In this study, we identified *P. putida* and *B. clasuii* as the potential biocontrol agents to control charcoal rot disease caused by *Macrophomina phaseolina* in soybean (*Glycine max* L.). The findings of the study can lead to the application of the identified biontrol agents to control charcoal rot disease in farmer's soybean fields as an ecofriendly and sustainable alternative to chemical pesticides.

2. Materials and Methods

2.1. Collection of Samples and Isolation of Bacteria

Soil and water samples were collected from Kondus hot spring (Ghanche District, 35°12' N, 76°21' E, >8000 MASL) and Garam Chashma (Chitral District, 36.1113° N, 72.1416° E, 1494 MASL) of Gilgit-Baltistan province of Pakistan. Water samples were obtained from hot springs of Gilgit and Chitral, whereas soil samples were collected from the close vicinities of these hot springs. Temperature and pH of water and soil were noted down at the collection sites before measuring pH, the soil was made free of residual plant roots, pebbles, and stones (Table S1).

Isolation of bacteria from soil and water samples was carried out by following Hassan et al. [11]. Pure cultures of isolates were obtained by using serial dilution and spread plate methods for soil and water samples and incubating them for 48 h at 30 °C. Morphologically distinct bacterial colonies were repeatedly streaked on LB agar plates for their complete purification. Each isolated strain was named and was observed for phenotypic and biochemical characteristics.

Different morphological attributes of a single colony of each strain were observed which include the shape, size, color, margins, opacity, elevation, consistency, and surface of the colony. Each strain was preserved into a sterilized 50% (v/v) glycerol solution and stored in a −20 °C refrigerator.

Bacterial strains were qualitatively evaluated for their plant growth promotion properties. Positive controls for each test were acquired from Applied Microbiology and Biotechnology Lab, COMSATS University, Islamabad.

Chrome azorole S (CAS) agar media developed by Schwyn and Neilands [13] was used for siderophore detection. A loop full of 24 h bacterial cultures was inoculated in the center of CAS agar plates and incubated at 30 °C for 5 d. Bacterial colonies with an orange-yellow-colored halo zone surrounded by green-colored zone were considered positive for siderophore production.

Phosphate solubilization activity was accessed on Pikovskaya (PVK) agar media [14]. A loop full of 24 h old bacterial cultures were inoculated in the center of PVK agar plates and incubated at 30 °C for 5 d. Strains positive for phosphate solubilization produced a clear halo zone around their colonies.

Screening of bacteria for potassium solubilization was done using Aleksandrov agar medium [15]. Bacteria were inoculated in the center of plates with a sterilized loop and incubated for 5 d at 30 °C. Potassium solubilizing strains produce a clear halo zone around their colonies.

Tris-mineral salts medium amended with ZnO was used for the assessment of bacterial zinc solubilization activity [16]. Plates were incubated at 30 °C for 3 d after the inoculation of bacteria with a sterilized loop in the center of plates. Development of a clear halo zone distinguished Zn solubilizers from non-solubilizers.

An alkaline picrate test was used for the evaluation of hydrogen cyanide (HCN) production by bacteria [17]. LB agar medium was amended with glycine and bacteria were streaked on it. A circular piece of sterilized Whatman filter paper no. 1 was flooded with 0.5% picric acid and 2% Na₂CO₃ solution and fixed to the underside of each Petri plate lid. Plates were incubated for 5 d at 30 °C.

Change in the color of filter paper from bright green to yellow-orange, red, or brown-reddish brown indicated positive results with weak, moderate, or strong HCN production ability, respectively.

2.2. Screening of Bacteria for Production of Hydrolytic Enzymes

Pontecorvo's minimal agar medium amended with starch was used for amylase detection by bacteria [18]. Overnight grown bacterial colonies were streaked in the form of a thick line and incubated at 30 °C for 2 d. 1% Lugol solution was dripped on the plates around colonies. The solution was left for 30 min on plates and then drained off. The color of the media turned dark blue on adding the Lugol. The development of a clear halo zone around bacterial streaks confirmed the respective strains as amylase producers.

Cellulase detection was also done by Pontecorvo's minimal agar medium amended with 1% carboxymethylcellulose (CMC) [18]. Bacteria were streaked for cellulase detection in the same way and employed for amylase detection. To detect cellulase production the plates were dripped with 1% Congo red solution for 30 min. After draining the Congo red solution, plates were flooded with 1 M NaCl for 60 min. The development of a clear halo zone around colonies indicated positive results for cellulase production.

Pectinase producing bacteria were identified by culturing bacterial strains on Pectinase screening agar medium (PSAM) according to the same conditions as for amylase and cellulase detection. Lugol (1%) was used as a detection solution. Strains positive for pectinase production produced a clear halo zone around them [19].

Nutrient agar media amended with 1% skim milk powder (skim milk agar) were used to detect protease activity by bacteria [20]. Both solutions, i.e., nutrient agar and skim milk, were prepared and autoclaved separately. These two were mixed before pouring. Bacteria were inoculated with a sterilized loop in the center of each plate and incubated for 2 d at 30 °C. The development of a clear halo zone around bacterial colonies indicated the positive results for protease production.

Clear halo zones produced by bacteria positive for phosphate, potassium, zinc solubilization, siderophore production, and hydrolytic enzymes were measured using the following formula [21].

$$SI = \text{Colony diameter (cm)} + \text{Halo zone diameter (cm)} / \text{Colony diameter (cm)} \quad (1)$$

2.3. Screening of Bacterial Isolates for Antagonism Against the Pathogen

Stock cultures of *M. phaseolina* were acquired from the Applied Microbiology and Biotechnology Lab, Comsats University, Islamabad. Potato dextrose agar (PDA) medium was used for the revival and maintenance of *M. phaseolina*. After fungal inoculation plates were sealed and incubated at 28 ± 2 °C for 7 d to obtain full growth. After careful observation pure cultures were selected for further use [22].

The dual culture technique was employed to access the antagonistic potential of isolated bacteria with slight modifications. Overnight grown bacterial isolates were streaked at the distance of 1 cm from the edge of PDA plates. Fungal mycelia were perpendicularly inoculated at the distance of 3 cm from the bacterial streak with a sterilized needle and incubated at 28 ± 2 °C for 7 d. PDA plates with inoculation of fungus only were used as a positive control. Percentage inhibition of fungal growth by bacteria was calculated by the formula given by Rais et al. [23].

$$PI = C - T / C \times 100, \quad (2)$$

where:

- PI = percent of radial mycelial growth inhibition;
- C = radial growth of the pathogen in the control plate;
- T = radial growth of the pathogen in dual culture.

2.4. In Vitro Experiment

An in vitro experiment was conducted to evaluate the effects of 5 selected antagonists isolates on germination and growth of soybean seeds under induced diseases stress. Plant growth parameters/biomass for all treatments were recorded and compared to select two best strains for pot experiment.

Seeds of soybean variety NARC-II (*M. phaseolina* susceptible) were obtained from NARC, Islamabad. Bacterial strains with efficient antifungal activity were selected for the in vitro experiment conducted in the Applied Microbiology and Biotechnology Lab, COMSATS University, Islamabad. In vitro seed germination assay was carried out to evaluate the germination rate of selected soybean variety, the effect of the pathogen on seed germination and growth, and the response of soybean seeds treated with selected bacterial strains towards the pathogen. A thick layer of sterilized and moistened cotton was placed in between two layers of sterilized and moistened filter papers in the plates. Sterilized water was used to moisten the contents of Petri plates. Treatments of the in vitro experiment included a control group, a fungal control, one seed treatment group for each selected bacterial strain, and a combination of the pathogen with each selected bacterial strain. Three replicates of each treatment were used. The experiment was done in laboratory conditions with an average temperature of 23 °C, photoperiod of 10 h light/14 h dark.

Seeds were decontaminated by being continuously shaken in 70% ethanol for 1 min and then in 4% NaOCl solution for 3 min followed by subsequent washing with sterile water at each step [24]. Seed bacterization at the rate of 10^6 CFU/mL was performed following the method of Zia et al. [22]. Bacterial strains were grown in LB broth for 24 h. Cultures were then centrifuged at 10,000 rpm for 10 min. Sterile double distilled water was added to each pellet in a volume to obtain same optical density (OD) of all strains at 600 nm on spectrophotometer. Sterilized soybean seeds were soaked in the resulting bacterial suspension for 4 h. Ten seeds for each treatment were immediately shifted to the Petri plates and kept in dark for 2 d. Distilled water was used for the watering of growing plants. The number of seeds germinated each day was recorded for 5 d.

Two-week-old plants were subjected to fungal stress following the method of Zia et al. [22] with slight modifications. Gelatin (0.5%) was used for harvesting fungal spores of 7 d old cultures of *M. phaseolina*. Spore density was adjusted to 10^5 /mL of the solution. This suspension (1.5 mL) was sprayed on each plate designated for fungal treatment and was covered with dark-colored plastic bags for 24 h to initiate fungal infection.

Plants were harvested after 4 d of fungal treatment. Plant growth parameters, i.e., root length, shoot length, fresh weight of roots, fresh weight of shoots, dry weight of roots, dry weight of shoots, and leaf area, were recorded. Dry weight was recorded after placing the roots and shoots of plants in oven at 60 °C for 2 d. Out of all bacterial strains used in the Petri plate experiment, two strains with the best disease suppression and plant growth promotion ability were selected for pot experiment.

2.5. Sequencing of 16S rRNA Gene of Potent Biocontrol Strains for Molecular Identification

DNA of potent biocontrol agents (CW1 and CW2) was extracted by standard cetyl trimethylammonium bromide (CTAB) technique of Sambrook and Russell [25]. Amplification of the 16S rRNA gene was done by using the reported primers and conditions as described by Yasmin et al. [24]. After that, amplified products were sequenced by using the commercial service of Macrogen Korea. Edited sequences were submitted to the gen bank based on maximum similarity to the strains already succumbed to the national center of biotechnology information (NCBI).

2.6. Pot Experiment

A completely randomized pot experiment was designed with 6 treatments, each with 3 replicates and each replicate had four plants per pot. The experiment was carried out in the greenhouse of COMSATS University, Islamabad (33.7294° N, 73.0931° E, Average temperature = 23 °C, humidity = 55%,

photoperiod = 10 h light/14 h dark). The soybeans were grown in its growing season (February–April 2019). Plastic pots (12 cm length × 15 cm width) were filled with 1.5 kg of sieved and an autoclaved mixture of sand and soil (1:3). The experimental design was comprised of 6 treatments including T1 = Control without any treatment (sterilized seeds only), T2 = Fungal control (plants subjected to fungal stress), T3 = Seeds bacterized with CW1, T4 = Seeds bacterized with CW2, T5 = CW1 + pathogen, T6 = CW2 + pathogen. Seeds were sterilized and bacterized according to the procedures adopted for the *in vitro* experiment.

Fungal spore suspension with a density of 10^5 /mL of the solution was prepared in distilled water [26]. The sorghum seeds were soaked in fungus culture and incubated for two weeks at 30 °C and mixed into the soil in the pots 4 h before sowing the seeds. The fungal inoculum was also prepared in a 5% gelatin solution and applied as a foliar spray on the 53rd day of germination. Pots were covered with plastic bags for 24 h to initiate fungal infection. Plants were harvested after the appearance of charcoal rot symptoms (7 d after fungal stress application). Before harvesting the number of flowers on soybean plants in all treatments were recorded. All the plant growth and biomass parameters were noted for pot experiments like those for the *in vitro* experiment. The biochemical post-harvest was done as follows.

2.6.1. Relative Water Content (RWC)

The RWC of leaves was measured by the procedure used by Weatherley [27]. Fresh leaves of plants from each treatment were weighed for the fresh weight (FW), turgid weight (TW), and dry weight (DW). Relative water content was calculated by using respective values in the formula:

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100 \quad (3)$$

2.6.2. Analysis of Photosynthetic Pigments

Concentrations of chlorophyll a, chlorophyll b, and carotenoids were determined by following the methods of Shoaf et al. [28]. A leaf sample (0.05 g) was placed in 10 mL of DMSO solution and incubated in a water bath for 4 h at 65 °C. Readings for chlorophyll a, chlorophyll b, and carotenoids were taken at 663 nm, 645 nm, and 480 nm, respectively.

2.6.3. Estimation of Proline Content

Estimation of proline in plant extracts was done by the following protocol described by Bates [29]. Leaves and roots (0.1 g) were separately ground in 1 mL of 80% ethanol. The solution used in the reaction was formed by mixing two solutions (A and B). Solution A was composed of 30 mL of pure acetic acid and 20 mL of distilled water. Solution B was formed by mixing 10 mL of ethanol with 40 mL of distilled water. These two solutions were mixed to make a new solution (C). The 1 g of ninhydrin was added into solution C (solution D). The reaction mixture was composed of 0.5 mL of sample extract and 1 mL of solution D. OD was measured at 520 nm.

2.6.4. Determination of Membrane Electrolytic Leakage

Membrane electrolytic leakage was determined by measuring leakage of electrolytic ions from leaves by the method of Rai [30]. Leaf discs (0.2 g) excluding the midrib were immersed in deionized water followed by immediate recording of initial water conductivity (initial cond.) using a conductivity meter. Tubes were then shaken gently for 3 h by placing in a shaking water bath at 25 °C and conductivity was measured again (cond. 3 h). After boiling in a water bath for 5 min again conductivity was measured to access maximum conductivity (max. cond.) Electrolytic leakage is ideally calculated as membrane stability index (MSI):

$$[(\text{cond. 3 h} - \text{initial cond.}) / (\text{max. cond.} - \text{initial cond.})] \times 100. \quad (4)$$

2.6.5. Analysis of Enzymatic Antioxidants

Preparation of Enzyme Extracts

Enzyme extracts for antioxidant analysis were obtained by grinding 0.5 g of root and shoot samples separately in 5 mL of 100 mM phosphate buffer (7.8) in a pre-chilled mortar and pestle. Ground samples were collected in falcon tubes and then subjected to centrifugation. The resulting supernatant was saved at 4°C for enzymatic antioxidant assays and the pellet was discarded.

Superoxide Dismutase (SOD)

SOD activity was measured by the method of Beyer et al. [31]. Composition of the reaction mixture was 100 µM EDTA- Na_2 , 130 µM methionine, 75 µM NBT, 20 µM Riboflavin, 0.25 mL distilled water, and 0.025 mL enzyme extract. The reference solution contained 2 mL of EDTA- Na_2 , methionine, and NBT mixture and 0.5 mL of riboflavin and enzyme extract each. The reference solution was placed in 100% dark and control and treatment solutions were kept under 15 W fluorescent lamps at 4000 lx to catalyze the reaction. After 20 min change in absorbance due to NBT degradation was measured spectrophotometrically at 560 nm and compared with blank.

Peroxidase (POD)

POD activity was accessed using the method of Vetter [32]. The reaction mixture was prepared by mixing 1.35 mL of MES buffer (pH 5.5), 5 µL H_2O_2 , 0.2 mL of 0.1% phenylenediamine, and 0.1 mL of enzyme extract. In blank mixture, enzyme extract was replaced with 0.5 mL of 0.1 M phosphate buffer with pH 7.

Catalase (CAT)

Method of Mendoza [33] with some modifications was employed for the determination of catalase activity. The reaction mixture was prepared by combining 2.8 mL of potassium phosphate buffer having pH 7.8, 0.1 mL H_2O_2 , and 0.1 mL of enzyme extract. H_2O_2 solution was freshly prepared immediately before use. The decline in absorbance values due to H_2O_2 degradation was recorded spectrophotometrically at 240 nm.

Ascorbate Peroxidase (APX)

Ascorbate peroxidase activity was measured by following the method of Starlin [34] with slight changes. The reaction mixture was made by a combination of 2.7 mL of potassium phosphate buffer (pH 7.8), 0.1 mL of 7.5 mM ascorbic acid, 0.1 mL of H_2O_2 , and 0.1 mL of enzyme extract. H_2O_2 was added as the last ingredient of the reaction mixture. The activity of APX was recorded from the decline in absorbance values due to the oxidation of ascorbate at 290 nm.

2.7. Quantification of Phytohormones

The detection and quantification of the salicylic acid (SA) and jasmonic acid (JA) were done by using the technique of Engelberth et al. [35]. The 1 g of frozen foliage were ground with 10 mL of 50 mM citric acid. Samples were centrifuged at 4000 g for 5 min at room temperature. Thereafter, the solvent was dried using a rotary thin film evaporator (RFE). Then phytohormones were extracted using half the volume of ethyl acetate of the sample four times and were completely dried down using RFE. To avoid loss of volatile compounds, 185 µL of citric acid, trisodium salt (1 M) was added to the final sample in airtight vial through a rubber septum. The samples were analyzed by high-performance liquid chromatography (HPLC) (Agilent 1100) using a C18 column with a mobile phase consisting of methanol acetic acid and water (30:1:70). SA and JA were detected at 280 nm. Detection and quantification of SA and JA were done based on their retention time and peak area regarding standards of respective phytohormones.

2.8. Data Analysis

The variations in the data set were statistically examined by the technique of analysis of variance (ANOVA) using the software Statistix (version 8.1). Three replicates of each treatment were analyzed for standard error. A comparison between the means of each treatment was done using the least significant difference (LSD) at ($p \leq 0.05$) [36]. The Principal Component Analysis was performed based on the correlation matrix using XLstat 2014 (<https://www.xlstat.com>).

3. Results

3.1. Plant Growth-Promoting (PGP) Traits of Bacteria Isolated from Hot Springs

Twenty bacterial isolates were obtained from water and soil samples of hot springs (Table S2). Gram's reaction and colony morphology of isolated strains showed marked visual differentiation in shape, size, margin, color, elevation, opacity, etc. (Table S2). Isolated bacteria were analyzed for their abilities to produce PGP determinants (Table 1). Among all the isolates, 90%, 55%, 30%, 75%, and 70% of isolates were positive for siderophores production, P, K, and Zn solubilization and production of HCN, respectively. The isolate CW2 showed maximum siderophore production, P, K, and Zn solubilization, respectively.

Table 1. Solubilization index produced by bacterial isolates and their HCN producing abilities.

Strain	Siderophore Production Index	Phosphate Solubilization Index	Potassium Solubilization Index	Zinc Solubilization Index	HCN Production Ability
GM1	–	2.32 ± 0.01 ^{ef}	–	–	+
GM2	–	–	–	–	+
GM3	4.45 ± 0.01 ^g	–	–	3.66 ± 0.33 ^g	+
GM4	4.25 ± 0.01 ^g	2.70 ± 0.01 ^d	–	7.91 ± 0.01 ^c	+
GM5	5.67 ± 0.33 ^d	4.11 ± 0.01 ^c	6.70 ± 0.01 ^b	3.12 ± 0.01 ^h	–
GM6	5.27 ± 1.41 ^{ef}	2.25 ± 0.01 ^{ef}	3.15 ± 0.01 ^d	5.15 ± 0.01 ^e	–
GW1	6.46 ± 0.01 ^c	2.46 ± 0.03 ^e	2.33 ± 0.01 ^g	4.07 ± 0.01 ^f	–
GW2	4.44 ± 0.01 ^g	2.25 ± 0.01 ^f	6.43 ± 0.01 ^c	9.33 ± 0.33 ^b	+
GW3	8.29 ± 0.01 ^b	2.32 ± 0.01 ^{ef}	–	3.30 ± 0.0 ^{gh}	–
CM1	5.04 ± 0.01 ^{ef}	–	–	–	+
CM2	4.37 ± 0.03 ^g	–	–	5.54 ± 0.01 ^d	+
CM3	5.40 ± 0.01 ^{de}	2.36 ± 0.03 ^{ef}	–	2.47 ± 0.01 ⁱ	+
CM4	8.63 ± 0.01 ^{ab}	–	–	3.33 ± 0.01 ^{gh}	–
CM5	3.86 ± 0.01 ^h	–	2.67 ± 0.01 ^f	5.33 ± 0.33 ^{de}	+
CM6	4.47 ± 0.01 ^g	–	–	5.17 ± 0.01 ^e	+
CW1	–	–	–	1.58 ± 0.09 ^j	+
CW2	8.67 ± 0.33 ^a	4.44 ± 0.01 ^b	7.65 ± 0.01 ^a	13.46 ± 0.03 ^a	+
CW3	–	2.17 ± 0.01 ^f	–	–	–
CW4	4.94 ± 0.01 ^f	–	–	–	–
CW5	4.36 ± 0.03 ^g	2.18 ± 0.01 ^f	–	9.13 ± 0.03 ^b	+
Positive Control	R2b	GuSM5	GuSM5	GuSM4	SLH4
	5.66 ± 0.33 ^d	4.89 ± 0.33 ^a	2.96 ± 0.01 ^e	5.13 ± 0.03 ^e	+++

Data are depicted as a mean of 3 replicates ± standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$). HCN = Hydrogen cyanide.

3.2. Hydrolytic Enzymes Production and Antagonistic Activity Against *M. phaseolina*

Of all the isolates tested 30%, 50%, 60%, and 45% of isolates were positive for amylase, cellulase, pectinase, and protease production, respectively (Table 2). Only four bacterial isolates (CM6, CW1, CW2, and CW3) were able to secrete all tested enzymes in the culture media. The bacterial isolate CW2 showed the maximum production of amylase, cellulase, pectinase, and protease enzymes in culture media (Figure 1).

Results showed that half of the total tested isolates showed antagonistic potential against the tested pathogen. Bacterial strains with more than 25% percentage inhibition against *M. phaseolina* were selected for further experiments. Selected strains include GM1, GW2, GW3, CW1, and CW2 (Table 2).

Table 2. Solubilization zones produced by bacterial isolates for different hydrolytic enzymes.

Strain	Amylase Production Index	Cellulase Production Index	Pectinase Production Index	Protease Production Index	Percentage Inhibition of <i>M. phaseolina</i>
GM1	2.56 ± 0.01 ^c	2.77 ± 0.01 ^c	3.32 ± 0.01 ^{bc}	3.35 ± 0.01 ^b	31.96 ± 1.14 ^b
GM2	–	–	–	–	–
GM3	–	2.23 ± 0.01 ^f	3.34 ± 0.01 ^b	2.37 ± 0.01 ^e	4.73 ± 0.14 ^h
GM4	–	–	–	–	3.57 ± 0.02 ^h
GM5	–	–	2.65 ± 0.01 ^{ef}	–	4.72 ± 0.03 ^h
GM6	–	–	–	–	–
GW1	–	–	–	–	–
GW2	–	–	–	–	27.12 ± 0.72 ^e
GW3	2.57 ± 0.01 ^{bc}	2.77 ± 0.01 ^c	2.58 ± 0.01 ^f	–	28.80 ± 0.92 ^d
CM1	–	2.23 ± 0.01 ^f	3.33 ± 0.33 ^{bc}	–	–
CM2	–	2.36 ± 0.06 ^e	2.47 ± 0.01 ^f	3.25 ± 0.01 ^{bc}	–
CM3	–	2.77 ± 0.01 ^c	2.89 ± 0.01 ^{de}	2.20 ± 0.01 ^e	–
CM4	–	–	–	–	8.22 ± 0.06 ^g
CM5	–	–	–	–	–
CM6	2.27 ± 0.01 ^d	2.66 ± 0.01 ^d	2.41 ± 0.01 ^f	3.33 ± 0.33 ^b	–
CW1	2.63 ± 0.08 ^b	2.87 ± 0.01 ^b	2.92 ± 0.01 ^{de}	2.63 ± 0.03 ^d	30.26 ± 1.13 ^c
CW2	2.92 ± 0.01 ^a	3.03 ± 0.01 ^a	3.68 ± 0.01 ^a	3.57 ± 0.01 ^a	41.7 ± 0.59 ^a
CW3	2.18 ± 0.01 ^e	2.77 ± 0.02 ^c	3.03 ± 0.01 ^{cd}	3.23 ± 0.01 ^{bc}	12.08 ± 0.68 ^f
CW4	–	–	3.33 ± 0.31 ^{bc}	3.05 ± 0.01 ^c	–
CW5	–	–	–	–	–

Data are depicted as a mean of 3 replicates ± standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

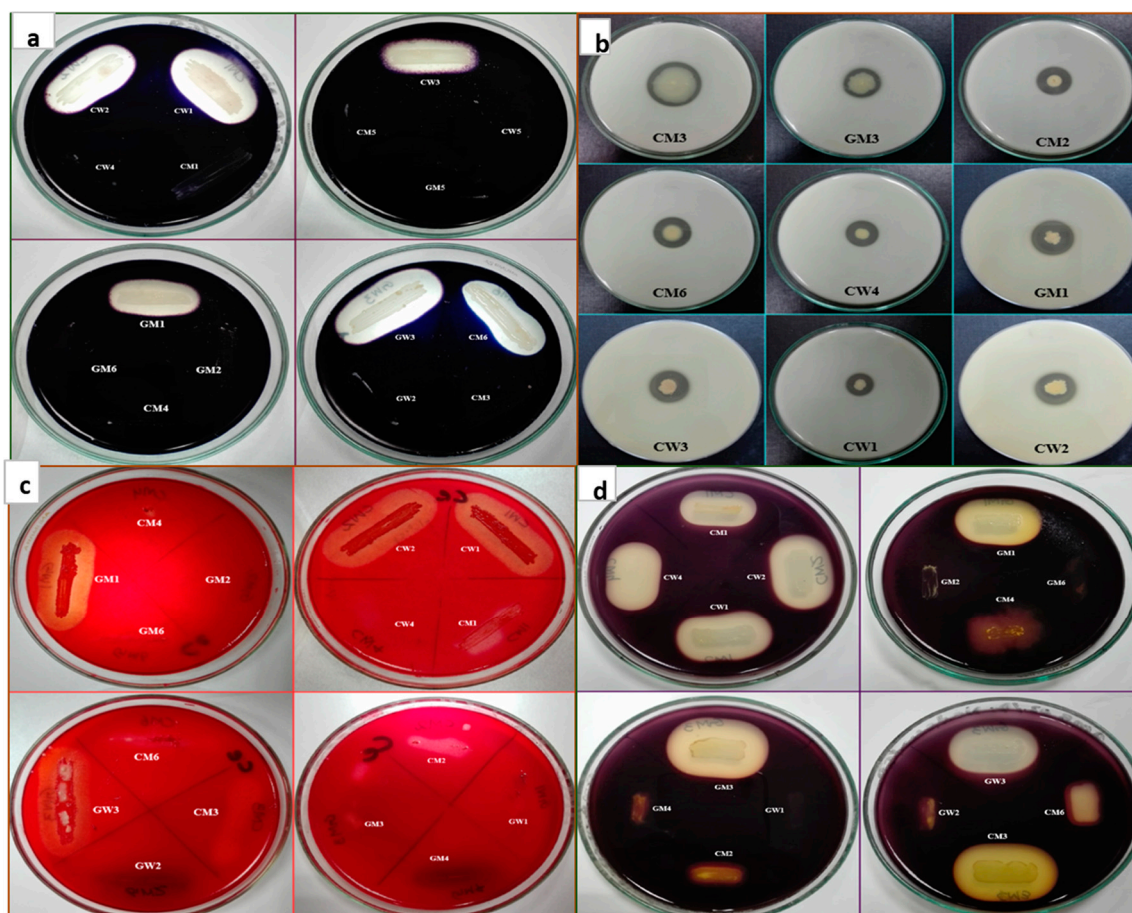


Figure 1. Hydrolytic enzymes production by of plant growth-promoting bacteria (PGPB): (a) Amylase production; (b) Protease production; (c) Cellulase production; (d) Pectinase production.

3.3. Post-Harvest Assays for In Vitro Experiment

Pathogen (*M. phaseolina*) control exhibited a marked reduction of soybean growth. Decrease in root and shoot length, fresh and dry weight and leaf area were observed in infected control compared to non-pathogen control. PGPB strains CW1 and CW2 imparted positive effects on root length, shoot length, fresh and dry weight, and leaf area compared to the untreated control under normal as well as disease stress.

Under stressed conditions, these two strains, PGPB strains CW1 and CW2, imparted a significant increase in length, fresh and dry weight of roots, and shoot of treated non-stressed and infected plants than the untreated control. In infected soybean plants, marked improved biomass was exhibited by the CW2 treated plants compared to the infected untreated control. Marked improved biomass was exhibited by the CW2 treated plants compared to the infected untreated control. The bacterial isolate CW2 performed best as compared to the other 4 PGPB strains and increased the shoot length by 200%, root length by 244%, and leaf area by 360% compared to the pathogen control (Table 3). A similar trend was observed for shoot dry weight and root dry weight, where CW2 showed an increase by 206%, and 134%, respectively, as compared to pathogen control (Table 3). Two isolates, CW1 and CW2, were selected based on their best performance for the pot experiment.

Table 3. Ex vivo analysis of PGPB isolates (CW1, CW2, GW2, GM1, and GW3) on the shoot and root length and leaf area and disease incidence under induced charcoal rot diseases caused by *Macrophomina phaseolina* in soybean (*Glycine max* L.).

Treatments	Shoot Length (cm)	Root Length (cm)	Leaf Area (cm ²)	Disease Incidence	Shoot Dry Weight (g)	Root Dry Weight (g)
Control	12 ± 0.62 ^h	11 ± 0.45 ^h	1.5 ± 0.13 ^g	0	0.74 ± 0.005 ^e	0.63 ± 0.007 ^h
MP	5 ± 0.26 ^j	4.5 ± 0.9 ⁱ	0.5 ± 0.14 ⁱ	75 ± 0.92 ^a	0.32 ± 0.007 ^j	0.32 ± 0.01 ^k
GM1	15 ± 0.92 ^e	12.5 ± 0.22 ^g	1.8 ± 0.08 ^d	0	0.8 ± 0.006 ^d	0.69 ± 0.03 ^g
GM1+ MP	11.7 ± 0.26 ^g	11 ± 0.26 ^h	0.5 ± 0.13 ⁱ	10 ± 0.12 ^d	0.4 ± 0.01 ⁱ	0.44 ± 0.01 ^j
GW2	17 ± 0.41 ^c	14 ± 0.71 ^d	1.6 ± 0.03 ^f	0	0.8 ± 0.01 ^d	0.74 ± 0.02 ^e
GW2+ MP	13 ± 0.57 ⁱ	13 ± 0.9 ^f	0.7 ± 0.05 ^h	12 ± 0.13 ^c	0.44 ± 0.01 ^h	0.43 ± 0.06 ^j
GW3	16 ± 0.26 ^d	13.5 ± 0.26 ^e	1.7 ± 0.13 ^e	0	0.85 ± 0.01 ^c	0.75 ± 0.02 ^d
GW3+ MP	12 ± 0.85 ^h	11 ± 0.49 ^h	0.8 ± 0.01 ^h	15 ± 0.11 ^b	0.56 ± 0.01 ^g	0.47 ± 0.1 ⁱ
CW1	18 ± 0.36 ^b	15 ± 0.55 ^c	2.4 ± 0.05 ^b	0	0.9 ± 0.01 ^b	0.87 ± 0.02 ^b
CW1+ MP	14 ± 0.26 ^g	14 ± 0.26 ^d	2 ± 0.13 ^d	5 ± 0.09 ^e	0.7 ± 0.01 ^f	0.7 ± 0.02 ^f
CW2	20 ± 0.55 ^a	16 ± 0.72 ^a	2.5 ± 0.12 ^a	0	0.8 ± 0.01 ^d	0.96 ± 0.01 ^a
CW2+ MP	15 ± 0.42 ^f	15.5 ± 0.81 ^b	2.3 ± 0.03 ^c	2 ± 0.07 ^f	0.98 ± 0.01 ^a	0.75 ± 0.11 ^c

Data are depicted as a mean of 3 replicates ± standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

3.4. Identification of Potent Biocontrol Agents

The 16SrRNA gene sequence analysis revealed similarity of these two potent biocontrol agents CW1 and CW2 with *P. putida* and *B. clausii*. Results of BLAST of 1529 bp sequence of bacterial isolate CW1 revealed 99% resemblance with *P. putida* (NC_021505.1). However, 1370 bp sequence of CW2 showed 99% close homology with *B. clausii* (NC_006582.1). The accession numbers for PGPB CW1 and CW2 were obtained from NCBI as MT604992 and MT604989, respectively.

Pot Experiment

Based on the results of the in vitro experiment, 2 PGPB strains, i.e., CW1 and CW2, were selected to access the charcoal rot disease suppression effects in a pot experiment. The symptoms of charcoal rot disease development on soybean leaves after 4 d of *M. phaseolina* inoculation include curling, chlorosis, necrosis, and wilting of leaves (Figure 2). Inoculation with PGPB CW1 and CW2 significantly reduced the incidence of the disease in soybean, however, CW2 showed more reduction in disease incidence by 97% as compared to pathogen control.

The number of flowers on pathogen control plants declined by 167% as compared to nonpathogenic control. Flowering first started in CW2 treated plants and pathogen treated plants showed initiation of flowering after all other treatments. A maximum number of flowers were observed on CW2 treated unstressed plants and their number increased by 70% as compared to untreated control. CW2 treated pathogen stress applied plants showed an increase in the number of flowers by 82% compared to the pathogen control (Figure 3).

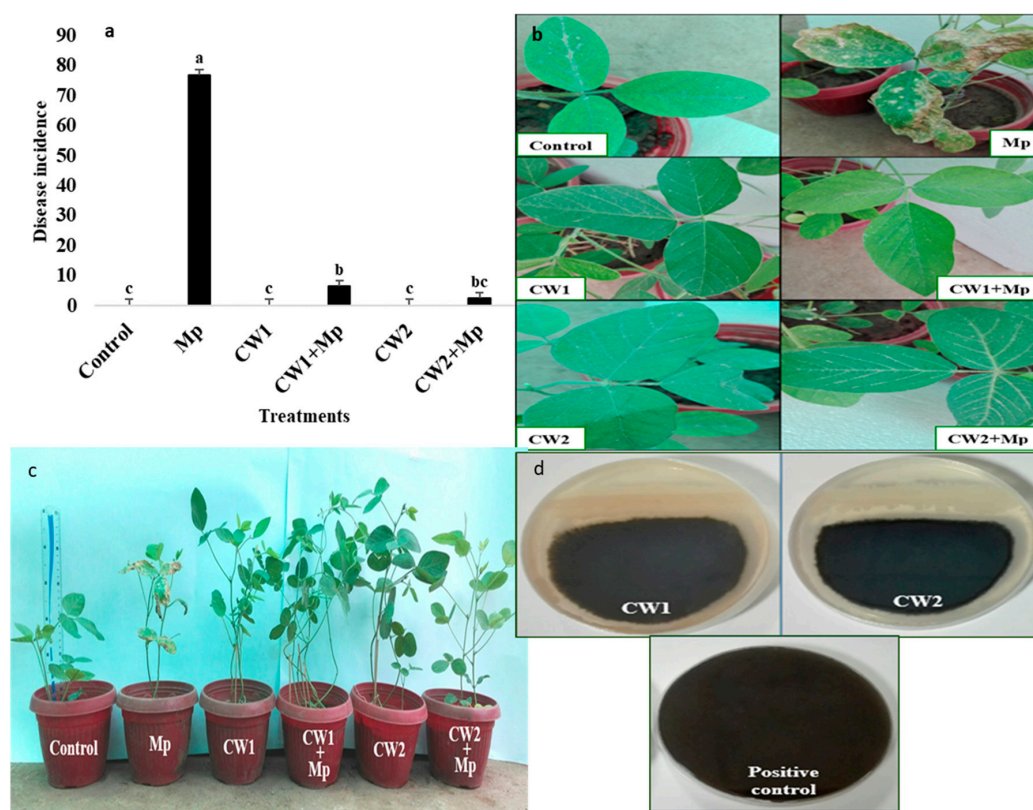


Figure 2. In vitro and in vivo experiments exhibited the potential of *Bacillus clausii* CW2 as biocontrol agents by inhibiting the growth of *Macrophomina phaseolina* and reducing charcoal rot disease incidence in soybean (*Glycine max* L.) plants. (a) Biological control effect of *Pseudomonas Putida* CW1 and *Bacillus clausii* CW2 to charcoal rot disease incidence of soybean (*Glycine max* L.) plants; (b) symptoms of charcoal rot diseases development on soybean leaves after inoculated with *Macrophomina phaseolina*; (c) effect of inoculation of *Pseudomonas Putida* CW1 and *Bacillus clausii* CW2 on soybean leaves after infection with *Macrophomina phaseolina* in a pot experiment; (d) The antagonistic potential of screened PGPB isolates against *Macrophomina phaseolina*.

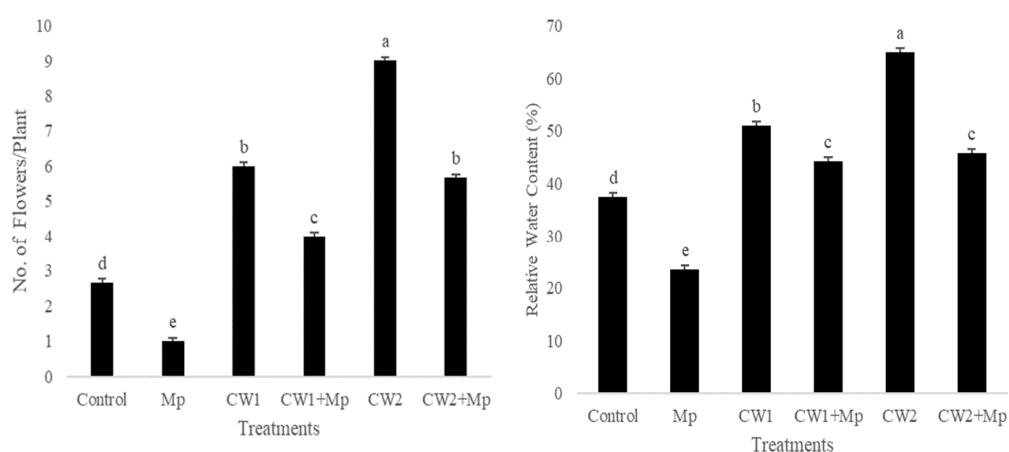


Figure 3. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2, on number of flowers and relative water content of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

A decrease of RWC values by 59% was seen for pathogen control in comparison to unstressed control. Both PGPB strains CW1 and CW2 manifested a significant increase in RWC values for treated plants under unstressed and pathogen stress conditions. Both strains also performed efficiently under pathogen stress and increased the RWC by 46% for CW1 and 48% for CW2 treated plants than the pathogen control (Figure 3).

Infected plants showed marked reduction in synthesis of photosynthetic pigments i.e., chlorophyll a, b, and carotenoids by 33%, 35%, and 28%, respectively as compared to non-pathogen control (Figure 4). Treatment of soybean seeds with PGPB strain CW2 manifested a significant increase in the chlorophyll a, b, and carotenoids content under non-stressed conditions that increased by 114.3%, 114% and 28.5% as compared to untreated control. Infected plants also showed increased values for chlorophyll a, b, and carotenoids content by treatment with CW2 that increased by 146%, 171%, and 38%, respectively, in comparison with infected control.

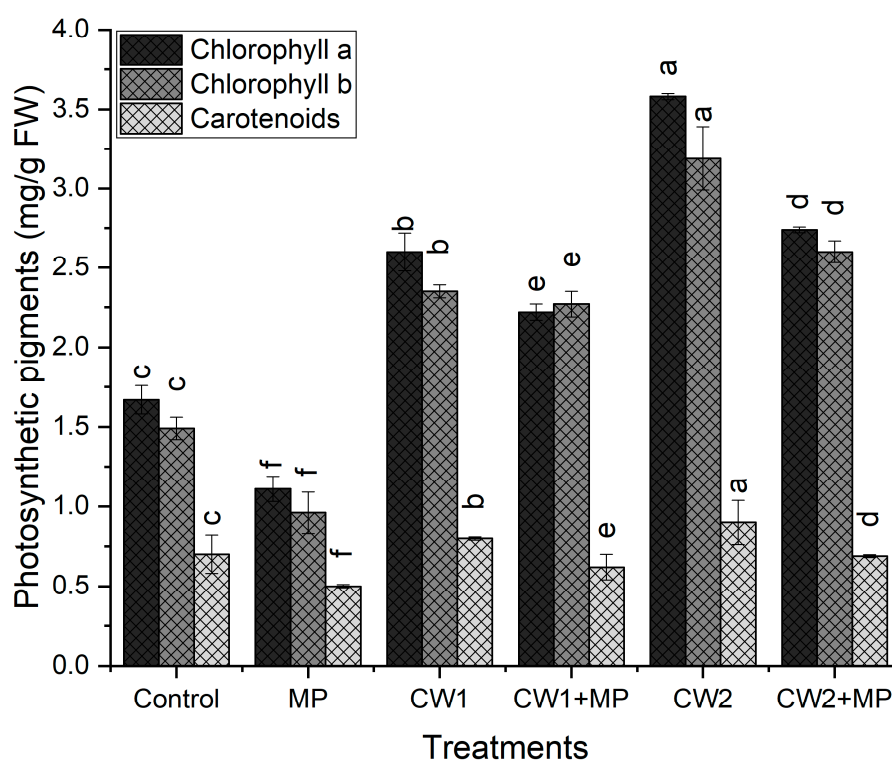


Figure 4. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2, on photosynthetic pigments of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

Proline function in the enhancement of soybean defense responses. Soybean plants infected with *M. phaseolina* showed an increased production of proline in shoots by 201% and in roots by 193% as compared to the healthy control (Figure 5). Inoculated treatments showed an increase in proline content of shoots and roots range from 114–211% and 180–206% in healthy plants. However, in case of pathogen stress, inoculated treatments showed a marked rise in proline content in shoots, and roots range from 40–73% and 51.6–89%, respectively than the infected control. Infected plants treated with PGPB CW2 showed a maximum increase in proline content of shoots and roots by 73% and 89%, respectively, compared to the pathogen control.

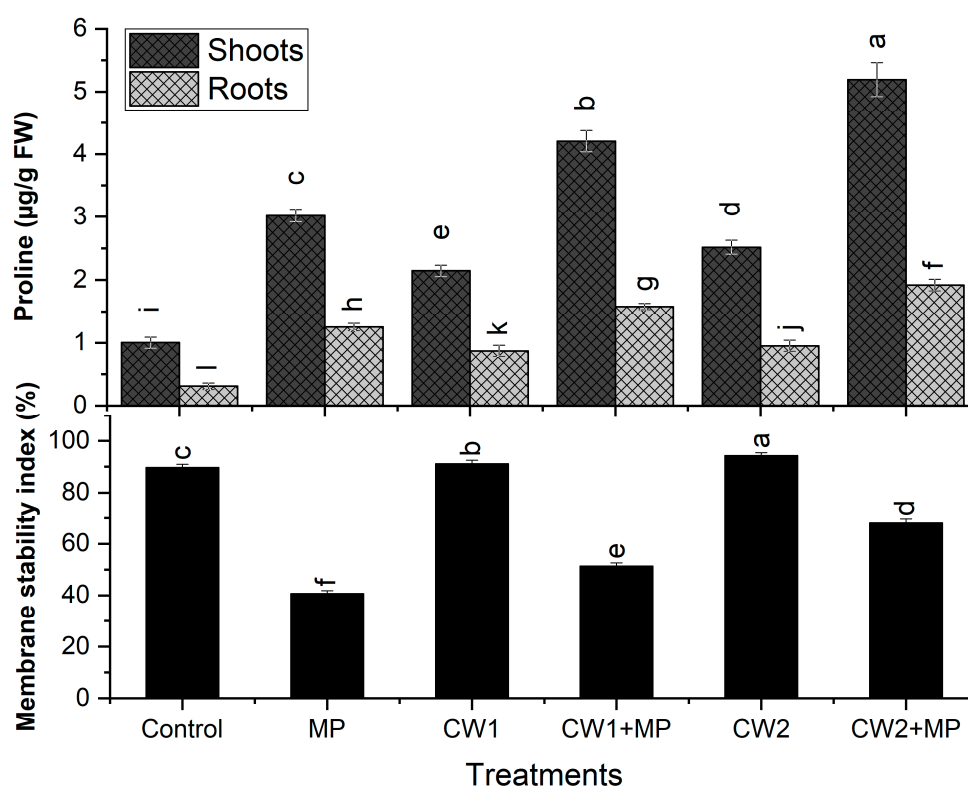


Figure 5. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2 on proline and membrane stability index of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

Membrane stability index (MSI) was determined to access the extent of damage caused by the pathogen to plant cell membranes. The minimum value of MSI was observed for pathogen treated plants showing the enhanced degree of damage caused by the pathogen to plant cell membranes. The bacterial isolate CW2 treated unstressed plants showed an increase of 5.1% in MSI as compared to control. However, under a stressed condition, CW2 treated plants showed a maximum increase in MSI values by 67% as compared to pathogen control (Figure 5).

Superoxide Dismutase (SOD)

A marked rise in superoxide dismutase (SOD) activity in shoots and roots of diseases plants by 110% and 66%, respectively, was observed as compared to the control. All the inoculated treatments showed an increase in SOD activity in shoots and roots ranging from 40–63% and 44–74% in non-stressed plants as compared to the non-treated control. However, in infected plants, PGPB treatments showed a further increase in SOD activity range from 220–356% and 165–207% in shoots and roots of soybean, respectively. Figure 6 represents the maximum increase in the SOD activity by CW2 treated stressed plants in shoots and roots by 356% and 208%, respectively, in comparison to pathogen control.

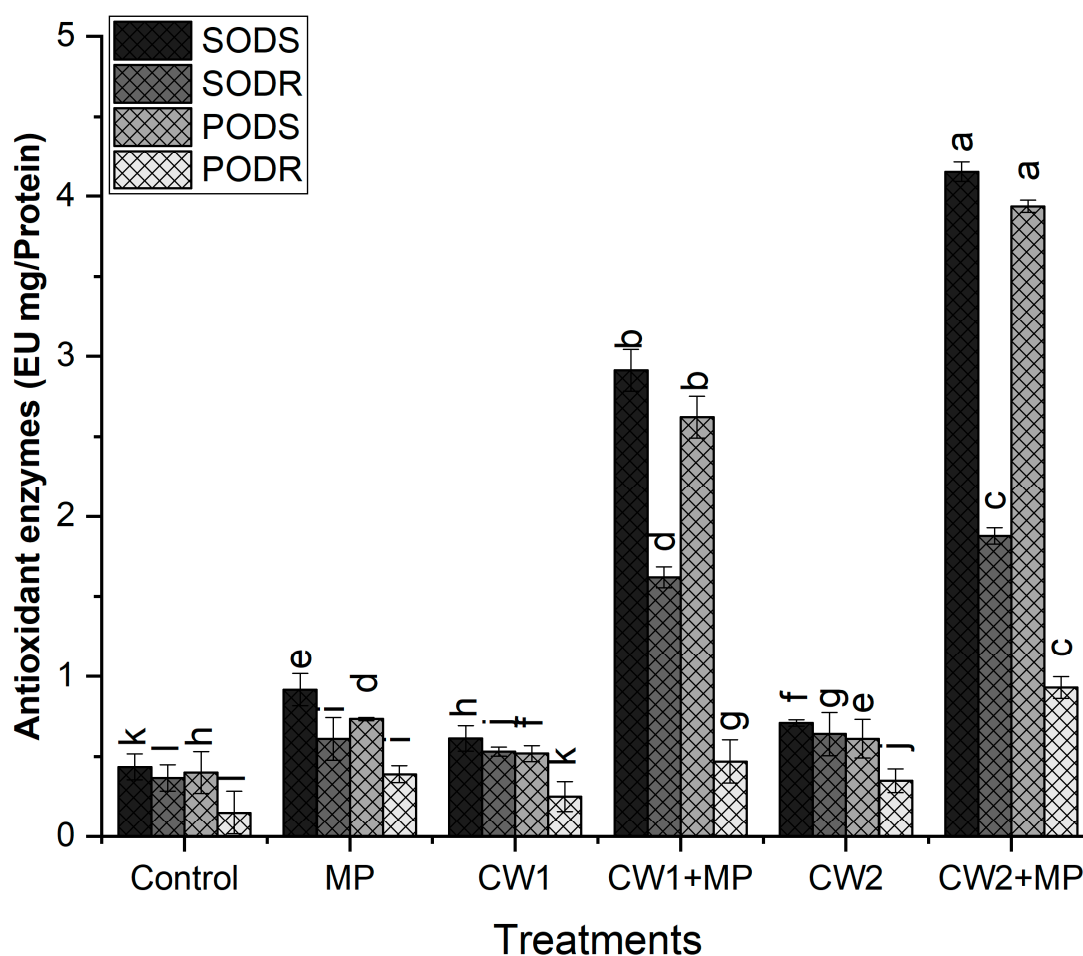


Figure 6. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2, on superoxide dismutase (SOD) and peroxidase (POD) activity of roots and shoots of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Whereas SODS and SODR is SOD activity in shoots and roots, PODS and PODR represent peroxidase activity of shoots and roots, respectively. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

Infected soybean plants showed a clear upsurge in peroxidase (POD) activity in shoots and roots by 82.4% and 160%, respectively, as compared to the healthy control. All the inoculated treatments showed an increase in POD activity in shoots and roots ranging from 29–51% and 67–133% in non-stressed plants as compared to the non-treated control. However, in infected plants, PGPB treatments showed a further increase in POD activity range from 259–439% and 20–138.6% in shoots and roots of soybean, respectively. The maximum increase in the POD activity by CW2 treated stressed plants in shoots and roots (439% and 138.6%, respectively) was observed as compared to pathogen control (Figure 6).

The catalase (CAT) activity of shoots and roots of infected soybean showed a marked increase by 59% and 72% respectively, as compared to healthy control. All the inoculated treatments showed an increase in CAT activity in shoots and roots ranging from 5–15% and 20–34.6% in non-stressed plants as compared to the non-treated control. However, in infected plants, PGPB treatments showed a further increase in CAT activity ranging from 199–255.8% and 43–80.8% in shoots and roots of soybean, respectively. The maximum increase in the CAT activity by CW2 treated stressed plants in shoots and roots was by 255.8% and 80.8%, respectively, in comparison to pathogen control (Figure 7).

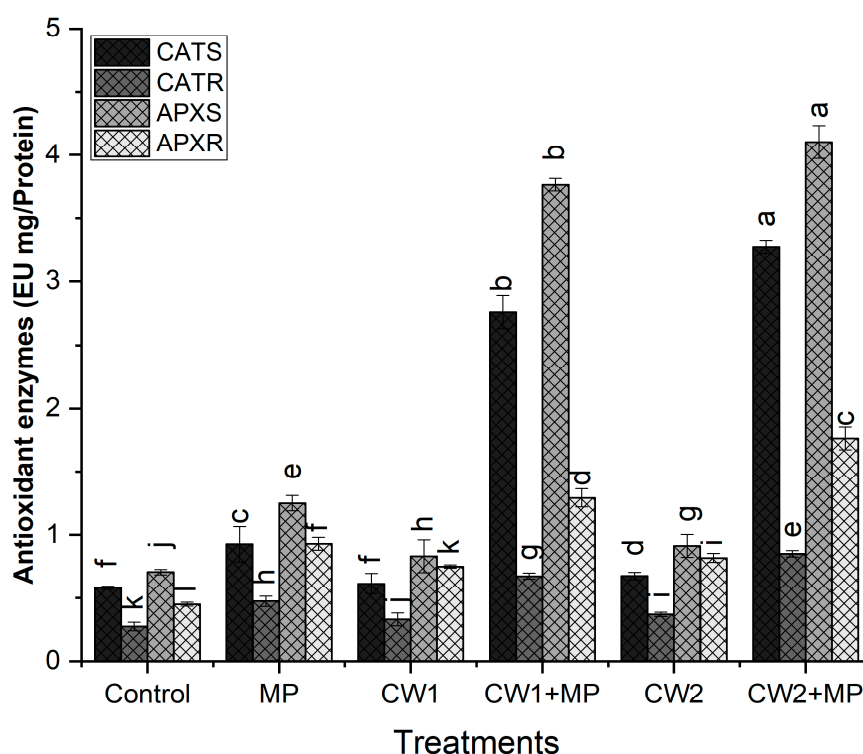


Figure 7. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2 on catalase (CAT) and ascorbate peroxidase (APX) activity of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Whereas CATS and CATR represent catalase activity of shoot and roots, and APXS and APXR represent ascorbate peroxidase activity of shoot and roots, respectively. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

Soybean plants infected with *M. phaseolina* showed an obvious rise in ascorbate peroxidase (APX) activity in shoots and roots by 77.3% and 106.4%, respectively, as compared to the healthy control. All the inoculated treatments showed an increase in APX activity in shoots and roots ranging from 17–29.4% and 66–82% in non-stressed plants as compared to the non-treated control. However, in infected plants, PGPB treatments showed a further increase in APX activity range from 201–228% and 39–90% in shoots and roots of soybean, respectively. Figure 7 represents the maximum increase in the APX activity by CW2 treated stressed plants in shoots and roots by 228% and 90%, respectively, in comparison to pathogen control.

An increase of 241% and 233% in salicylic acid (SA) and jasmonic acid (JA) content was observed as compared to healthy control. All the inoculated treatments showed an increase in SA and JA content ranging from 258–257% and 273–300%, respectively, in non-stressed plants as compared to the non-treated control. However, in infected plants, PGPB treatments showed a further increase in SA and JA content (50–60%) of soybean plants, respectively. The maximum increase in the SA and JA content was exhibited by CW2 inoculated infected plants by 75% and 60%, respectively, in comparison with pathogen control (Figure 8).

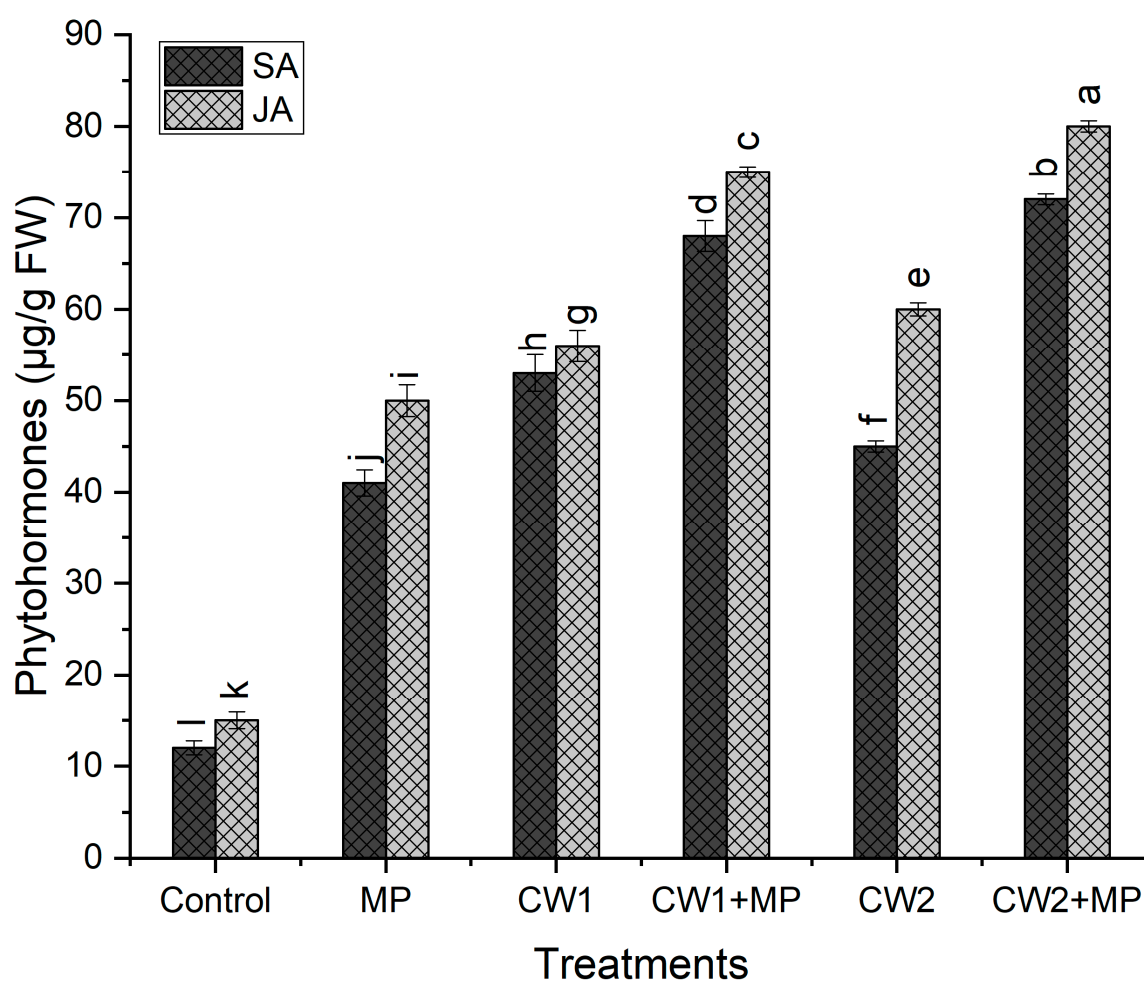


Figure 8. Effect of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2 on salicylic acid (SA) and jasmonic acid (JA) of soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. Data are depicted as a mean of 3 replicates \pm standard error of means. Different letters indicate a statistically significant difference between treatments ($p \leq 0.05$).

Principal Component Analysis (PCA)

Patterns of variation and dependencies among individual parameters were assessed using the correlation matrix-based principal component analysis (PCA). The PCA transformed the eleven parameters into a smaller number of principal components (PCs) and categorized them into groups.

Out of the 5 principal components PCs, the first two PCs (PC1 and PC2) were statistically significant with eigenvalues > 1 and accounted for 69.8% and 25% (commutatively 94.8%) variation, respectively (Figure 8). Thus, PCA summarized 94.8% variation of the 14 parameters in two PCs and categorized them based on their mutual relationship (Figure 9). All parameters except MSI, carotenoids, chlorophyll a and chlorophyll b were more related to PC1 showing the positive relationship among themselves. The parameters MSI, carotenoids, chlorophyll a, and chlorophyll b were more related to PC2. The parameters JA, SA, proline (roots), and proline (shoot) were highly correlated with each other and were more influenced by the treatment CW2 + MP. The parameters SODR, SODS, APXR, APXS, CATR, and CATS grouped and were more affected by the treatment CW1 + MP.

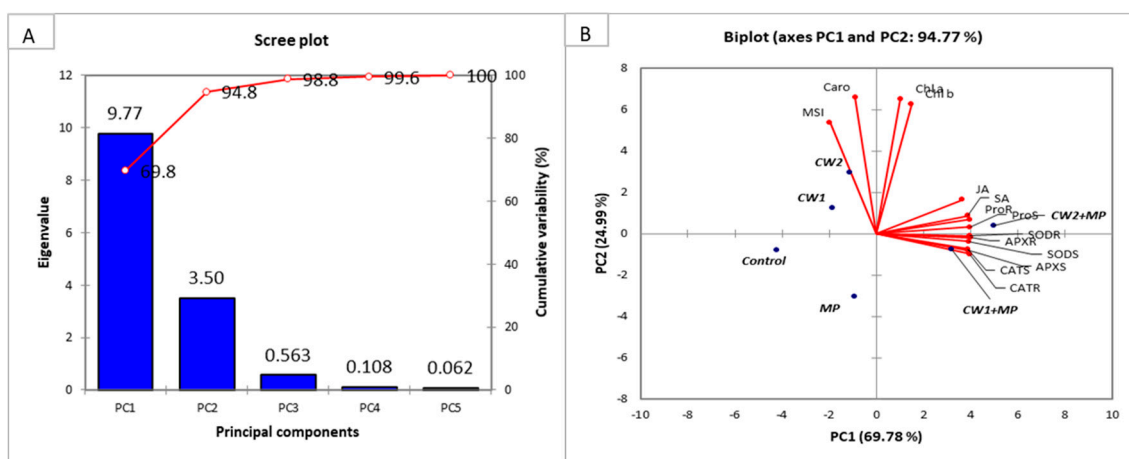


Figure 9. Principle component analysis (PCA) based on photosynthetic pigments, stress determinants, and antioxidant enzymes of *Pseudomonas Putida* CW1, *Bacillus clausii* CW2 treated soybean (*Glycine max* L.) under induced charcoal rot diseases caused by *Macrophomina phaseolina* in a pot experiment. (A) Scree plot showing the reduction of 14 parameters into 5 PCs and their eigenvalues (B) Biplot showing parameter distribution between PC1 and PC2.

4. Discussion

In our study, we isolated bacterial strains from hot springs, screened them for plant growth promotion properties, and applied them on charcoal rot susceptible soybean variety NARC-II to observe their effects on disease control by modulating ROS scavenging system in soybean plants. Plant growth promoting bacteria (PGPB) isolated from hot springs and their vicinities are reported to be efficient plant growth-promoting agents [37,38]. In this study, 20 bacterial strains were isolated. Diverse morphologies of our isolated strains indicated that extreme environments like hot springs harbor distinct types of bacteria which may prove to be efficient plant growth promoters.

Metabolites produced by PGPB have an intrinsic relationship with their antagonistic potential. *P.putida* (CW2) produces all tested metabolites P, K, Zn, siderophores, and hydrolytic enzymes (amylase, pectinase, protease, chitinase) while *B. clausii* (CW1) was positive for potassium and zinc solubilization and siderophore production. Siderophores are ferric ions binding proteins secreted by PGPB as a mechanism of biocontrol against fungi by causing deficiency of iron for them, hence restricting their advancement of spore formation, change of fungal cell morphology, and decline in nucleotide synthesis [13,39]. Zinc is an important micronutrient as it is required for proper health and chlorophyll content of plants. Here, 75% of our isolated strains were positive for the solubilization of zinc which showed their potential for making zinc available for plants when they are facing zinc deficiency as indicated by different studies showing that zinc content of PGPB inoculated plants increased as compared to non-inoculated plants [16,40]. Phosphorous is the most important nutrient for plants after nitrogen and participates in all vital processes in plants. Among our isolated bacterial strains, 55% were phosphate solubilizers which indicates that extreme environments can also serve as good sources of biotic plant growth promoters as indicated by previous studies [41,42].

Production of hydrolytic enzymes by bacteria makes them efficient disease suppressors when they colonize the roots or vicinities of plant roots. Among all the isolates tested for production of pectinase, 60% of them were positive. Pectic enzymes are important for plant growth as they play a role in the elongation and growth of plant cells along with the ripening of fruits [43]. Among our isolated strains, 45% were positive for producing protease enzymes. This bacterial protease can boost the immune system of plants and allow them to withstand stress conditions. Proteases are the enzymes that play their role in various important processes of plants such as development, reproduction, growth, immunity, photosynthesis, and programmed cell death [44,45]. We found that 30% of tested isolates were amylase producers. The importance of the amylase enzyme for plants is well established [46].

This enzyme plays a key role in the mobilization of starch in developing plant seeds. Bacterial amylase may help plants to utilize their starch contents more effectively.

Subsequently, cellulase is another important enzyme detected in 50% of our bacterial isolates. Cellulase producing bacteria can be efficient litter degrading organisms and besides that, they can restrict the growth of fungal pathogens [47]. The possession of plant growth promotion traits in addition to the production of hydrolytic enzymes makes these bacterial strains promising disease suppressors and plant growth enhancers [20,45].

We found that the effects of *B. clausii* regarding plant biomass, photosynthetic pigments, antioxidant status, phytohormone production, and disease suppression were more pronounced as compared to that of *P. putida*. Soil- or seed-borne diseases substantially reduce the biomass of plant roots which results in impaired nutrient and water uptake from soil resulting in stunted growth and plant death [48]. In vitro and greenhouse testing of our newly isolated strains, *P. putida* and *B. clausii* produced prominent results regarding the root length of plants in the case of nonpathogenic conditions as well as in case of fungal stress on plants thereby increasing plant survival rates. Similar results were reported in many studies by the application of PGPB in stressed and non-stressed conditions in soybean and other plants [6,49].

Both bacterial strains increased the biomass of soybean. Similar results are reported in various studies concerning the positive effects of PGPB on stressed and unstressed plants [23,50,51]. The positive results for root and shoot weights are correlated with the previous studies involving the exploitation of PGPB to reduce disease severity and at the same time strengthen their growth parameters [6,23,51]. Adequate leaf area for plants facilitates the absorption of sunlight and results in the promotion of photosynthetic processes in plants to produce an enhanced amount of photosynthetic products which increase the degree of well-being for plants. In the present study, an increase in inoculated soybean biomass, leaf area, as well as photosynthetic pigments, was notable in both in vitro and natural environmental conditions by treatment of plants with *P. putida* and *B. clausii* in stressed and non-stressed circumstances. PGPB are well-studied for their biocontrol and plant foliar mass improvements as confirmed by previously published literature [52,53]. Photosynthesis is the vital process for plant life which is badly disturbed due to fungal infection as a result of damage to plant photosynthetic pigments and enzymes causing chlorosis and necrosis. However, treatment of soybean seeds with PGPR strains increased the concentration of photosynthetic pigment in stressed and unstressed plants as indicated by already published literature for biotic stresses [54].

During the pot experiment, flowering was induced earlier in our PGPB treated unstressed and stressed plants in comparison to non-pathogen and pathogen controls. Similar positive results of PGPB on the flowering of strawberry and mango plants were reported [55,56]. Infection of soybean plants with *M. phaseolina* cause wilting as a primary disease symptom due to abundant formation of fungal microsclerotia which block the water transport passages of plants. The relative water content (RWC) of leaves is measured to determine the hydration status of leaves as compared to a maximum water holding potential of leaves. It represents the intensity of biotic or abiotic stresses on plants which cause water deficit and also depicts the degree of disease severity. We found that the RWC content of *B. clausii* treated plants was higher in stressed and non-stressed conditions which indicates that this strain was able to limit the advancement of a fungal pathogen in the plant body. Moreover, it also helped the plants to retain the water content necessary for proper functioning as indicated by previous studies regarding RWC of plants subjected to various types of stresses [24,57].

Membrane electrolytic leakage is a measure of the damage caused by stress to plant cell membranes. Electrolytic leakage is the function of the permeability of plant cellular membranes. Higher values for electrolytic leakage are an indication of increased membrane permeability due to damage caused by a stress [58]. In our study, the highest values for membrane electrolytic leakage were observed for pathogen control. However, electrolytic leakage values for *P. putida* and *B. clausii* treated plants were lower as compared to stressed and non-stressed controls indicating the initiation of disease suppression mechanisms in plants under stressed conditions and improving the functioning of plants under non-stressed conditions. Our findings are correlated with previous findings of Dixit et al. [59]

who found reduced electrolyte leakage was correlated with resistance of *Arabidopsis* plants to drought and *P. syringae*.

Proline is an amino acid that plays a beneficial role in plants against biotic and abiotic stresses. It is an efficient osmolyte and besides this, it also plays other significant roles in stressed plants, i.e., it acts as an antioxidant molecule, signaling molecule, and a metal chelator [24,60]. The results for the proline contents of charcoal rot stressed plants were more pronounced in the case of *B. clausii* treated plants as compared to that of *P. putida*. The findings are in line with [61] who reported the interaction of spermidine and proline to induce tolerance in pepper plants against root rot disease.

Plants respond to biological agents through induced systemic resistance (ISR). PGPB provoke systemic resistance in plants in response to pathogens by initiating production of ROS scavenging enzymes and defense-related phytohormones [8,12,51,62]. Non-enzymatic antioxidants such as proline and enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) take part in ROS scavenging. SOD acts as the first line of defense in stressed plants as its catalysis breakdown of superoxide radical into hydrogen peroxide and molecular oxygen [23]. We found that PGPB strain *B. clausii* showed maximum increased synthesis of SOD in fungal stressed plants which efficiently disintegrated superoxide radicals into their components and reduced disease incidence by 97% as compared to infected control. Many research articles reported the increase in SOD activity after treatment of plants with PGPB infected with a pathogen which provides similar results as we obtained in our study [12,23,51].

The hydrogen peroxide formed by SOD during the disintegration of superoxide radicals is degraded into oxygen and water by the CAT enzyme. *B. clausii* increased the production of CAT in plants under stress conditions. Our findings proved that PGPB can induce activated antioxidant activities in stressed plants to reduce damage caused by diseases. Similar effects of PGPB on catalase in diseased plants are reported [6]. The role of POD and APX is mainly the degradation of hydrogen peroxide radicals; their activities were boosted in stressed plants as compared to control substantially by *P. putida* as compared to *B. clausii* whose effect was less pronounced; these results are same as indicated by different studies on various plant stresses involving the use of different PGPB [6,12,23,51].

Our results demonstrated that the quantity of SA and JA showed a pronounced rise in *M. phaseolina* infected soybean plants. To the best of our knowledge, this is the first report showing a rise in SA and JA in *M. phaseolina* infected plants. The SA and JA played an important role in plant defense against charcoal rot diseases in soybean. This is an important finding in the understanding of the positive interaction of phytohormones with antioxidants to enhance the resistance of soybean plants against *M. phaseolina*. This is consistent with what was found in the previous finding of Agarwal et al. [62] who reported upregulation in the expression of SA, antioxidant (SOD and CAT), and pathogenesis-related genes (PR-1) in transgenic tobacco infected with *M. phaseolina*. Our results go beyond previous studies, showing that inoculation with *P. putida* and *B. clausii* exhibited further increase in SA and JA content which confer resistance to soybean plants against charcoal rot disease. A positive correlation was observed between antioxidant enzymes SOD, POD, CAT, APX, and phytohormones SA and JA as a complex interrelation of biochemical processes involved in the resistance of plant species. Figure 9 represents the PCA analysis which showed a 94.8% variation of the 14 parameters in two PCs. The PCA analysis showed that parameters JA, SA, proline (roots), and proline (shoot) were also highly correlated with each other and were more influenced by the treatment CW2 + MP. The parameters SODR, SODS, APXR, APXS, CATR, and CATS grouped together and were more affected by the treatment CW1 + MP. Gupta [63] reported that different phytohormones showed complicated convergent and divergent responses to combat single and multiple stresses in plants. Overall, PGPB-mediated systemic resistance in soybean is the result of combined effect of phytohormones, antioxidants, and osmolytes that decrease the disease incidence and improve the resistance and growth of plants.

5. Conclusions

In conclusion, the newly isolated strains *P. putida* and *B. clausii* produced induce systemic tolerance against *M. phaseolina* by activation of several defense mechanisms in soybean plants. However, our results provide a rationale to understand the biocontrol agent-mediated complex interaction between antioxidant enzymes, osmolytes, phytohormones, and photosynthetic pigments to elicit charcoal rot disease incidence in the soybean plant. Still, their effects are required to be further explored for a complete understanding of their positive effects on plants for sustainable disease control measures. After robust field trials, the identified biocontrol agents for controlling charcoal rot disease in soybean could be used as cheaper and ecofriendly biopesticides leading to sustainable agricultural practices.

Supplementary Materials: The supplementary materials are available online at <http://www.mdpi.com/2071-1050/12/17/6856/s1>.

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