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Serratia marcescens promotes growth and induces systemic resistance in *Glycine max* L.

Maitry R. Prajapati[1], Vasudev R. Thakkar[1*], Ramalingam B. Subramanian[1]

Post Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India.

***Corresponding Author:**
Prof. Vasudev R. Thakkar
E mail: vr_thakkar@spuvvn.edu,vasuthakkar@gmail.com
Phone: +91-2692-234402, 940 803 4512
Fax: +91-2692-231041

ABSTRACT

Plant Growth Promoting Rhizobacteria, one of the best alternatives of chemical fertilizers, induce broad-spectrum, systemic resistance in plants against biotic stress. Glycine max L. is a nutritionally important legume affected by several pathogens. The Fusarium species causes severe yield loss of Soybean around the world. We report here plant growth-promoting characteristics of a bacterium (MP3), isolated from the rhizosphere of Soybean. Isolate MP3 improved the percentage of germination of uninfected as well as F. oxysporum infected Soybean seedlings from 36.66 % to 56.44%. The treatment of MP3 isolate also increased the dry weight of fungal infected plants from 0.453 g to 0.929 g and number of leaves from 12.11 to 24.36 as measured after 35 days of growth. As MP3 promoted the growth of infected plants also, elicitation of resistance was evident, which was corroborated by finding increased activities of the defense-related enzymes in germinated seedlings. MP3 was identified by biochemical and molecular characterization as Serratia marcescens.

Keywords: Serratia marcescens, Soybean plants, Fusarium oxysporum, ISR

INTRODUCTION

Soil microorganisms play important roles in biogeochemical cycles by decomposing organic matter for their nutrition acquisition and metabolism and contribute to soil fertility. In rhizospheric soil, microbes thrive as free as well as symbiotic in nature, some of which help the plants to grow better are called Plant Growth Promoting Rhizobacteria (PGPR). They are also known as Plant Health Promoting (PHPB) Bacteria (1). PGPR promotes the growth of plants by direct and indirect mechanisms. Direct mechanism includes production of phytohormones such as IAA, Gibberellins, Cytokinins, and Abscisic acid, solubilization of phosphate, potassium, zinc, etc. and fixation of atmospheric nitrogen. The indirect mechanism includes production of Siderophores, competition of nutrients, and biocontrol (inhibiting the growth of pathogen). PGPR also elicits immune systems in plants, known as Induced Systemic Resistance (ISR), which is different from the resistance developed in plants upon pathogenic attack, called Systemic Acquired Resistance (SAR), however both types of resistance activate pathogenesis-related proteins (PR proteins). Since many types of PR proteins are produced by plants, they had been initially classified into five families, PR1 to PR5, and later many were added,

up to 16 and more. Commonly expressed PR proteins in plants include PR-2, PR3 & PR4, which are hydrolytic enzymes involved in the degradation of the cell wall of pathogens like β 1-3 glucanase, Chitinase, etc. (2). The major pathways that are activated in plants for the defence purpose include Phenylpropanoid, Lipoxygenase (LOX), Sesquiterpene, Oxylipin, etc. Estimating the activities of enzymes involved in these pathways provide an estimate of the activation of defence mechanism in plants. Some of these enzymes are activated by the pathogenic attack, for example, LOX, PO, and PPO are activated during the response of plants upon pathogen ingress, called hypersensitive response (HR). Phenyl propanoid and oxylipin pathways also lead to production of antimicrobial compounds (3, 4).

Soybean (*Glycine max L.*, family Fabaceae), also known as "Golden bean", is a versatile crop having applications in food, feed, and other industries. Globally it contributes 25% in vegetable oil production. It also provides about two-thirds of the world's protein concentrate for livestock feeding and it is a valuable ingredient in formulated feeds for poultry and fish. Annually about 85% of the world's soybeans are processed into meal and oil. Soybean plants are affected by many diseases, such as seed diseases, seedling diseases, soybean cyst, sudden death syndrome, *Fusarium* root rot, brown spot, etc. (5). The pathogenic organisms include *Alterneria spp., Cercospora spp., Fusarium spp., Cladosporium, Phomopsis spp., Rhizoctonia spp., Pythium spp, Heterodera glycines,* and *Septoria glycines* (5, 6, 7). Bacteria earlier reported as PGPR for Soybean are *Azotobacter, Serratia, Azospirillum, Bacillus, Caulobacter, Chromobacterium, Agrobacterium, Erwinia, Flavobacterium, Arthrobacter, Micrococcus, Pseudomonas,* and *Burkholderia* (8).

Local Soybean varieties (grown in Gujarat region) are Gujarat soybean 1 & 2, JS – 335. The major pathogen causing seed and seedling diseases as well as root rot locally is *F. oxysporum*. In the present work, we have studied the efficacy of a PGPR (MP3) on growth and activation of the defense system in Soybean plants against *F. oxysporum*.

Materials and Methods

Fusarium oxysporum was procured from MTCC, Chandigarh, India. The Fungus was grown and maintained in Potato Dextrose agar medium. Isolate MP3, previously isolated from the rhizospheric soil of *Glycine max* L., was maintained in our lab on King's B agar medium. Soybean variety JS-335 was purchased from a local agro shop, Anand, Gujarat (India).

Effect of MP3 isolate on germinated seedlings of *Glycine max* L.

Germination assay was carried out by the method described earlier (9). Soybean seedlings were provided four treatments, A – control (No

treatments), B – treatment with bacterium isolate, MP3 C – challenged by *F. oxysporum,* and D – bacterium treated as well as fungal infected.

Three hundred seeds of soybean were washed in sterile distilled water three times followed by disinfection by 0.1% Mercury chloride for one minute. Seeds were again washed with sterile distilled water to remove the excess of mercury chloride on the surface of seeds and divided into two parts. One part (150 seeds) was treated with MP3 bacterial suspension containing 1×10^8 cells/ml and kept aside for two hours to coat with MP3 bacteria. The rest of the seeds, again parted into two. One (75 seeds) was imbibed in sterile distilled water which served as control and the rest (75 seeds) were infected with *F. oxysporum* spores (1×10^4 spores/ ml).

Half quantity (75 seeds) of dried seeds (MP3 coated) were transferred to a fresh beaker and added with fungal spore suspension to challenge them. Seeds were incubated for one hour followed by drying for another hour.

Five seeds of each treatment group were carefully placed using forceps into the petri plates over layered with damp sterile Whatman filter paper no.1 and incubated at 25 ± 2 ˚C temperature for 12 days. Plates were observed day to day and poured with the required amount of water. Sample of soybean seedlings from each treatment including zero-day was collected and stored at -20 ˚C for further analysis. On the twelfth day of germination root length, shoot length, fresh weight, dry weight, and percent germination were recorded. Vigor of seedlings was calculated using following equation.

Vigor = [Mean root length + Mean shoot length] x Germination Percentage

Effect of MP3 isolate on the growth of *Glycine max* L. plants

Seeds were treated in four different groups as explained above in germination assay. Twelve pots containing approximately 3 kg garden soil were sown with treated seeds by preparing a pit of 1 cm away from the surface and then covered with the soil. Water was sprinkled over it until seeds germinated to seedlings and then each pot was regularly watered sufficiently up to 35 days. When seeds turned to plants, root length, shoot length, fresh weight, dry weight, and number of leaves of each plant were recorded.

Defense-related enzyme studies.

Enzymes such as Phenyl ammonia lyase, Lipoxygenase, Peroxidase, Polyphenol oxidase, and Catalase were studied.

Extraction of enzyme.

Enzyme extraction buffer was prepared by following the method of Jiang and Zhang (9). The buffer contained 50mM sodium phosphate pH 7.0, 1 mM EDTA, 0.6% PVP and 1 mM PMSF. One gram of Soybean seedlings were crushed in a pre-chilled mortar pestle and added with 5 ml of the extraction buffer. The homogenate was centrifuged at 4 ˚C at 7000 rpm for 15 min. The supernatant was carefully transferred to fresh

Eppendorf tubes and stored at 4 ˚C for further analysis. Protein content was measured by Lowry's method to calculate the specific activity of enzymes.

Phenyl ammonia lyase (PAL) [EC 4.3.1.5]

The activity of PAL was measured by preparing a reagent mixture of 0.4 ml of enzyme extract, 0.5 ml of 0.1 M borate buffer mixed (pH 8.8) with 12 mM phenylalanine. It was allowed to incubate for 30 min at 30 ˚C. The product formed was measured by taking OD at 290 nm against a blank. Trans cinnamic formed as a product was calculated using extinction coefficient 9630 M^{-1} (10).

Lipoxygenase (LOX) [EC 1.13.11.12]

LOX activity was assayed using a reaction mixture containing 5µl of 25 mM substrate solution (linoleic acid), the 20 µl enzyme extract, and 2.975 ml of phosphate buffer pH 6.0 (10mM) solution to make up the final volume to 3ml. LOX activity was measured at an absorbance at 234 nm after one minute of incubation. Its specific activity was defined as µmol of conjugated diene Hydroperoxy-9,11-octadecadienoic acid (HPOD) produced per mg of protein per minute and calculated using a molar coefficient of 25000M^{-1} cm^{-1} (11).

Peroxidase (PO) [EC 1.11.1.7]

Peroxidase enzyme activity was measured following the method of Patel *et. al*, (2015). The substrate was prepared using 1.5 ml of 0.05 M pyrogallol, 0.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). and 1% hydrogen peroxide. After addition of 0.5 ml of enzyme extract, change in OD was recorded every 30 seconds for 3 min. at 420 nm and was expressed ΔAbsorbance/ min/ mg of protein.

Polyphenol oxidase (PPO) [EC 1.14.18.1]

The reaction mixture for PPO activity consisted of 200 µL of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µL of 0.01 M catechol was added and the change in O.D. was recorded at 30 s interval up to 3 min at 495 nm. The enzyme activity was expressed as changes in O.D. per minute at 495 nm (10).

Catalase (CAT) [EC 1.11.1.6]

Catalase activity was estimated by preparing 3 ml of the system. 800 µl of 200 mM of hydrogen peroxide was followed by 200 µl of crude enzyme in 100 mM potassium phosphate buffer. The reaction was measured at 240 nm (12).

Characterization of isolate MP3

Isolate MP3 was characterized by the morphological, biochemical, and molecular methods.

Morphological characteristics and Gram's Nature

Colony characteristics were observed and recorded. For Gram staining, the freshly grown bacterial colony was transferred to sterile distilled water to make a uniform suspension. A loop of

suspension was spread on a clear glass slide and allowed it to air dry. The slide was fixed by heating over a flame. Crystal violet solution was drained over a slide for 1 min followed by gram's iodine for 30 seconds. An excess amount of stain was poured off and the slide was gently washed under tap water. The slide was again washed with 90% alcohol to remove the unbound crystal violet - iodine complex from the cell wall of bacteria. Then the slide was covered with safranin, a counterstain, for 2 min. The stain was gently removed and the slide was washed with tap water and allowed it to dry. The slide was examined under a light microscope using 40X and 100X lenses. Violet color cells were identified as Gram-positive and pink color cells as Gram-negative. Observations were recorded.

Biochemical characterization of isolate MP3

Biochemical characteristics of the isolate were measured for its identification using a ready test obtained from Hi-media, Mumbai, India. The bacterial colony was point inoculated on a ready strip and was incubated for 24 hr. The results were recorded.

Molecular Characterization of MP3

DNA of isolate MP3 was extracted using a method described by Sambrook and Green (13). Isolated DNA was amplified and the PCR product was sent to 16s rRNA identification at Eurofines, Bengaluru, India. The obtained sequence was aligned with known bacterial sequences on NCBI using software MEGA7. The Gene sequence was submitted to Genebank.

Statistical analysis

Statistical analysis was done on a totally randomized sample using SPSS software version.22 (SPSS Inc.). The statistical analysis comprised of three replicates of each sample. The one-way ANOVA was carried out for the experiment. The significant difference was confirmed by Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Results

Effect of MP3 isolate on the growth of *Glycine max* L seedlings

The effect of isolate MP3 on germination of *Glycine max* L. was studied by coating of freshly grown bacterial cell suspension on seeds of Soybean and observation of germinated seedlings. Percent germination of control, as well as *F. oxysporum* challenged seeds were increased from 46.66 % in control to 55.77% in MP3 coated seeds and 56.44 % in MP3 coated plus *F. oxysporum* challenged seeds (Table 1). MP3 improved the percent germination as well as all the vegetative parameters like root length, shoot length, fresh weight, dry weight, and vigor of Soybean seedlings (Fig 1). MP3 was found to increase root length, shoot length, fresh weight, and dry weight of seedlings significantly. It increased the vigor of soybean seedlings from 181.1 (control) to 302 (MP3) treated seedlings. Moreover, MP3 also significantly promoted the

vegetative growth and vigor of Soybean seedlings infected by *F. oxysporum.*

Fig.1 shows germination assay of Soybean seeds (JS-335 variety), where bacterial treated and bacterial treated + fungal infected seeds exhibit higher root length, shoot length.

Effect of MP3 on the growth of *Glycine max* L. plants:

The growth of bacterial treated plants was found better than the control and pathogenic fungus challenged and bacterial treated plants. It was observed that root length, shoot length, fresh weight, dry weight, and the number of leaves in Soybean plants increased significantly in the presence of MP3 isolate. It was also found to increase even in the presence of fungal pathogen *F. oxysporum.* Thus results of the pot assay corroborated the results observed in the germination assay.

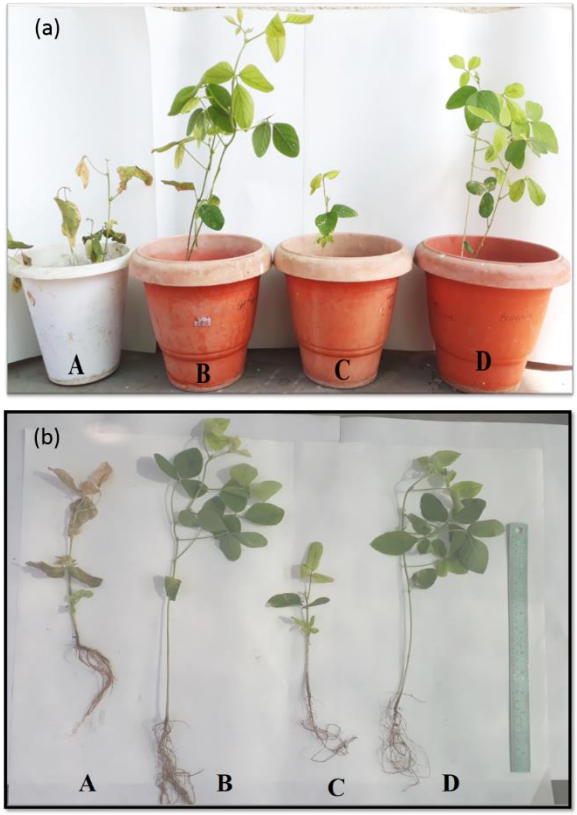

Fig. 2 Pot assay of *Glycine max* L (JS-335). (a) Differential growth of differently treated plants (b) Plants showing vegetative growth, where, A – control, B – MP3 treated, C – Fungal infected (*F.oxysporum*), D – MP3 treated+ fungal infected (*F.oxysporum*).

Effect of MP3 on the induction of defense-related enzymes

PAL activity

Phenylalanine ammonia lyase (PAL), the key enzyme in the phenylpropanoid pathway, was assayed from control and treated germinated seedlings. In fungal infected seedlings, PAL activity was found lower than control, indicating lack of phenol synthesis and defence response.

	Germination%	Root Length	Shoot Length	Fresh Weight	Dry Weight	Vigor
Control	46.66 ± 15.27^{ab}	2.80 ± 0.74^{cd}	0.93 ± 1.20^{a}	0.344 ± 0.14^{ab}	0.122 ± 0.11^{a}	181.12
MP3	55.77 ± 18.85^{ab}	$3.71 \pm .14^{abcd}$	1.51 ± 0.83^{a}	0.404 ± 0.14^{ab}	0.149 ± 0.07^{a}	302
Fungus	36.66 ± 18.55^{b}	2.21 ± 1.17^{d}	1.07 ± 1.63^{a}	0.286 ± 0.14^{b}	0.081 ± 0.06^{a}	132.67
MP3 + F	56.44 ± 14.86^{ab}	$3.43 \pm .40^{abcd}$	1.74 ± 1.81^{a}	0.341 ± 0.17^{ab}	0.120 ± 0.05^{a}	312.69

(Significance at $p \geq 0.005$, Duncan's DMRT test)

Table 1 Vegetative parameters of twelve-days-grown germination seedlings. It indicates that MP3 treated seedlings significantly enhanced the overall growth of soybeans.

	Root Length	Shoot Length	Fresh Weight	Dry Weight	Number of leaves
Control	13.49 ± 1.74^{ef}	6.70 ± 1.28^{ab}	1.638 ± 0.14^{de}	0.535 ± 0.02^{fg}	16.77 ± 1.07^{f}
MP3	19.33 ± 0.90^{bc}	8.53 ± 2.03^{ab}	1.760 ± 0.07^{cd}	0.909 ± 0.08^{d}	18.94 ± 1.29^{ef}
Fungus	11.07 ± 1.97^{f}	4.40 ± 1.19^{b}	1.082 ± 0.12^{f}	0.453 ± 0.04^{g}	12.11 ± 1.83^{g}
MP3 + F	18.40 ± 2.82^{bc}	10.41 ± 4.67^{ab}	1.976 ± 0.47^{bc}	0.929 ± 0.08^{d}	24.36 ± 1.76^{bc}

(Significance at $p \geq 0.005$, Duncan's DMRT test)

Table 2 Pot assay results of vegetative growth parameters of 35-days-grown plants. Root and shoot length, fresh weight, dry weight, and number of leaves of Soybean plants treated with MP3 bacterial isolate were significantly higher than the control. All these parameters were also increased even in the presence of fungal pathogen *F.oxysporum*

PAL activity was increased in MP3 treated uninfected as well as infected seedlings, indicating elicitation of plant defence. In MP3 treated seedlings increase in PAL activity was observed much earlier, but in fungal infected MP3 treated seedlings, it increased from the fourth day onwards, showing significance of MP3 in early mounting of resistance. Higher PAL activity was maintained further as measured up to eight days.

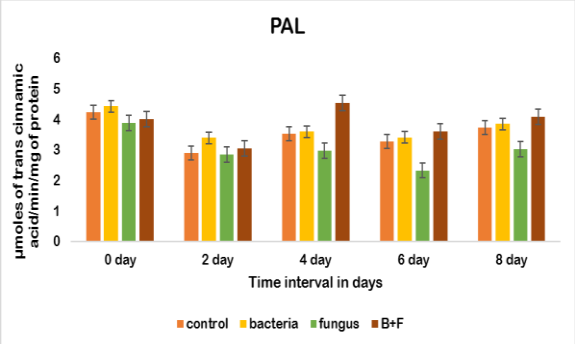

Fig. 3 PAL activity was estimated from germinated seedlings of soybeans grown for different number of days. Infected as well as uninfected seedlings treated with MP3 bacterial isolate showed higher PAL activity. Error bar indicates SD value.

LOX Activity

LOX activity was found to be elevated in combined treatment of MP3 and *F. oxysporum* in Soybean seedlings rapidly, but sustained only for two days, whereas only MP3 treatment showed delayed and lesser induction of the LOX activity except on the fourth day.

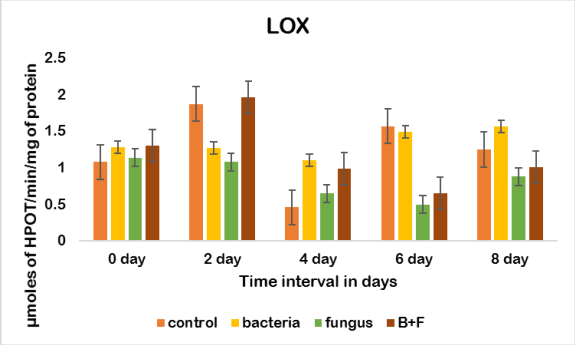

Fig.4 LOX activity from different-days-old Soybean seedlings. Treatment of MP3 isolate induced LOX activity from the fourth day, however, combined treatment of MP3 isolate and *F. oxysporum* showed higher LOX activity from day 2.

PO Activity

A significant increase in peroxidase activity in MP3 treated uninfected as well as infected Soybean seedlings were observed, indicating the role of MP3 in the induction of systemic resistance in *G. max* L. In seedlings infected with *F. oxysporum* only (not treated with the isolate MP3), there was no increase in peroxidase (Fig 5).

PPO Activity

A significant increase in PPO activity was found due to MP3 bacterial treatment in both fungal infected and uninfected Soybean seedlings, although infection of *F. oxysporum* also induced PPO activity to some extent (Fig 6). The increase in PPO activity supports the observation that MP3 mounts ISR in *G. max* L. seedlings and is backed up by similar observations in ISR generated in other plants (14, 15).

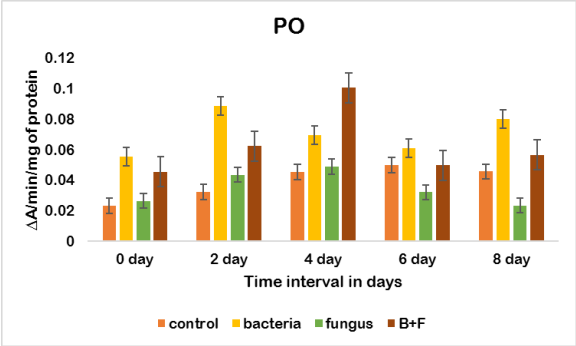

Fig.5 Activity of PO enzyme from different-days-old Soybean seedlings. Bacterial isolate MP3 induced the PO activity in both uninfected as well as infected plants. Bars in sequence: Control, Bacteria (MP3) treated, fungus infected and Bacteria treated as well as fungal infected (B+F).

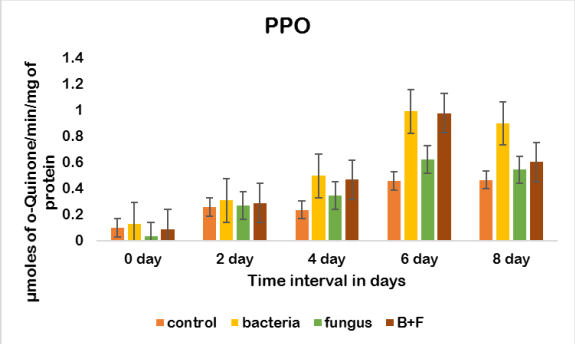

Fig.6 Activity of PPO enzyme from different-days-old Soybean seedlings. Activity of PPO was found to increase rapidly due to the treatment of PGPR MP3.

Catalase Activity

The activity of catalase was found to be different in comparison to controls on different days, probably due to varied amount of reactive species in plant tissues.

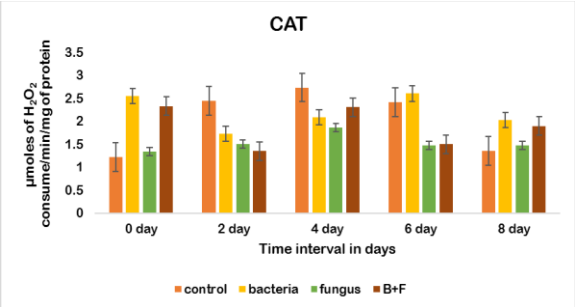

Fig.7 Activity of Catalase enzyme from different-days-old Soybean seedlings. Catalase was differentially induced in MP3 bacteria treated seedlings.

Identification of Isolate MP3

Morphological characteristics and Gram's Nature of MP3 Isolate.

Morphological characteristics of the freshly grown isolated colony of MP3 on King's B agar medium were observed and the observations were recorded (Table 3). Isolate MP3 was found to be Gram-negative tiny rod-shaped bacteria (Fig 8).

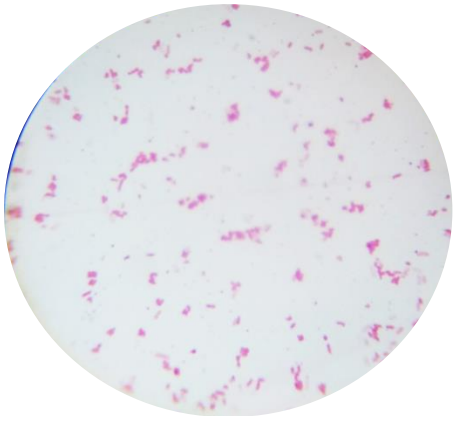

Fig.8 Gram staining of MP3

Biochemical characteristics of MP3 Isolate.

Isolate MP3 was found positive for Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginase, Glucose, Arabinose, Trehalose, Mannitol, Sorbitol, and Sucrose. Biochemical characteristics were found to match with bacterial genera *Serratia*.

Molecular characterization of MP3 Isolate.

Genomic DNA was isolated from MP3 isolate and the 16s rRNA partial gene sequence was amplified by PCR (Fig 9 a & b). Sequencing and alignment of the 16s r RNA gene using BLAST on NCBI showed 99% similarity with *S. marcensecns*. The Gene sequence was submitted to Genebank and the accession number obtained was MK073018. The phylogenetic neighbour joining tree was prepared using MEGA7 software. Bacterial 16s rRNA gene sequence was found to be closely related to *S. marcensecns* with high bootstrap value (Fig 10). The tree was prepared by a bootstrap bar of 0.005 basepairs.

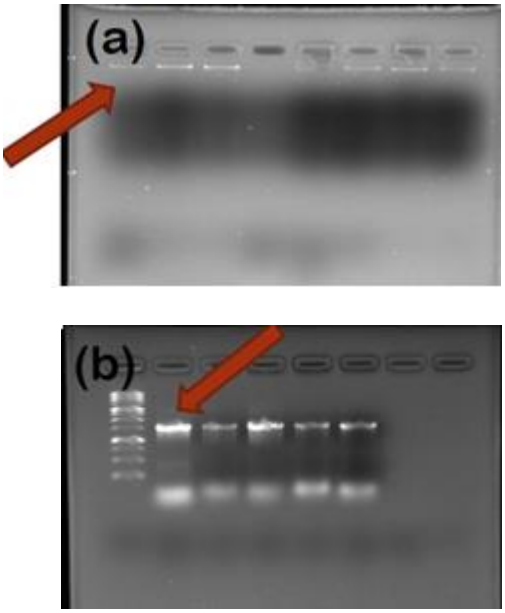

Fig.9 (a) DNA band on 0.8 % Agarose gel. (b) PCR band of 1500 bp on 0.8 % Agarose gel.

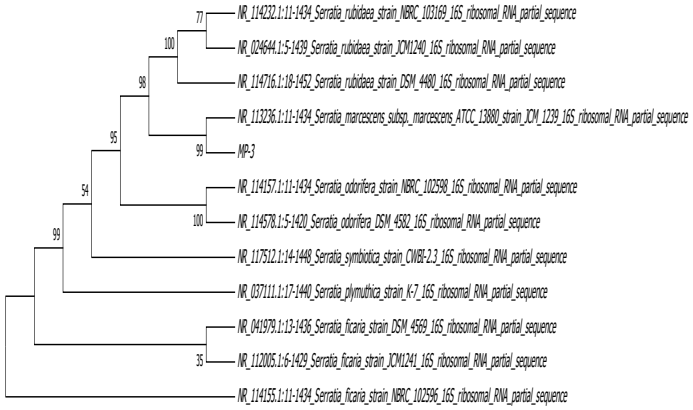

Fig 10 Position of MP3 bacterial isolate in a neighbour-joining tree.

Discussion

In the present study, we have characterized plant growth-promoting characteristics of one of the isolates, MP3 isolated from the rhizosphere of *Glycine max* L. plants. MP3, identified as *S. marcescens* improved the germination of Soybean seedlings, promoted the growth of plants, and controlled one of the most important pathogens of Soybean, *Fusarium oxysporum*. It was observed that seedlings and plants infected with *F. oxysporum* neither grew well nor showed induction of systemic resistance, whereas if co-treated with *S. marcescens*, they showed improved growth and better mounting of ISR. *S. marcescens* is earlier reported to promote the growth of coconut plants (8).

Synthesis and accumulation of phenols by phenylpropanoid pathway is the primary

inducible defense response in plants against a number of biotic and abiotic stresses leading to the synthesis of defense metabolites like phytoalexins, coumarins, flavonoids, and precursors of lignin and suberin (12, 15, 16,). The activity of the first enzyme of this pathway, PAL was induced by *S. marcescens,* which is one of the factors for the mounting of resistance and uninhibited growth of *F. oxysporum* infected Soybean plants (Figure 2). An increase in PAL activity due to the induction of systemic resistance in *Glycine max* L. infected with *F. oxysporum* has been reported earlier (15, 16). Lipoxygenase enzyme, responsible for lipid peroxidation, loss of membrane integrity, and electrolyte leakage is induced in a number of plant-pathogen interactions (4). LOX activity was not induced upon treatment of this bacterium, however it did increase upon combined treatment with *F. oxysporum*. These results are corroborated with many reports showing that LOX is generally activated by the necrotrophic pathogen. *F. oxysporum* being a hemibiotroph (initially biotrophic but later necrotrophic) was able to activate LOX (17). Activities of PO and PPO were induced in bacterium treated seedlings. An increase in PO and PPO activity as a part of ISR has been reported in many plants infested with *F. oxysporum* (14, 15). Catalase activity was found varying on different days. Catalase along with other antioxidant enzymes clears ROS in plant tissues infected with pathogens (generated during HR). As catalase transforms reactive hydrogen peroxide into water, its activity depends upon the amount of ROS levels in plant cells and different plant tissues express different catalase activity in response to *Fusarium* infection (10, 12, 15).

Tests of Biochemical characteristics of the isolate identified that it belongs to genus *Serratia,* based on positive results for Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginase, Glucose, Arabinose, Trehalose, Mannitol, Sorbitol, and Sucrose, as well as literature support (18). Based on this as well as on the alignment of the 16s rRNA gene with bacterial sequences on NCBI, this isolate was identified as *Serratia marcescens. S. marcescens* has been previously reported as PGPR and showed similar biochemical characteristics (18), however, this is the first report of *S. marcescens* promoting the growth of *Glycine max* L. plants.

Conclusion

A PGPR isolate MP3 increases the germination, promotes growth, and induces

the defense mechanism against *F. oxysporum* in *Glycine max* L. plants. Using biochemical and molecular characterization, it was identified as *S. marcescens*. Based on the present studies it can be concluded that *S. marcescens* can be used as a PGPR in Soybean plants and as a biocontrol agent against *F. oxysporum* after confirmation of its performance at the field level.

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Bacteria Name	**Size**	**Shape**	**Edge**	**Elevation**	**Surface**	**Consistency**	**Pigment**
Mp3	Small	Round	Entire	Colonial	Alveoli	Moist	Orange, Red to Maroon

Table 3 MP3 Morphological characteristics