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Mutation Based Strain Improvement of Pgpr Isolates (*Pseudomonas fluorescens* & *Bacillus subtilis*) for the Improvement of Growth and Yield of Paddy (*Oryza sativa* L.)

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Abstract: Plant growth promoting rhizobacteria (PGPR) offers an attractive way to replace chemical fertilizer, pesticides and supplements; most of the isolates result in a significant increase in plant growth and PGPR help in the disease control in plants. In the present study, strain improvement of PGPR isolates were carried out by mutation for the improvement of growth and yield of Paddy. The plant growth promoting substances produced by UV and EMS mutated isolates and phosphate solubilization was estimated. The EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* showed more plant growth promoting substances and phosphate solubilization when compared to the UV mutated *Pseudomonas fluorescens* and *Bacillus subtilis*. The antagonistic activity of wild and mutated of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Pyricularia oryzae* was investigated. The mutated strains showed more zone of inhibition when compared to wild strain. Among the mutated strains, EMS mutated *Pseudomonas fluorescens* showed maximum zone of inhibition when compared to UV mutated bacterial isolates. The effect EMS mutated strains of *Pseudomonas fluorescens* and *Bacillus subtilis* on plant growth stimulation was studied under pot culture condition. The plant growth promoting characteristics, biochemical constituents and plant defensive enzymes were analyzed. The seed treatment with combined application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* increased plant growth promoting characteristics, biochemical constituents and plant defensive enzymes.

Key words: Paddy · *Pseudomonas fluorescens* · *Bacillus subtilis* · *Pyricularia oryzae* · Antagonistic Activity · UV Mutation and EMS Mutation

INTRODUCTION

Agriculture is heavily dependent on the use of chemical fertilizers and pesticides to achieve higher yields. This dependence is associated with problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities that otherwise support crop production. Hence, crop production and pest and disease management have to be achieved in shorter intervals of time with fewer detrimental inputs. The use of bioresource to replace chemical fertilizers and pesticides is growing. In this context, plant growth promoting microorganisms are often novel and potential tools to provide substantial benefits to agriculture.

Cereals are the world's major source of food for human nutrition and rice (*Oryza sativa* L.) is one of the predominant cereal crop and represent staple diet for more than two fifth of the world population. To feed the ever increasing human population, the world's annual rice production must be increased from 560 million tonnes to 760 million tonnes by 2020. The future increase in rice

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production has to come from the same or even reduced land area and the productivity yield (per ha) must be greatly enhanced by providing additional nutrient input and through effective control of phytopathogens. Blast disease of rice caused by *Pyricularia oryzae* is one of the most destructive fungal diseases of rice causing loss up to 90% and has a ubiquitous occurrence in almost all the rice growing countries [1].

Rice is an important agricultural crop in India which provides cheap carbohydrate source for human consumption in many countries including India. Rice is the seed of the monocot plant *Oryza sativa*. As a cereal grain, it is the most important staple food for a large part of the world's human population. It is the grain with the second highest worldwide production. Rice is normally grown as an annual plant, although in tropical areas it can survive as a perennial and can produce a ratoon crop for up to 30 years. Rice cultivation is well-suited to countries and regions with low labour costs and high rainfall, as it is labour-intensive to cultivate and requires ample water. Rice can be grown practically anywhere, even on a steep hill or mountain.

Diseases are very common in plants and are responsible for the loss of approximately one third of the crop yield. Chemical pesticides that control plant diseases have become a threat to health and the environment and hence being banned worldwide. This has increased the interest in biocontrol of plant diseases. PGPR mediated agriculture is now gaining worldwide importance and acceptance for an increasing number of crops and managed ecosystems as the safe method of pest control. Eventhough, chemical control of the rice diseases is quite effective; their consistent use on a regular basis is undesirable from economic and environmental considerations. The persistent, injudicious use of chemicals has toxic effects on non -target microorganisms and can cause undesirable changes in the environment. Moreover, these synthetic chemicals are too expensive for the resource poor farmers of Asia [2] and the possible emergence of fungicide resistant pathogens can also occur. These considerations have promoted a search for biocontrol agents, which are effective against the rice pathogens.

Biocontrol has untapped potential and was underused, under exploited, underestimated, often untried and therefore unproven. The new tools of recombinant DNA technology, mathematical modeling and computer technology combination with a continuation of the more classical approaches such as importation and release of natural enemies and improved germplasm, breeding and field testing should quickly move biocontrol research and technology into a new era. Although activity and effects of biocontrol have been reported for a number of antagonists, the underlying mechanisms are not fully understood. This deficiency in our knowledge often hinders attempts to optimize the biological activity by employing tailored application strategies. Several mechanisms of plant - microbe interaction may participate in the association and affect plant growth, including IAA, Siderophore production and biocontrol against *Pyricularia oryzae*. Thus, the Plant Growth Promoting Rhizobacteria (PGPR) affect the plant growth through direct promotion by producing plant growth promoting substances and through indirect promotion by acting against plant pathogenic microorganisms [3].

Plant growth promoting rhizobacteria (PGPR) are free - living, soil - borne bacteria, which enhance the growth of the plant either directly or indirectly [3, 4]. The direct mechanisms involve nitrogen fixation, phosphorus solubilization, HCN production, production of phytohormones such as auxins, cytokinins and gibberellins and lowering of ethylene concentration [4, 5]. Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas* and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth [6]. There are many reports on plant growth promotion and yield enhancement by plant growth promoting rhizobacteria (PGPR) [7]. The mechanisms of plant growth promotion by PGPR include: the ability to produce phytohormones, N_2 fixation, antagonism against phytopathogens and solubilization of insoluble phosphates [8]. It was also suggested that the PGPR can also prevent the deleterious effects of stresses from the environment [9].

Many soil microorganisms possess multiple beneficial traits of nutrient mobilization, production of plant growth promoting substances and biocontrol ability [10]. Plant growth promoting rhizobacteria (PGPR), a group of root associated bacteria, intimately interact with the plant roots and consequently influence plant health and soil fertility. They offer an excellent combination of traits useful in disease control and plant growth promotion. Amongst the PGPRs, *Pseudomonas fluorescens* and *Bacillus subtilis* have emerged as the largest and potentially the most promising group of PGPR with their rapid growth, simple nutritional requirements, ability to utilize diverse organic substrates and mobility. *Pseudomonas fluorescens* and *Bacillus subtilis* produce

highly potent broad spectrum antifungal molecules against various phytopathogens, thus acting as effective biocontrol agents [11]. The present study was aimed to improve the strains of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates for enhancing its biocontrol potential against Paddy (*Oryza sativa*) disease *Pyricularia oryzae* and maximization of paddy yield.

MATERIALS AND METHODS

Strain Improvement of *Pseudomonas fluorescence* and *Bacillus subtilis* by Mutagenesis

UV Mutagenesis: The UV Mutagenesis of the culture of *Pseudomonas fluorescens* and *Bacillus subtilis* was carried out by following the method of Miller [12]. During UV mutagenesis, the culture was exposed to short wavelength UV light (260 nm) from a distance of 60 cm for various time intervals (for 0.1% survivors). From the serial dilutions of the mutagenized culture, 0.1 ml was plated onto Nutrient agar plates. Throughout UV mutagenesis, dark condition was maintained to prevent photo reactivation.

Ethyl Methane Sulfonate (EMS) Mutagenesis: The EMS Mutagenesis of the culture of *Pseudomonas fluorescens* and *Bacillus subtilis* was carried out by following the method of Miller [12]. The bacterial cells were washed thrice with sterile 0.1 M of Tris – HCl buffer (pH 7.5) and resuspended in 1.5 ml of same buffer. One ml aliquots of the washed cells were transferred to 1.5 ml micro centrifuge tubes and kept on ice. A total of 15 µl of EMS was added and incubated on a shaker water bath at 30°C for various time intervals. The cells were washed with 0.1 M of Tris HCl buffer (pH 7.5) and resuspended in 1.0 ml buffer. The mutagenized samples were then grown for 24 hrs after which it was serially diluted and plated onto Nutrient agar plates.

Estimation of Plant Growth Promoting Substances Produced by Mutated *Pseudomonas fluorescence* and *Bacillus subtilis* Isolates

Estimation of Indole Acetic Acid (IAA): The Indole acetic acid produced by mutated (UV and EMS) PGPR isolates were estimated by following the procedure of Gorden and Paleg [13].

Estimation of Gibberellic Acid (GA_3): The Gibberellic acid production by mutated (UV and EMS) PGPR isolates was estimated by following the procedure of Borrow *et al.* [14].

Estimation of Siderophore Production: The Siderophore production by mutated (UV and EMS) PGPR isolates was estimated by following the procedure of Reeves *et al*. [15].

Screening of Mutated *Pseudomonas fluorescens* and *Bacillus subtilis* for Phosphate Solubilization: The screening of Phosphate solubilization by mutated (UV and EMS) PGPR isolates was determined by following the procedure of Pikovskaya [16].

Detection of Antagonistic Activity of Wild and Mutant Strains of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Pyricularia oryzae*: A loopful of cultures of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates were transferred aseptically to the centre of PDA plates which have been pre-inoculated with *Pyricularia oryzae*. The plates were incubated at 28±2°C for 7 days. After the incubation period, the diameter of inhibition zone was measured. Three replications were maintained for each isolate.

Developing Liquid Formulation of Mutant Strains of *Pseudomonas fluorescens* and *Bacillus subtilis* with Chemical Amendments: For developing liquid formulation of *Pseudomonas fluorescens* and *Bacillus subtilis,* Nutrient broth was prepared and the standardized dosage of chemical amendments *viz*., trehalose at 15 mM, polyvinyl pyrollidone (PVP) at 2% and glycerol at 15mM was added to one liter of broth separately. One ml of log phase culture of mutated *Pseudomonas fluorescens* and *Bacillus subtilis* was inoculated individually in each broth. An uninoculated control will be maintained for each broth and the flasks will be incubated at room temperature. The rice seeds were soaked into the mutated *Pseudomonas fluorescens* and *Bacillus subtilis* and then used for the Pot culture experiment.

Effect of Mutant Strains of *Pseudomonas fluorescens* and *Bacillus subtilis* on the Growth and Yield of Paddy (ADT 43): The pot culture experiment was conducted to study the effect of mutant strains of *Pseudomonas fluorescens* and *Bacillus subtilis* on the growth and yield of paddy var ADT 43. The study was conducted at Department of Microbiology, Annamalai University, Annamalai Nagar. The soil used in the pot culture experiment was clay loamy in nature. The experiment was arranged in Randomized Block Design (RBD) with three replications. For sowing in inoculated pots, paddy seeds were soaked with trehalose at 15 mM, polyvinyl pyrollidone (PVP) at 2% and glycerol for 30 min in

different formulations (20 ml/kg of seeds), (Spacing, 15 cm × 10 cm: 3 seedlings/hill and 12 seedlings/pot). Gap filling was done after 10 DAS. The fertilizer schedule of 100:50:50 (100% NPK/ha) was followed for control plots while other treatments followed 75% of recommended dose of N. The entire dose of P_2O_5 and K_2O has applied basally as super phosphate and muriate of potash, respectively. The crop was given hand weeding on 30th DAS and well protected against pests and diseases. A water level of 5cm depth was maintained through the crop period. Five representative samples of plant hills in each pot were pegmarked for periodical observation. The treatments were: T_1 – Control (100% N), T_2 – EMS mutated *Pseudomonas fluorescens* + 75%, T_3 – EMS mutated *Bacillus subtilis* + 75% N and T_4 – EMS mutated *Pseudomonas fluorescens* + EMS mutated *Bacillus subtilis* + 75% N.

Biometric observation and yield parameters

Effect of Plant Height: The height of the plants in each treatment was measured at 30th day after transplantation (DAT). The mean value of the plants from 3 replications was recorded.

Effect of Dry Weight of Root and Shoot: The dry weight of root and shoot was taken at 30th day after transplantation (DAT). The plant samples were drawn, washed, air dried and later dried to a constant weight in an oven at 60°C. The mean oven dry weight of the samples was recorded.

Total Chlorophyll Content of Rice Leaves: One gram of fresh leaves was cut into small pieces, homogenized with excess acetone in a clean mortar and pestle and the extract was decanted by using Whatman No. 42 filter paper. The extraction was repeated again with 80 percent acetone, the contents decanted and the rice was washed with acetone until colourless. The filtrates were pooled, volume made up to 100 ml in a volumetric flask. Then, 5 ml of the extract was diluted to 50 ml with acetone (80%). The absorbance of this extract was measured at 645 nm and 663 nm and the total chlorophyll content rice leaves determined by using the following formula.

$$\text{Total Chlorophyll (mg/g)} = \frac{20.2\ A645 + 8.02\ A663}{a \times 1000 \times w}$$

a = length of light path in the cell; v = volume of the extract in ml; w = fresh weight of the sample in g.

'N' Content of Plant: The plant samples were collected on 30th day after transplantation (DAT), washed in water, air dried and later dried to a constant weight in an oven at 60°C. Then, they were powdered, sieved and 100 mg of sample was taken for analysis. The total nitrogen content was estimated by Microkjeldahl method.

Grain and Straw Yield of Rice: The matured crop was harvested, hand threshed, winnowed and sun dried. The dried grains from each treatment were weight and recorded. After threshing, the rice straw was subjected to Sun dry and the weight was recorded.

Estimation of Biochemical Constituents: Plant sample material from each treatment was taken at 0,7,14 and 21 DAT were collected and chopped and they were subjected to 80% ethanol extraction procedure by Mahadevan and Sridharan [17] was used for the estimation of reducing sugars, total phenol and enzymatic activity.

Quantitative Estimation of Sugars

Reducing Sugars: Reducing sugar present in the extraction was estimated according to Nelson [18] method.

Non-Reducing Sugars: Non- reducing sugars with ethanol extract hydrolyzed and the total sugars were estimated by employing Nelson's method. The total reducing sugars were calculated as glucose equivalents. The final concentration was calculated by deducting the reducing sugar present in the unhydrolyzed original sample from the reducing sugar present in the hydrolyzed sample.

Hydrolysis of non-reducing sugars was carried out according to Inman [19].

Quantitative Estimation of Phenols: Total phenols were estimated by employing Folin- ciocalteau reagent [20].

Enzyme Assay: One gram of the leaf was cut into small bits, crushed in chilled 0.1M sodium phosphate buffer pH 7.1 and the volume was made upto 5 ml with the buffer, centrifuged at 2100 rpm, for 30 min and the supernatant was used as the enzyme source and all the assays *viz.*, polyphenol oxidase and peroxidase were performed in a UV Spectrophotometer at 28±1°C [21]. The activity of polyphenol oxidase was estimated by the method of Matta and Diamond [22] and Peroxidase by Hampton [23]. The enzyme activity in the sample was expressed in terms of unit/minute/ mg of protein.

Statistical Analysis: The experimental result were statistically analysed in completely randomized design (CRD) as per the procedure described by Gomez [24].

RESULTS AND DISCUSSION

Blast disease of rice, caused by *Pyricularia oryzae*, is one of the most destructive phytopathogen of rice and has a ubiquitous occurrence in all the rice growing countries, under severe infection; it causes a yield loss upto 90 percent [1]. In this present study, the survey was conducted at ten locations in Cuddalore district of Tamil Nadu comprising Annamalai Nagar, Bhuvanagiri, Kurinjipadi, Vadalur, Neyveli, Keelamoongiladi, Vayalore, Sivayam, Kannangudi and Mangalam. In each and every location of the survey area a field which has been under long behind mono culture practice was selected.

Plant growth promoting rhizobacteria (PGPR) may promote growth directly by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus and potassium, production of siderophore that solublize and sequester iron, or production of plant growth regulators [25]. Some bacteria support plant growth indirectly, by improving growth restricting conditions either *via* production of antagonistic substances or by inducing host resistance towards plant pathogens. Since associative interactions of plant and microorganisms must have come into existence as a result of co-evaluation, the use of either former or latter groups of bioinoculants form one of the vital components for a long term sustainable agriculture system [26].

In the present research, the plant growth promoting substances (Indole acetic acid, Gibberellic acid and Siderophores) produced by UV and EMS mutated PGPR isolates and phosphate solubilization was estimated and the results were given in Table – 1. The EMS mutated *Pseudomonas fluorescens* (EMS – PF) and *Bacillus subtilis* (EMS – BS) showed more plant growth promoting substances and phosphate solubilization when compared to the UV mutated *Pseudomonas fluorescens* (UV – PF) and *Bacillus subtilis* (UV – BS). The results of the present study was in line with the research of Saranraj *et al*. [27] Sivasakthi *et al*. [28], Usharani *et al*. [29] Kanchana *et al*. [30] and Usharani *et al*. [31].

Mandira Kochar *et al*. [32] analyzed the biocontrol strain *Pseudomonas fluorescens* Psd for indole-3-acetic acid (IAA) biosynthesis and studied the effect of its consequent manipulation on its plant-growth-promoting (PGP) potential. While the indole pyruvic acid (IPyA) pathway commonly associated with PGP bacteria was lacking, the indole acetamide (IAM) pathway generally observed in phytopathogens was expressed in strain Psd. Overexpression of IAM pathway genes *iaaM-iaaH*, from *Pseudomonas syringae* subsp. *savastanoi* drastically increased IAA levels and showed a detrimental effect on sorghum root development.

Paul and Sharma [33] reported through an experiment that the *Pseudomonas* is the most abundant auxin producer micro-organism. Growth regulators especially IAA (Indole-3-Acetic Acid), often affects the root systematic features such as root primary growth, side-root formation and root hairs. Auxins are a group of herbal hormones which IAA is the most important of them.

Alstrom [34] isolate a growth promontory fluorescent *Pseudomonas* having the potential of phosphate solubilization and siderophore production. Misra and Ahmed [35] reported higher solubilization of phosphate by *Rhizobium phaseoli* than *Pseudomonas* spp. Dave and Patel [36] found that various compounds of carbon and nitrogen sources were tested for their effect on solubilization of tricalcium phosphate and rock phosphate by *Pseudomonas fluourescence*. Saranraj *et al*. [27] reported that the cold tolerant mutants of *Pseudomonas fluorescence* strains GRSI. PRS9 showed an effect solubilization ability and subsequent effect on plant growth promotion under *in vitro* and situ conditions. Sivasakthi *et al*. [28] examined the phosphate solubilization by different, bacterial culture *viz*., *Bacillus*, *Pseudomonas*, *Azotobacter*, *Rhizobium* and *Azospirillum* isolate and *Rhizobium* showed high tricalcium phosphate solubilizing ability in both solid and liquid medium.

The antagonistic activity of wild and mutated (UV and EMS mutated) of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Pyricularia oryzae* was investigated and the results were showed in Table – 2. The mutated strains showed more zone of inhibition when compared to wild strain. Among the mutated strains, EMS mutated *Pseudomonas fluorescens* (30 mm in dm) showed

Table 1: Plant growth promoting substances produced by mutated PGPR isolates

S. No	Plant growth promoting substances	EMS mutated *Pseudomonas fluorescens*	UV mutated *Pseudomonas fluorescens*	EMS mutated *Bacillus subtilis*	UV mutated *Bacillus subtilis*
1	Indole Acetic Acid (IAA) (µg/ml)	37.26	34.11	33.88	30.05
2	Gibberellic Acid (GA_3) (µg/ml)	8.90	7.60	6.72	6.12
3	Siderophore production (µg/ml)	13.60	12.53	10.15	9.91
4	Phosphate solubilization	+++	+++	+++	++

Table 2: Antagonistic activity of wild and mutated strains of PGPR isolates against *Pyricularia oryzae*

S. No	Mutated PGPR isolates	Zone of inhibition (mm)
1	*Pseudomonas fluorescence*	10
2	*Bacillus subtilis*	8
3	EMS Mutated *Pseudomonas fluorescence* (EMS-PF)	30
4	UV Mutated *Pseudomonas fluorescence* (UV-PF)	28
5	EMS Mutated *Bacillus subtilis* (EMS-BS)	23
6	UV Mutated *Bacillus subtilis* (UV-BS)	20

Table 3: Effect of EMS mutated PGPR strains application on the enhancement of growth parameters in Paddy (ADT 43)

		Dry weight (g plant $^{-1}$)			
Treatment	Plant height (cm)	Root	Shoot	Chlorophyll content (mg g^{-1} of leaf)	'N' content of plant (%)
T_1	68.65	1.12	4.41	2.47	1.15
T_2	78.79	1.55	5.51	2.54	1.52
T_3	71.82	1.34	5.37	2.50	1.48
T_4	88.50	1.78	6.75	2.94	1.88
SE_D	4.39	0.14	0.48	0.11	0.14
CD (P = 0.05)	8.80	0.39	0.96	0.23	0.30

Table 4: Effect of EMS mutated PGPR strains application on the Grain and Straw yield of Paddy (ADT 43)

Treatment	Grain yield (g pot^{-1})	Straw yield (g pot^{-1})
T_1	25.34	38.70
T_2	38.42	53.04
T_3	36.86	51.44
T_4	46.57	62.56
SE_D	4.37	4.90
CD (P = 0.05)	8.80	9.82

maximum zone of inhibition when compared to UV mutated bacterial isolates. The results of the present study was in line with the research of Saranraj *et al*. [27], Sivasakthi *et al*. [28], Usharani *et al*. [29], Kanchana *et al*. [30] and Usharani *et al*. [31].

The effect EMS mutated strains of *Pseudomonas fluorescens* and *Bacillus subtilis* on plant growth stimulation was studied under Pot culture condition. The plant growth promoting characteristics *viz*., plant height, root and shoot dry weight, chlorophyll content and 'N' content were studied and results are presented in Table - 3. The seed treatment with combined application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* increased the plant height (88.5 cm), dry weight of root (1.78 g $plant^{-1}$), shoot dry weight (6.75 g $plant^{-1}$), chlorophyll content (2.94 mg g^{-1}) and 'N' content (1.88 mg g^{-1}) to a higher level followed by *Pseudomonas fluorescence* alone, *Bacillus subtilis* alone and control treatments. The same trend was also recorded with yield parameters. The treatment of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis*, increased grain yield (46.57 g pot^{-1}) and the straw yield (62.56 g pot^{-1}) to a maximum level when compared to other treatments (Table – 4).

The use of *Pseudomonas fluorescens* for increasing the crop production is an attractive approach in the modern system of sustainable agriculture [37]. The increase in growth might be associated with secretion of auxins, gibberellins and cytokines [38]. Moreover, *Pseudomonas fluorescens* application was also found to be more effective for the biocontrol *Pyricularia oryzae* in rice crop [39]. The result of the present study also indicated the effectiveness of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* to augment the plant growth by secreting the phytohormones and suppress the blast disease pathogen through ISR mediated biocontrol. The increase in grain yield due to the application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* might be associated with the increased plant growth and decreased incidence. However, the pot culture experiment of the present study clearly revealed the importance of additional EMS mutated *Bacillus subtilis* requirement for the effective induction of systemic resistance in rice plant against *Pyricularia oryzae* incidence.

The reducing and non-reducing sugar level of plant material sample was found to reduce throughout the sampling period in all the treatments. The reducing and non-reducing sugar level profoundly reduced due the

Table 5: Changes in reducing sugar content of paddy as influenced by the application of EMS mutated PGPR strains

	Reducing sugar content (mg g^{-1})			
Treatment	0 day	7 day	14 day	21 day
T_1	36.70	33.54	32.03	30.25
T_2	34.65	28.11	26.20	23.92
T_3	35.72	31.20	29.34	26.72
T_4	32.52	23.33	17.69	16.90
SE_D	0.89	2.20	3.11	2.82
CD (P = 0.05)	1.80	4.42	6.25	5.65

Table 6: Changes in Non-Reducing sugar content of paddy as influenced by the application of EMS mutated PGPR strains

	Non-Reducing sugar (mg g^{-1})			
Treatment	0 day	7 day	14 day	21 day
T_1	21.14	24.81	26.22	27.37
T_2	18.02	21.67	23.35	24.06
T_3	19.61	22.52	24.06	25.80
T_4	15.01	16.92	18.46	19.02
SE_D	1.31	1.65	1.63	1.81
CD (P = 0.05)	2.62	3.30	3.26	3.63

Table 7: Effect of EMS mutated PGPR isolates on the improvement of Total phenol content in Paddy (ADT 43)

	Total phenol (mg g^{-1})			
Treatment	0 day	7 day	14 day	21 day
T_1	2.24	2.59	2.90	3.11
T_2	2.70	3.15	3.40	3.61
T_3	2.47	2.95	3.02	3.21
T_4	3.66	4.02	4.55	4.60
SE_D	0.31	0.30	0.37	0.34
CD (P = 0.05)	0.62	0.60	0.75	0.68

application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* treatments and the results are presented in Table - 5 and Table - 6. It was found that application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application reduced the reducing and non-reducing sugar level of rice plant to a higher level when compared to other treatments. In the present study, the reducing and non-reducing sugar level decreased to a higher rate due to EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application followed by EMS mutated *Pseudomonas fluorescence* alone and EMS mutated *Bacillus subtilis* alone treatments. But, the reduction effect was more pronounced due to the combined inoculation of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application and challenge inoculation of *Pyricularia oryzae* in paddy.

Post- infectional drop in the sugar reserve of the host plant was documented by several workers [21, 40]. The reduction in reducing sugar level might be due to (1) Oxidation to meet the energy requirements for various resistant reactions, (2) Polymerization of reducing sugar to starch and (3) Utilization of reducing sugars to starch for the synthesis of phenolic compounds through shikimic pathway. Usharani *et al*. [31] suggested that blast might be regarded as "high sugar" disease. Rice leaves contain glucose, fructose, sucrose, methanol and starch which might serve as the carbon sources for the pathogens. The entry and establishment of the pathogen was reduced to a higher level, the post infectional drop in sugar content due to PPFM and SA treatment and in conformity with above findings.

The total phenol content was found to increase gradually throughout the sampling period in all the treatments. The total phenol content was profoundly increased by the combined application of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* treatment when compared to other treatments and control. The results of total phenol content were presented in Table - 7. In the present study, EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application during the challenge inoculation of *Pyricularia oryzae* augmented the total phenol content to

Table 8: Changes in Peroxidase content of paddy as influenced by the application of EMS mutated PGPR strains

	Peroxidase (unit/min/mg of protein)			
Treatment	0 day	7 day	14 day	21 day
T_1	1.02	18.54	118.85	76.92
T_2	1.17	21.04	120.03	79.28
T_3	1.10	20.75	119.69	78.13
T_4	2.97	22.37	121.53	81.29
SE_D	0.46	0.79	0.55	0.92
CD (P = 0.05)	0.92	1.68	1.12	1.86

Table 9: Changes in Polyphenol oxidase content of paddy as influenced by the application of EMS mutated PGPR strains

	Polyphenol oxidase (unit/min/mg of protein)			
Treatment	0 day	7 day	14 day	21 day
T_1	2.37	2.31	2.80	15.62
T_2	2.52	4.51	24.40	18.71
T_3	2.45	4.32	22.40	18.14
T_4	4.45	5.80	25.48	19.29
SE_D	0.50	0.72	5.36	0.80
CD (P = 0.05)	1.00	1.11	10.74	1.60

a higher level when compared to EMS mutated *Pseudomonas fluorescens* inoculation alone treatment and emphasized the importance to build up the bacterial numbers in rice phyllosphere in order to augment the total phenolic content of the rice plant. In the same manner, EMS *Pseudomonas fluorescens* and *Bacillus subtilis* application along with challenge inoculation of *Pyricularia oryzae* augmented the total phenol content of rice to a higher level when compared to EMS mutated *Bacillus subtilis* application alone.

Accumulation of phenolic compounds plays a major role in the resistance of plants against phytopathogens. A variety of phenol and related derivatives conferring protection and the physiological and biochemical aspects of resistance to blast disease has been reported by Chen *et al*. [41]. Application of PGPR isolates increased the total phenolic content of the groundnut plant during the challenge inoculation with *Cercospora personatum* [42]. The results of the present study clearly revealed the importance of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* against the control of *Pyricularia oryzae* on the accumulation of phenolic compounds in rice plants which is known to play a key role in the augmentation of ISR and in conformity with the above findings of Meyer and Hofte [43] and M'Piga [44].

Several studies have revealed the ability of *Pseudomonas fluorescence* to induce systemic resistance against many phytopathogens. The application of *Pseudomonas fluorescence* triggered a set of plant defense reaction in rice against many phytopathogens [44]. Phenolic compounds accumulated with lumen of host cells due to *Pseudomonas fluorescence* and treatment followed by challenge inoculation with *Fusarium oxysporum f.sp lycopersici* [44]. Direct toxicity to the pathogens or oxidation of phenolic compounds and their toxic derivatives (Quinines) might be explained as a mechanism by which phenols confer resistance to plants. Moreover, *Bacillus subtilis* acts as systemic signal molecules in inducing the disease resistance of plants [39].

The effect of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* on the production of plant defense enzymes, namely, peroxidase (PO) and polyphenol oxidase (PPO) in rice plants was estimated and the results are presented in Table - 8 and Table - 9. Application of EMS mutated *Pseudomonas fluorescens* alone was found to augment the plant defense enzyme activities to a higher level. But, the effect was more pronounced during the application of EMS mutated *Pseudomonas fluorescens* along with *Bacillus subtilis* application. It has been known that these two enzymes play a key role in inducing the systemic resistance of rice plants against phytopathogens by degrading the phenolic substances into toxic quinones. The enzymes peroxidases (PO) and polyphenol oxidase (PPO) are the two key enzymes for the oxidation of phenolic compounds in plants and the resulting quinines were effective inhibitors of SH-group of enzymes, which might be inhibiting the pathogens. Higher peroxidases activity was noticed in cucumber roots treated with *Pseudomonas corrugata* and challenge inoculation with *Pythium aphanidermation* [41].

In the present study, inoculation of EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application and challenge inoculation of *Pyricularia oryzae* augmented the peroxidases and polyphenol oxidase activity, host defense related enzymes, in rice plants. Sridhar and Mahadevan [45] reported the increased activity of peroxidase on pathogen infection might be required for an additional deposition of lignin around the lesions induced by pathogen. Rice leaves infected with *Pyricularia oryzae* exhibited increase in peroxidase activity [46]. *Bacillus subtilis* treated plants showed an increase in peroxidases and polyphenol oxidase activity whereas *Cercospora personatum* was challenge inoculated in ground nut [42]. Polyphenol oxidase is a copper containing enzyme oxisizing phenolics to highly toxic to quinines and conferred disease resistance. The increase in the peroxidases and polyphenol oxidase activity due to EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* application and challenge inoculation of *Pyricularia oryzae* are in conformity with the above findings.

CONCLUSION

From this present study, it was concluded that the EMS mutated *Pseudomonas fluorescence* showed maximum plant growth promoting substances, phosphate solubilization when compared to UV mutated isolates. It also showed maximum zone of inhibition against *Pyricularia oryzae*. The treatment containing EMS mutated *Pseudomonas fluorescens* and *Bacillus subtilis* showed maximum plant height, root and shoot dry weight, chlorophyll content, nitrogen content, grain yield, straw yield, reducing sugar level, non – reducing sugar level, phenol content, polyphenol oxidase and peroxidase. Further work on the enhancement of this strain's antagonistic activity and characterization of the mechanism of action is currently underway.

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