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Research Article

EVALUATION OF BIOFERTILIZER POTENTIALITY OF AMMONIA EXCRETORY MULTIPLE HERBICIDE RESISTANT STRAIN OF *ANABAENA VARIABILIS* ON GROWTH AND PRODUCTIVITY OF RICE

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ABSTRACT
Rice deserves a special status among cereals as world's most important wetland crop. The inoculation of nitrogen fixing cyanobacteria is an alternative and sustainable source of nitrogen to increase the rice productivity. Contribution of diazotrophic cyanobacteria to the nitrogen fertility of paddy fields and there by to the productivity and yields of rice crop result from mineralization of their biomass and not from immediate availability of fixed molecular nitrogen. The success of the wet land rice agriculture is heavily dependent on the extensive use of herbicides for selective elimination of weeds of rice-crops. Herbicides are not only detrimental to weeds but to biofertilizer strains of cyanobacteria also. The strains of free living cyanobacteria defective in ammonia assimilation leading to extracellular liberation of N_2 - derived ammonia, and tolerating common rice field herbicides would be ideal for use as biofertilizer in rice field. In this study the effect of ammonia excretory multiple herbicide resistant mutant strain of diazotrophic cyanobacterium *Anabaena variabilis* [*Av* (MHR-Eda)^r] as biofertilizer on growth and yield attributes of rice crop (IR-36) were evaluated. The ammonia excretory multiple herbicide resistant mutant strain treated rice plant exhibited 26% increase in seed germination, 45% increase in seedling growth and 83% increase in grain yield as compared to untreated control. The maximum range of growth and yields parameters were recorded more in this mutant strain when compared to other treatments. The results suggest that the application of this improved cyanobacterial biofertilizer strain significantly enhances growth and productivity of rice plant.

Key Words**:** *Ammonia Excretion, Biofertilizer, Cyanobacteria, Herbicide Resistant, Rice Productivity*

INTRODUCTION
Rice *(Oryza sativa)*, the most widely grown food grain crop, serves as the staple food for about half of the population in world. Rice is one of the first leading ancient cultivated crops of the world. The cultivation of rice is increasing tremendously. In terms of area 55% of the total cultivated land is under rice cultivation. This global grain provides 35-80% of total calorie uptake to more than 2.7 billion people (Gorantla *et al.,* 2005).
Cyanobacteria or blue green algae (BGA) are alternative source of nitrogen to the chemical fertilizers. The choice of biological fertilizer is due to eco-friendly, fuel independent, cost effective and easily availability. The agronomic potential of BGA in rice cultivation was recognized in 1938 by De who attributed the natural fertility of tropical rice field due to nitrogen fixing cyanobacteria. Beneficial effects of diazotrophic cyanobacteria in increasing soil fertility and crop productivity in rice agriculture have been identified, studied and well documented (Singh, 1961; Venkataraman, 1972; Roger, 1995; Hegde *et al.,* 1999; Paudel *et al.,* 2012).
Although rice fields provide favourable environment for the development of cyanobacteria, the biologically fixed nitrogen by BGA becomes available to rice plant in gradual manner through oxidation and decomposition. However, under field conditions, only a part of this fixed nitrogen is available to the rice plants, some being either reincorporated by the microflora or volatilised. Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of the rice plants. The breakthrough for this problem will be the development of cyanobacterial strains, which release ammonium continuously into the field. Sporadic reports are available regarding isolation and

characterization of MSX (L-methionine-D, L- sulfoximine), EDA (ethylene diamine) resistant strains defective in GS (Glutamine synthetase) activity capable of excreting N_2- derived ammonium into external environment (Spiller *et al.,* 1986; Mahasnesh *et al.*, 1994; Healy *et al.*, 2003). The isolation of such ammonia excretory mutants leads to the incorporation of single essential feature into any ideal biofertilizer strain.

However, the success of the wet land rice agriculture is heavily dependent on the extensive use of herbicides for selective elimination of weeds of rice-crops. Laboratory studies on the effect of various herbicides on diazotrophic cyanobacteria have shown them to be inhibitory on different metabolic processes (Leganes and Fernandez-Valientl, 1992; Fairchild *et al.*, 1998; Aslim and Ozturk, 2009). Reports are also available regarding isolation of herbicide (s) resistant strains of N_2-fixing cyanobacteria (Singh *et al.*, 1986; Vaishampayan *et al.*, 2000; Singh *et al.*, 2012).

Unfortunately little efforts were made to incorporate most of these essential features in an ideal biofertilizer inoculum (Modi *et al.,* 1991) and have not been systematically and comprehensively tested outdoors for real practical purposes. Thus the concept of using N_2-fixing cyanobacteria as nitrogenous biofertilizer is not fully explored and major short comings are still limiting the wide and effective utilization of this bio-fertilization technology.

Hence attempt was made to isolate a spontaneous mutant of a local rice isolate *Anabaena variabilis* exhibiting resistance to six commonly used rice field herbicides viz. Arozin, Alachlor, Butachlor, 2,4-D, Atrazine and DCMU (Singh *et al.,* 2011; Singh *et al.*, 2012). Further this multiple herbicide resistant strain was screened for the presence of spontaneous mutant resistant to growth inhibitory concentrations of EDA (ethylene diamine) for the isolation of ammonia excretory strain. The biofertilizer potentiality of the developed mutant strain was evaluated in open air pot experiments. The ammonia excretory multiple herbicide resistant mutant strain [*Av* (MHR-Eda)^r] exhibited highest capability of promoting growth and grain yield of rice plants.

MATERIALS AND METHODS

Organism and Growth Conditions

The axenic clonal culture of wild type N_2-fixing cyanobacterium *Anabaena variabilis*, a rice field isolate, its spontaneous mutant [*Av* (MHR)^r] exhibiting resistance to the lethal dosages of Arozin, Alachlor, Butachlor, 2,4-D, Atrazine and DCMU (Singh *et al.*, 2011; Singh *et al.*, 2012) was cultivated in BG_{11} medium (Rippka *et al.*, 1979) devoid of any combined nitrogen source (N_2-medium). Cultures were incubated in an air-conditioned culture room maintained at 25± 1°C fitted with cool day fluorescent light. The photon flux density of light on the surface of the vessel was 45 $\mu Em^{-2} s^{-1}$ for 18 h d^{-1}.

Isolation of EDA (Ethylene diamine) Resistant Mutant of Multiple Herbicide Resistant Mutant Strain of A. Variabilis

The wild type and multiple herbicide resistant mutant strain [*Av* (MHR)^r] did not survive beyond a concentration of 10 mg L^{-1} and 15 mg L^{-1} respectively of EDA accordingly, its diazotrophically grown cultures (5.0x10^7 CFU) were seeded in diazotrophic nutrient plate containing growth inhibitory concentration of EDA to select out spontaneously occurring resistant mutant clones.

Colonies of mutant appearing on the nutrient plates containing EDA were tested 3-4 times for their stability by streaking them on fresh nutrient plates containing same concentration of EDA. Stable mutant clones thus obtained were grown and maintained in N_2-medium under photoautotrophic growth conditions as described above.

Soil and Rice Variety

For outdoor pot experiments sandy loam soil collected from agricultural field was used. The soil was cleared by removing plant debris, roots and rock particles. It was finally milled in a uniform mixture. The seeds of dwarf variety of rice (*Oryza sativa* IR-36) for biofertilization were procured from the Rice Research Center, Department of Plant Breeding and Genetics, Jawaharlal Nehru Agriculture University, Jabalpur, Madhya Pradesh, India.

Weather Conditions

The climate of Jabalpur (Madhya Pradesh) India is sub-tropical. The average annual rainfall is 1300-1400 mm, mostly concentrated from July-October. Accordingly, pot experiments were conducted during this rice growing season. The weather was mostly cloudy with temperature ranging from 25°C-35°C.

Impact of Cyanobacterial Inoculants on Seed Germination, Seedling Growth and Plant Productivity

The experiment was set in petriplates containing soil. The soil was moistened with water and in each petriplate 20 seeds were sown. Each petriplate was treated separately with 10 ml (3.0 µg chl *a* ml^{-1}) of wild type, mutants and one plate was treated with 1 mM of urea. Petriplate containing only seeds without any treatment served as control. After five days, germinated seeds in each set of three petriplates were counted. The numbers of seeds germinated was expressed as percentage of those in control plates.

For seedling growth the experiment was set up in plastic pots (11 cms x 6 cms) containing soil and 20 seeds each. The soil was moistened with water. Each pot in triplicate was treated individually with 10 ml (3.0 µg chl *a* ml^{-1}) of wild type, mutants and one plastic pot was treated with 1 mM of urea. Pot containing only seeds without any treatment served as the control. These pots were kept under outdoor conditions. After 12 days, morphology and height of the plants were recorded as a measure of the growth potential.

For plant productivity the experiment was conducted in earthen pots (22.86 cm x 22.86 cm). Finely milled, cleaned sandy loamy soil was filled in these pots, weighing approximately 8 kg for each pot. The pots were flooded with water, creating a waterlogged condition. Pots were divided in two sets in triplicate. One set was treated with the recommended field dosages of herbicides and the other was not exposed to any herbicide. In each pot, 10 seedlings of same height (15-17 cm) already growing in the same environment were transplanted. Each pot was inoculated individually with 100 ml (7.0 µg chl *a* ml^{-1}) of wild type, *Av*$(MHR)^r$, *Av*$(MHR\text{-}Eda)^r$ and one pot was supplemented with 1 mM of urea. Pots containing only seeds without any treatment served as the control. First inocula delivery was made 6 days after transplanting the seedlings. Thereafter, two more applications were made at an interval of 20 days, similar to the first application. All the pots were grown in open air exposed to natural climatic conditions with regular watering.

After a growth period of 90 days from the time of transplanting, the pre harvest data were collected for further analysis. Following parameters were examined for the rice crop:

(i) Plant height (ii) Flag leaf length and width (iii) Number of tillers per plant (iv) Number of seeds per panicle (v) Number of seeds per plant (vi) Weight of straw per plant (vii) Grain yield per plant.

Statistical Analysis

The data shown are mean values ± standard errors. Analysis of variance (ANOVA) was used to assess the significance (p=0.05) of the mean values of treatments and control of grain yield and the differences were compared using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Isolation of EDA (Ethylene diamine) Resistant Mutant of Multiple Herbicide Resistant Strains of A. Variabilis

Spontaneous mutation frequency leading to production of EDA resistant in multiple herbicide mutants was 1.5 x 10^{-7}. The mutant was stable and showed normal heterocyst frequency and ammonium repressible nature of heterocyst formation and nitrogenase activity. Growth measured in terms of chlorophyll *a* showed that *Av* $(MHR\text{-}Eda)^r$ exhibited faster growth rate than wild type as well as to *Av* $(MHR)^r$. The specific growth rate constant calculated for *Av* $(MHR\text{-}Eda)^r$, *Av* $(MHR)^r$ and *Av* (P) is 1.34, 1.23, 1.07 and doubling time is 17.9, 19.5, 22.4 hrs. respectively.

Influence of bio Inoculants on Seed Germination, Seedling Growth and Plant Productivity

The results indicate (Fig-1) that there was significant increase in rate of rice seed germination over control in all the treatments. *Av*$(MHR\text{-}Eda)^r$ treated seeds exhibited highest enhancement (26%) in germination of rice over control.

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The results of seedling growth (Fig-1) indicate that *Av*(MHR)^r inoculated pots showed 20% increase over control. However, the rice seedling height achieved by urea and *Av*(MHR-Eda)^r treated pots exhibited 45% increase over untreated control seedlings.

Influence of Bio Inoculants on Growth and Yield of Rice Plant

Maximum height of plants were achieved in soil inoculated with *Av*(MHR-Eda)^r followed by urea, *Av*(MHR)^r and *Av*(P) (Fig-2). Flag leaf length was also highest in the plants inoculated with ammonia excretory multiple herbicide resistant mutants exceeding that in plants treated with either urea or other strains (Figure 3). The number of tillers were significantly higher in plants treated with urea, *Av*(MHR-Eda)^r than plants inoculated with wild type *A. variabilis* consequently the quantity of straw produced per plant followed a similar pattern. There was 44% increase in the seeds per panicle in *Av*(MHR-Eda)^r treated plants followed by urea, *Av*(MHR)^r and *Av* (P) over untreated control. Tremendous increase in the number of seeds per plant was recorded in the plants inoculated with ammonia excretory multiple herbicide resistant mutant in comparison to untreated control. Lowest increase (18%) in grain yield was recorded in plants inoculated with *Av* (P) followed by urea (75%), *Av*(MHR)^r (76%), and *Av*(MHR-Eda)^r (83%) inoculated plants over untreated control plants (Table-1). The application of diazotrophic cyanobacteria as biofertilizers in the cultivation of wet-land rice has a beneficial effect on the growth and yield (Venkataraman, 1979; Watanabe and Roger, 1984; Grant *et al.*, 1986). The paddy field ecosystem provides a favourable environment for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and nutrient availability. Thus the use of cyanobacteria as a biofertilizer for rice fields is very promising but limited due to fluctuation in quality and quantity of inoculum and its physiological attributes in varied agroecological regions.

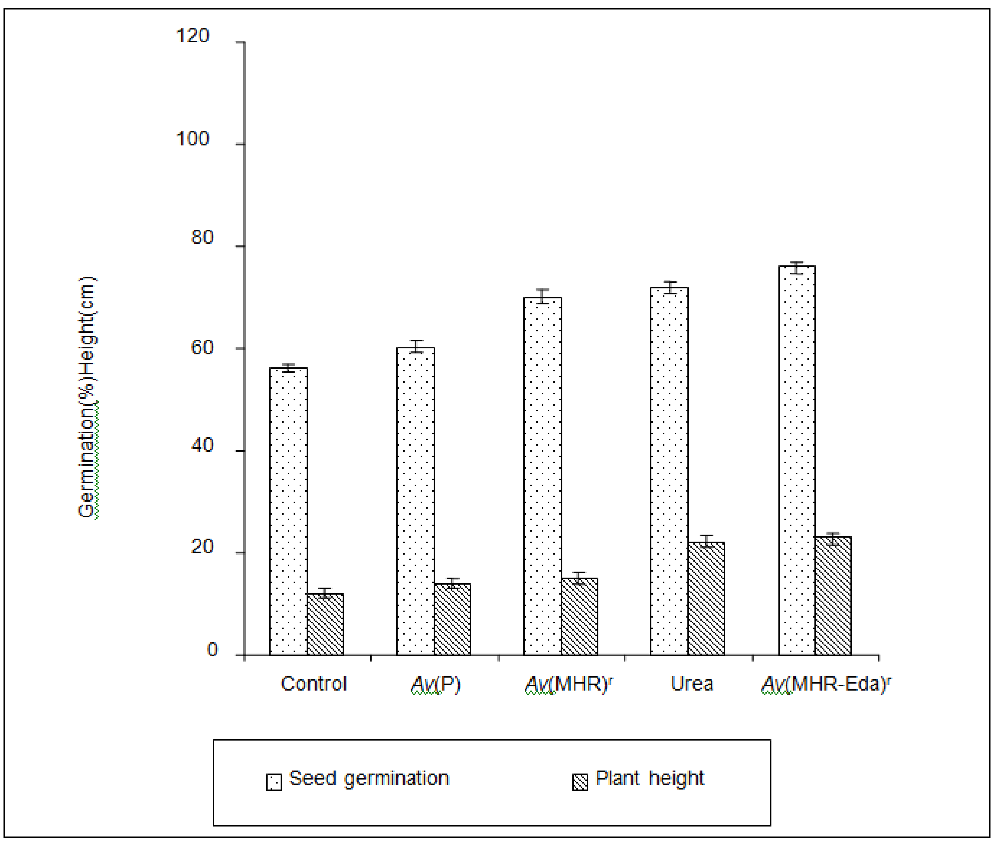

Figure 1: Impact of wild type and mutant strains on seed germination and on the average height of rice seedlings (bars indicating standard errors)

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Table 1: Effect of wild type and mutant strains on overall productivity of *Oryza sativa* IR-36. Data was collected 90 days after transplanting the seedlings in pots

Treatments	**No. of Tillers/ Plant**	**No. of Seeds/ Panicle**	**No. of Seeds/ Plant**	**Wt. of Straw/ Plant (gm)**	**Grain Yield/ Plant (gm)**
Control	3±0.2	78±1.2	202±2.2	33.5±2.1	3.2±1.3 a
Urea	6± 0.3	125±1.8	549±1.8	125.0±1.9	12.9±0.8 gh
Av(P)	4±0.2	90±2.3	219±1.6	57.0±1.6	3.9±0.8 a
Av(MHR) ^{r}	7± 0.3	131± 2.0	554±2.4	127.0±2.7	13.5±0.5 gh
Av(MHR) Eda^{r}	8 ± 0.2	140 ±2.5	575±2.0	137.5± 2.2	18.7±1.2 h

Each value is an average (± SEM) of three independent experimental determinations.
Different letters in the grain yield column denote statistically significant differences among treatments according to Duncan's Multiple Range Test at p=0.05.

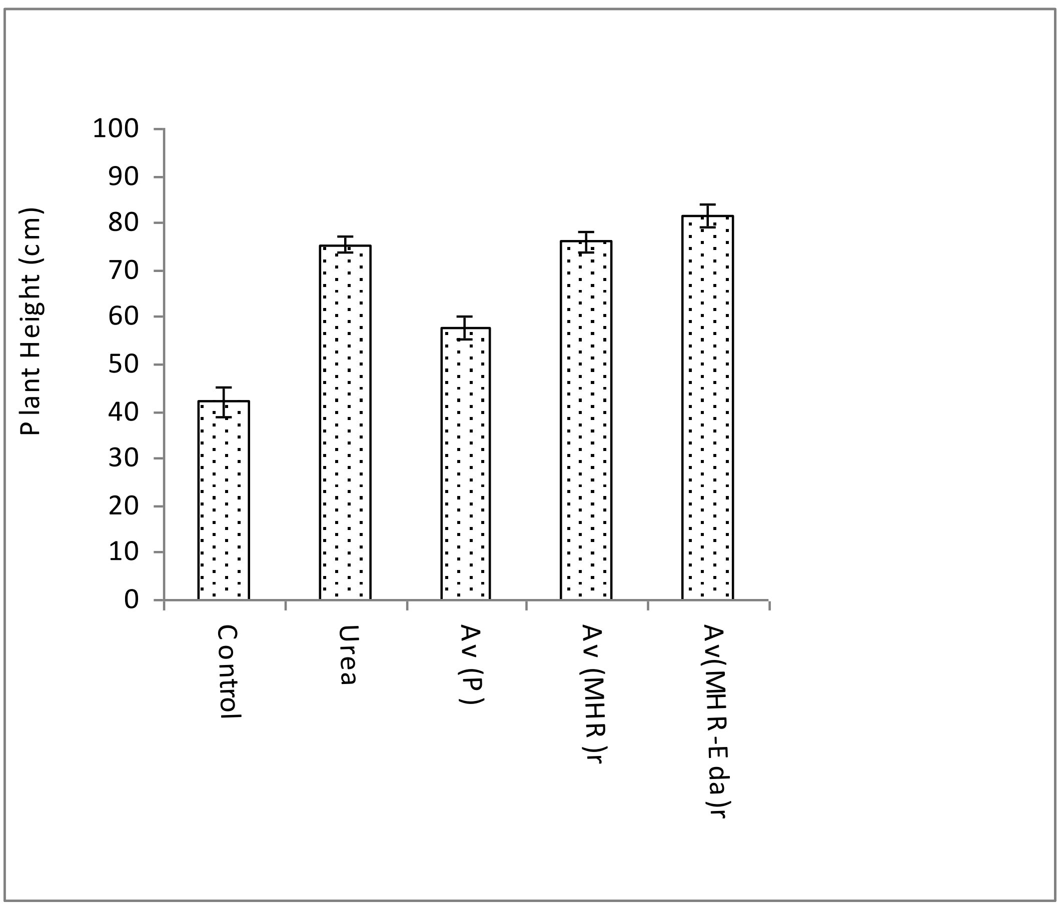

Figure 2: Impact of wild type and mutant strains on height of rice plants (bars indicating standard errors)

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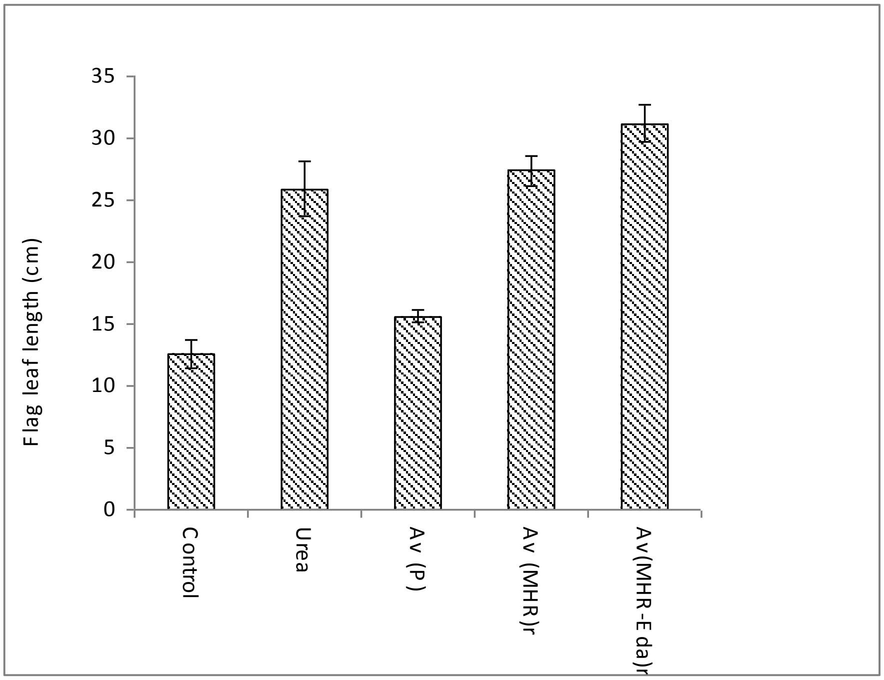

Figure 3: Impact of wild type and mutant strains on flag leaf length of rice plants (bars indicating standard errors)

In the present study there was significant enhancement in seed germination and in height of seedling over control in all the treatments. This could be due to the excretion of extra cellular substances (Adam, 1999; Ordog, 1999). Cyanobacteria excrete a great number of substances that influence plant growth and development (Rodriguez *et al.,* 2006). These micro-organisms have been reported to benefit plants by producing growth-promoting regulators (the nature of which resembles gibberellin and auxin), vitamins, amino acids, polypeptides, antibacterial and antifungal substances that exert phytopathogen biocontrol and polymers, especially exopolysaccharides, that improve soil structure and exoenzyme activity. While working on the algae of Indian paddy fields, Gupta and Lata (1964) observed that cyanobacteria accelerated seed germination and promoted seedling growth. In addition, they also observed that both the yield and the quality of the grains were improved in proteins content. It seems very likely that the beneficial effect of the algae on the rice crop may not be restricted to their capacity to fix atmospheric nitrogen alone, but also they have additional beneficial roles, such as releasing of bioactive substances.

It is also evident from the results that algalization increased the growth and yield of plants. Mechanisms used by microbes to stimulate plant growth include biofertilization (increasing the supply of mineral nutrients to the plant), biological control (elimination of the plant enemies including microbial pathogens, insects and weeds) and direct plant growth production by delivering plant growth hormones (Lugtenberg *et al.,* 1991). Biofertilization techniques using cyanobacteria are recommended for increasing the rate of seed germination and growth parameters of many plants (Sharma *et al.,* 2012).

The stimulative and profoundly significant impact of cyanobacterialization through use of ammonia excretory multiple herbicide resistant strain of diazotrophic cyanobacterium *Anabaena variabilis* was found to be evident on seed germination, seedling growth and grain yield of rice over the control. Ammonia excreting mutants of cyanobacteria have been isolated for cyanobacteria like *Nostoc muscorum, Anabaena variabilis* (Thomas *et al.,* 1991; Spiller *et al.,* 1986; Healy *et al.,* 2003) and the inoculation of such mutants to rice and wheat plants in outdoor pots has been found to produce more panicles and grain yield than plants inoculated with the parent strain (Kamuru *et al.,* 1997). Moreover mostly biofertilizer potentials of wild type cyanobacterial strains have been evaluated (Relwani and Subrahmanyan, 1963; Venkataraman, 1979; Selvi and Sivakumar, 2012) and few reports of herbicide resistant mutants (Vaishampayan and Hemantaranjan, 1998; Singh and Datta, 2007) but no ammonia excretory herbicide resistant strain seem to have been evaluated.

Thus, it may be concluded that the enhancement in seed germination, seedling growth and grain yield of rice plants over the uninoculated control plants clearly demonstrates the competence and biofertilizer potentials of such mutationally improved strain.

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