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ISOLATION OF PARAQUAT RESISTANT MUTANT OF A CYANOBACTERIAL BIOFERTILIZER *Nostoc linckia* WITH ABILITY TO DEREPRESS NITROGEN FIXATION UNDER AMMONIA MEDIATED CONDITION

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Abstract: *Nostoc linckia, a nitrogen fixing cyanobacterium, was found growing luxuriantly in the paddy fields of Barh (Patna, Bihar) during rice growing season. The cyanobacterium, in our preliminary experiments, exhibited strong potentiality to serve as bio-N-fertilizer alternative to chemical-N-fertilizers. The experiments also indicated that this efficiency of the cyanobacterium is neutralized due to frequent employment of a bipyridylium herbicide paraquat in the paddy fields, which caused massive lysis of the cyanobacterial cells above 50 ppm doses resulting in their ultimate death. The nitrogen fixing ability of N. linckia was also repressed in the presence of chemical-N-fertilizers in the paddy fields.*

With a view to inducing paraquat resistance coupled with nitrogenase derepression the unicelled N. linckia cultures were treated with 120ppm of MNNG for 30 minutes, washed thoroughly and spread over solid Chu10(M) medium containing 50 or 75 ppm of paraquat supplemented with 1mM ammonium chloride and 0.2mg/mL sodium azide. The treated cultures were incubated under standard cultural conditions. After 30 days of incubation a few cyanobacterial colonies found growing on the agar plates were isolated and grown in fresh Chu10 (M) N_2 medium up to ten successive generations and then transferred in media containing 50/75 ppm paraquat and 1mM NH_4Cl. The colonies exhibiting normal growth in paraquat containing media and forming heterocysts in the presence of ammonium medium were isolated and designated as "Paraquat Resistant, Nitrogenase Derepressed Mutants." Such mutants can be used as efficient biofertilizer even in the paraquat treated; chemical nitrogen supplied paddy fields for better yield of rice since the farmers are not intended to discontinue the employment of pesticides and nitrogenous fertilizers during rice cultivation in fear of suspected reduced paddy yield.

Keywords*: Paraquat Resistance, Nitrogenase derepression, Nostoc linckia.*

Introduction: Farmers apply synthetic chemical fertilizers in the paddy field as the availability of sufficient amount of nitrogen boosts the productivity of rice per unit area. But the escalating cost of manufacture of fertilizers as well as their transportation to the crop fields is becoming an economical constrains for the poor farmers. Thus the concept of replacing chemical fertilizers by naturally growing eco-friendly biofertilizers is gaining momentum. The role of nitrogen-fixing cyanobacteria as alternative source of nitrogen supplement in the rice fields has been realized [1-5]. Advocated for the utilization of algal biofertilizers for rice crops as a supplement to chemical fertilizers for the safety of humen health and environment [6].

Initial screening experiments confirmed that the nitrogen–fixing cyanobacterium *N. Linckia* has strong potentiality to act as bio-N-fertilizer, alternative to chemical-N- fertilizers during rice cultivation in the paddy fields of Barh [7]. However, the cyanobacterial strains were found to be very sensitive to the herbicide paraquat which is frequently employed in this locality for weed control in the paddy fields [7] as a result of which not only the beneficial effects of these eco-friendly, naturally growing organisms is adversely affected but also the essentiality of application of large doses of ecologically harmful synthetic-N-fertilizers is supposed to be enhanced to sustain the better yield of rice [8]. Also have shown the damaging

effects of pesticides on the growth and DNA organization of cyanobacterial species growing alongwith rice cultivars [9-11]. The application of large doses of synthetic-N-fertilizers results in the repression of nitrogen fixing activity of the few surviving cyanobacterial cells and under such situation the surviving cyanobacterial cells are forced to become nitrogen consumers to maintain their own growth in contrast to nitrogen suppliers to the rice plants [12]. Hence it is desirable to develop ammonia derepressible paraquat resistant cyanobacterial mutants for algalization of wet rice fields so that the improved cyanobacterial strains could serve as viable and efficient bio-N-fertilizers in herbicide treated paddy fields [13, 14, 15].

The present study was, thus, undertaken with a view to making an attempt to isolate certain ammonia derepressible and/or paraquat resistant mutants of *N. linckia* so that their genetically improved strains could be employed as efficient bio-N-fertilizers in the paraquat treated paddy fields.

Materials and Methods

Nostoc linckia is a photoautotrophic, filamentous, heterocystous, nitrogen-fixing cyanobacterial species was collected from local rice fields , isolated to unialgal , axenic state and grown in Chu 10(M) medium under controlled cultural conditions (Temperature 28 ±2 ^{o}C, Light Intensity 2500 lux, L/D cycle - 14h/10h.).

With a view to isolating paraquat resistant mutants of *N. linckia*, spontaneous as well as induced mutagenesis techniques were employed. For spontaneous mutation the exponentially growing NO_3^-containing liquid mediated algal cultures were harvested, washed thoroughly in N_2 medium to remove the traces of nitrate, recentrifuged and then fragmented into unicells with the help of sterile glass beads, rewashed and then transferred to sterile N_2 medium to make a homogeneous suspension. The suspension was left overnight and then equal amount of each algal suspension (1mL) was spread separately over N_2 medium solidified by 1.2% difco-bacto-agar supplemented with 20, 50 or 75ppm of paraquat. 50 petri dishes were used in each set of experiment and the culture plates were incubated under standard culture conditions for 30 days. Algal colonies appearing on the treatment plates during incubation period were scored. Suspension of the same algal populations diluted to 1:1000 was spread on paraquat free N_2 medium for scoring total number of "Colony Forming Units" (CFU) in control. Frequency of paraquat resistant spontaneous mutants was determined by counting the total number of mutant colonies in proportion to the total number of CFU multiplied by 1000 [16,17].

For induction of Paraquat resistance with or without nitrogenase derepression in *N. linckia*a potent mutagen for cyanobacteria MNNG (N-methyl-N-nitro-N-nitrosoguanidine) [18, 19] and sodium azide suggested [20] were selected.

Initially the effects of different concentrations of MNNG and sodium azide on the survival of the parent cyanobacterial strains were examined. For this the exponentially growing liquid mediated cultures were harvested, fragmented into unicells and treated with different concentrations of MNNG or sodium azide for 30 minutes. The treated organisms as well as their untreated controls were washed thoroughly and spread over different sets of solid N_2 medium (1.2% difco-bacto-agar). After a fortnight of incubation viable cyanobacterial colonies appearing on the variously treated samples were scored and compared with control to determine the percentage of survival [21]. Afterwards, 20, 50 and 75% survival doses of MNNG and sodium azide were selected and used to induce desired mutations.

The unicelled algal cultures were treated with the selected doses of MNNG for 30 minutes, washed thoroughly with N_2 medium and then spread separately on N_2 and NO^-_3 containing solid media supplemented with 20,50 or 75 ppm of paraquat. Untreated cultures were spread over paraquat free solid media as control. After 30 days of incubation under standard conditions the algal colonies appearing on the paraquat containing plates were scored and compared with control.

For the selection of nitrogenase derepressed mutants, the MNNG treated algal cultures were spread over the agar medium containing 1mM NH_4Cl supplemented with 0.2 mg/mL sodium azide as suggested [22] and after 30 days of incubation under standard conditions, the algal colonies appearing on the treatment plates were scored. The mutant colonies appearing on the different treatment plates with the following markers were selected.

1. Mutation for paraquat resistance leading to a constant supply of reductants through the intact photosynthetic system for nitrogen- fixation irrespective of paraquat treatment.

2. Mutation for derepressed nitrogenase for constitutive nitrogen-fixation irrespective of the level of fixed nitrogen in the external environment.

The selected mutant colonies having the above markers were inoculated on fresh solid and liquid N_2 as well as NO_3^-media. Ten subsequent transfers were made in the two nitrogen containing media in both solid and liquid culture conditions to ascertain the stability of the mutants. The isolated mutants which maintained paraquat resistance and/or heterocyst formation in NO_3^-media even after ten such transfers were considered to be stable paraquat resistant/ nitrogen derepressed mutants. Such isolates were characterized for their growth and heterocyst frequency in N_2 and NO_3^- media supplemented or unsupplemented with paraquat. A clonal population of mutants was raised from the stable cultures in N_2 or NO_3^- medium without paraquat.

Results

The survival pattern of *N. linckia* treated with different concentrations of paraquat and MNNG is illustrated in table 1 whereas its survival pattern in N_2 media containing graded concentrations of sodium azide are represented in table 2.

Table 1: Percent survival of *Nostoc linckia* treated with different concentrations of paraquat and MNNG in N_2 medium.

Concentration of Paraquat(ppm)	Percent survival	
	Paraquant Treated	MNNG Treated
5	76.2 ± 1.12	97.2 ± 1.46
10	64.6 ± 1.64	90.6 ± 2.31
20	50.2 ± 2.04	84.7 ±1.15
50	29.3 ± 1.18	80.0 ± 1.48
75	0.8 ± 0.04	70.4 ± 1.34
100	0.0	62.1 ± 0.36
120	0.0	50.1 ± 1.24
150	0.0	20.2 ± 1.88

Table 2: Percent survival of *Nostoc linckia* in N_2 medium containing different concentrations of sodium azide.

Concentration of sodium azide (mg/ mL)	Percent survival
	Nostoc linckia
0.01	99.2 ± 1.02
0.02	96.4 ± 1.12
0.05	84.6 ± 1.16
0.10	72.3 ± 1.12
0.20	50.6 ±1.23
0.50	23.2 ± 1.06
1.00	4.2 ± 1.04

N. linckia exhibited 50% survival in N_2 medium when treated with 20 ppm of paraquat whereas at 50 ppm level the survival was 30%.The algal strain exhibited negligible survival at 75 ppm level while at 100 ppm it could not survive at all.

When *N. linckia* was treated with different concentrations of MNNG, ranging from 5 ppm to 150 ppm, the survival was 50.1 at 120 ppm dose. The lower doses supported better growth of the alga than the respective paraquat doses. As regards to the toxicity of sodium azide, about 50% survival of the alga was obtained in the N_2 medium added with 0.2 mg/mL of sodium azide (Table 2).

On the basis of survival pattern the 20, 50 and 75 ppm levels of paraquat were selected for an attempt to isolate paraquat resistant mutants of *N. linckia* either through spontaneous mutagenesis or through induction with 120 ppm of MNNG. In order to isolate nitrogenase derepressed mutants, 0.2 mg/mL dose of sodium azide was used as marker in 1mM ammonium chloride containing medium.

When cells of *N. linckia* either treated or untreated with 120 ppm of MNNG were inoculated on solid N_2 media containing 20, 50 or 75 ppm of paraquat a few cyanobacterial colonies were found to be growing on agar plates within 20 to 30 days of incubation under standard condition. The colonies appearing without MNNG treatment were considered as spontaneous paraquat resistant mutants whereas those appearing from MNNG treated populations were designated as induced paraquat resistant mutants. They were referred to as SpR_{20}, SpR_{50}, SpR_{75}, Ipr_{20}, IpR_{50} and IpR_{75} on the basis of the doses of paraquat for which the resistance was developed. The colonies appearing from MNNG treated populations on media containing sodium azide and ammonium chloride were termed as $IpRNd_{20}$ and $IpRNd_{50}$. The number of such

colonies was scored and their mutation frequencies were estimated which are illustrated in Tables 3 and 4.

Table 3: **Mutation frequencies of *Nostoc linckia* scored on solid N_2 medium containing different concentrations of Paraquat.**

Concentration of Paraquat (ppm)	Mutation Frequencies	
	Untreated with MNNG	Treated with MNNG
20	$4.6 \pm 1.5 \times 10^{-7}$	$5.2 \pm 1.6 \times 10^{-3}$
50	$3.4 \pm 1.3 \times 10^{-7}$	$5.1 \pm 1.2 \times 10^{-3}$
75	$1.7 \pm 0.7 \times 10^{-9}$	$3.8 \pm 1.4 \times 10^{-4}$

Table 4: Mutation frequencies of *Nostoc linckia* treated with 120 ppm of MNNG and grown on solid NH_4^+ medium supplemented with 0.2 mg/mL sodium azide and different concentrations of Paraquat.

Concentration of Paraquat (ppm)	Mutation Frequencies
	Nostoc linckia
20	$4.4 \pm 1.6 \times 10^{-5}$
50	$3.2 \pm 1.2 \times 10^{-5}$
75	Nil

The cyanobacterial species *N. linckia* produced paraquat resistant mutants both in spontaneous and Induced manners at each of the paraquat doses of 20,50 and 75 ppm, although there was decrease in the mutation frequency with increase in paraquat doses. The frequency of MNNG treated populations (IpR) was higher than the spontaneously developed populations (SpR) at each dose of paraquat.

When MNNG treated populations of *N. linckia* was grown on plates containing ammonium chloride and sodium azide supplemented N_2 media, some mutant colonies were found to be growing at 20 and 50 ppm paraquat containing media but no colony could develop on 75 ppm paraquat containing media. These colonies represented the nitrogenase derepressed mutants of *N. linckia*. However, the frequencies of IpRNd were marginally lower than IpR at 20 and 50 ppm levels respectively. The isolated mutant colonies were tested for their growth behaviour and heterocyst forming ability in N_2 and NO_3^- media and the results are illustrated in Tables 5 &6 .

Table 5: Growth and heterocyst frequencies of Paraquat resistant mutants of *Nostoc linckia* in N_2 medium after ten successive generations growth in Paraquat free medium.

Concentration of Paraquat (ppm)	Optical Density (663 mm)	Heterocyst Frequency
0 CONTROL	0.48 ± 0.015	4.6 ± 0.21
20	0.49 ± 0.016	4.5 ± 0.22
50	0.45 ± 0.011	4.5 ± 0.18
75	0.44 ± 0.012	4.3 ± 0.16

Table 6: Growth and heterocyst frequencies of Paraquat resistant nitrogenase derepressed mutants of *Nostoc linckia* in NO_3^- medium after ten successive generations growth in Paraquat free medium.

Concentration of Paraquat (ppm)	*Nostoc linckia*	
	Optical Density (663 mm)	Heterocyst Frequency
0 CONTROL	0.61 ± 0.14	0.0
20	0.62 ± 0.18	4.8 ± 0.22
50	0.62 ± 0.21	4.8 ± 0.22
75	0.58 ± 0.61	4.2 ± 0.18

The mutants grew well in both the culture media and formed heterocysts almost like their parents grown as control. However, only the nitrogenase derepressed mutants formed heterocysts in NO_3^- media. The growth patterns were almost similar for both spontaneous and induced mutants in different nitrogen media. The selected mutants were grown in paraquat free medium for ten successive generations and there after some algal cells were transferred in paraquat containing media. The mutants grew well forming heterocysts as screened after their isolation and thus maintained their paraquat resistant nature.

Discussion

The experimental results confirm the earlier findings that the herbicide paraquat is very toxic to the cyanobacterial biofertilizer *N. linckia*. The herbicide appears to be even more toxic than MNNG and sodium azide since these latter two chemicals have caused 50% killing of the cyanobacterial cells at the treatment doses of 120 ppm and 200 ppm levels respectively whereas the paraquat treatment at only 20 ppm level has given similar killing effect. Also found paraquat more toxic than MNNG for *Nostoc muscorum* [23]. However, paraquat does not appear to impart any negative effect on the photosynthetic activity and nitrogen metabolism

of the cyanobacterial species since it is able to form heterocysts in paraquat supplemented N_2 medium but failed to do so when grown in paraquat added NO_3^- medium. As proposed [24] the toxicity of paraquat is due to massive lysis of the cyanobacterial cells by hydroxyl or peroxy radicals which are produced inside the living cells through oxidation of paraquat. Moreover, incubation of *N. linckia* cells in paraquat supplemented medium up to 30 days have resulted in the development of some spontaneous mutants resistant to 20,50 and 75 ppm doses of paraquat. Such mutants have also developed at higher frequencies when the cyanobacterial species was treated with a potent chemical mutagen MNNG. The origin of paraquat resistant mutants either through spontaneous or induced mutagenesis might be due to alteration in cellular permeation or synthesis of certain enzyme inside the paraquat resistant mutants which may metabolize the toxic radicals produced during paraquat metabolism in culture condition to cyanobacterial cells against the lytic effects. Also succeeded in isolating a variety of mutants of *Nostoc muscorum* through treatment with MNNG which were resistant to streptomycin and methylamine [23].

MNNG treatment has also given rise to some mutants of *N. linckia* which are not only resistant to paraquat but are also able to form heterocyst in NO_3^- media, a feature which is lacking in their parental strains. The heterocyst frequencies are almost equal to those formed by the parental strains grown in N_2 medium. The mutants have thus acquired the capacity to derepress nitrogenase and carry out nitrogen fixation in the presence of nitrogen in the external environment. Such mutants were earlier isolated from wild type strains of *N. muscorum* [20, 25] and an isolate of *A. azollae* [26]. The technique employed in the present experiment was based on the findings [22] that azide or cyanide may be reduced to ammonium as a result of nitrogenase action and these compounds were proved to be possible selective reagents to either isolate *nif* mutants or mutants derepressed for nitrogenase synthesis with the excess of ammonium. In the selection of derepressed mutants the wild type would be killed by cyanide- or azide supplemented medium with ammonium as the nitrogen source. A derepressed mutant is, therefore, forced to evolve nitrogenase even in ammonium medium in order to detoxify azide or cyanide by reducing it to ammonia. Succeeded in isolating amitrole-resistant mutant of *N. muscorum* showing substantial derepression of nitrogenase activity under ammonium supplemented conditions and also supported increase in paddy yield [27].

On the basis of all these observations it can be speculated that the paraquat resistant mutants with or without capability to derepress nitrogenase activity, isolated during the present course of investigations, might be a short in the arms of rice cultivators for enhancing their crop yield in paraquat treated crop fields, even in the presence of certain nitrogenous fertilizers.

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