Original article

Comparative nitrogen fixation by mesophilic (HTS) vis-à-vis thermotolerant mutants (HTR) of *Azotobacter chroococcum* at high temperature and their effect on cotton biomass

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Abstract

Introduction and objective: The amount of nitrogen fixed by *Azotobacter* varied from 0.02-0.25 KgN/ha/d and has been reported to be variable with various physiological and environmental factors. The nitrogen fixation by mesophilic soil isolates and their analogue resistant mutants of *Azotobacter chroococcum* was compared with their thermotolerant mutants at three different temperatures (30°C, 37°C, 42°C).

Material and methods: Mutants spontaneously resistant to methylammonium chloride were derived originally from a soil isolate of *A. chroococcum.* Ammonia excretion ability of different strains of *A. chroococcum* was determined by the method of Chaney and Marbach (1962). Nitrogenase activity of different strains of *A. chroococcum* was assayed by growing the cultures in the Burk medium. Effect of *Azotobacter* inoculation on cotton biomass was followed.

Results: Growth and ammonia excretion were related in both mesophilic as well as thermotolerant mutants at 30°C but not at elevated temperature (42°C). A thermotolerant strain (HTR71) has excreted as much as 24.1µg ammonia/ml of culture broth in a sucrose supplemented synthetic Jensen's medium at elevated temperature of 37°C under stationary conditions of growth while a thermotolerant mutant (HTR54) showed nitrogenase activity of 46.13 nmoles C_2H_4 /h/mg protein at 37°C while Mac68, a mesophilic strain from which HTR54 is derived by mutation has nitrogenase activity of 35.07 nmoles C_2H_4 /h/mg protein. Thermotolerant mutants showed better performance over mesophilic strains on the cotton biomass under pot house conditions.

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Conclusion: The present study suggest that these thermotolerant mutants would be the ideal diazotroph to be used as biofertilizers for crops in semiarid regions having high temperature (40^{o}C or above).

Keywords: *Azotobacter chroococcum;* Diazotroph; Mesophilic; Thermotolerant; Biofertilizers

Introduction

The capacity to fix nitrogen is widely distributed in phyla of Bacteria and Archaea. Among bacteria, *Azotobacter* is one of the best fixer of nitrogen**.** The amount of nitrogen fixed by *Azotobacter* varied from 0.02-0.25 KgN/ha/d and has been reported to be variable with crop, soil status and environmental conditions [1-3]. Soil conditions like available nitrogen, soil moisture and soil temperature are known to affect various physiological properties [4].

In nitrogen fixing bacteria, the immediate product of nitrogen fixation is ammonia which goes into cellular synthesis through transamination reaction [5,6]. Produced ammonia is rapidly metabolized through ammonia assimilating enzymes and normally is not excreted into growth medium. However, ammonia synthesis and its subsequent assimilation are two different processes brought about at different sites. Thus, if the former process is relatively rapid as compared to the assimilation process, cells might release ammonia into growth medium [7]. Moreover, ammonia synthesis and its utilization are highly controlled and regulated processes [8].

Nitrogen fixing microorganism can satisfy their nitrogen requirement by metabolizing a variety of compounds. Their most important property is the ability to reduce dinitrogen with consumption of ATP, through enzyme complex nitrogenase [9,10]. It also reduces acetylene to ethylene, a property used to estimate the enzyme nitrogenase by gas liquid chromatography (GLC). Acetylene reduction assay method is a rapid technique to quantify the amount of nitrogen fixed [11] *in vitro* and in rhizosphere. The synthesis of nitrogenase and its activity are regulated at several levels. Nitrogenase synthesis is controlled by the oxygen and molybdenum concentration [12], temperature [13] and NH_4^+ supply. Regulation of nitrogenase activity includes feed back inhibition by ADP and carbamyl phosphate, enzymatic covalent modification of one nitrogenase component [14], reversible switch off by O_2 [15] and control of reduction flow [16].

$N_2+8H^++8e^- +16Mg\ ATP \longrightarrow 2NH_3+H_2+16\ Mg\ ADP+16Pi$

Semiarid soils are deficient in nitrogen and supply of nitrogen is dependent to a great extent on microbial nitrogen fixation. Nitrogen fixation in crops grown in semi-arid regions is subjected to the extremes of temperature in summer months. In tropics and subtropics, soil temperature might reach to 40-45^{o}C exposing *Azotobacter* inoculant to temperature ranging from 20-45^{o}C for different varying length of time [4]. Many nitrogen fixing bacteria such as *Azospirilium brasilense* [13] and diazotrophic filamentous cyanobacterium *Trichodesmium* [17] have been analyzed to understand nitrogen fixation at high temperature. *Azotobacter* biofertilizers in combination with *Azospirilium* revealed higher percentages of N and protein in plant tissues [18].

The capacity to fix nitrogen has long been considered to be absent from the *Pseudomonas* genus. But, by genome sequencing it is confirmed that it contains genes involved in broad utilization of carbon sources, nitrogen fixation,

denitrification, degradation of aromatic compounds, biosynthesis of polyhydroxy-butyrate and multiple pathways of protection against environmental stress [19].

The cellulolytic fungus *Trichoderma*, *Aspergillus* with strains of nitrogen-fixing bacteria *Bacillus cereus* var. mycoides fixed nitrogen two to four times higher than upon the inoculation of the strains of *B. cereus* var. mycoides L1 only [20]. Most of the isolates and standard analogue resistant mutants of *A. chroococcum* being supplied to farmers as biofertilizers are mesophilic [2]. So, high temperature in semiarid regions has necessitated the use of thermotolerant mutants of *A. chroococcum* for use as inoculant for different crops. This is also important because of their involvement in the system of sustainable agriculture.

The present investigation was therefore, undertaken to study the nitrogen fixation at different temperatures in isolated thermotolerant mutants of *A. chroococcum* and mesophilic strains and their effect on cotton biomass.

Materials and methods

Two soil isolates [1(1), 6(2)], two mutants spontaneously resistant to methylammonium chloride (Mac27, Mac68) derived originally from a soil isolate of *A. chroococcum* (strain 103) and four high temperature (42^{o}C) resistant mutants (HTR51, HTR54, HTR57, HTR71) derived from Mac68 strain of *A. chroococcum* were procured from culture collection, Department of Microbiology, Chaudhary Charan Singh Haryana Agricultural University, Hisar (India).

All soil isolates and mutants were grown for 2-3 days at 30^{o}C on slants of modified Burk medium [21] and stored at 4^{o}C. Cotton (*Gossypium hirsutum*) cultivar H777 was obtained from cotton section, Department of Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar (India). Nitrogen and hydrogen gases used in the nitrogenase assay were from Indian oxygen limited, New Delhi.

Methods

To study growth characteristics under stationary conditions at different temperatures, a loopful inoculum of pure culture was inoculated into 30ml Jensen's broth [22] and incubated at 30^{o}C on shaker till log phase is reached. 1ml of log phase cells were transferred to 250ml Erlenmeyer flask containing 100ml of Jensen's broth and growth was followed by aperiod of 48h under stationary conditions of growth. The samples were withdrawn at a regular interval of four days and growth curve was prepared by taking viable count on Jensen's agar plates [22]. For determining growth under shake culture conditions, another set of flasks were treated as above except kept on reciprocal rotary shaker (225rpm) at different temperatures of 30, 37 and 42^{o}C.

Production and estimation of ammonia

Ammonia excretion ability of different strains of *A. chroococcum* was studied by growing the culture in triplicate. The flasks were inoculated and incubated for 20 days at different temperatures of 30, 37 and 42^{o}C. Aliquots of broth culture (5ml) were removed at regular intervals of 4 days, centrifuged at 10,000rpm for 30mins and the ammonia released in the supernatant was determined by the method [23] as described follow: Reagents (g/l): A) phenol 50; sodium nitroprusside 0.25; B) sodium hydroxide 25; sodium hypochlorite 2.1; A', B'-reagents A and B were diluted five times with distilled water.

One milliliter of supernatant, 2.5ml of reagent A' and 2.5ml of reagent B' were combined. The color was allowed to

develop for 30mins with occasional shaking. Color intensity was read at 625nm against a reagent blank using spectrophotometer. Ammonium sulfate was used as a standard. Results were expressed as µg of ammonia released per ml of culture supernatant.

Estimation of nitrogenase activity

Nitrogenase activity of different strains of *A. chroococcum* was assayed by growing the cultures in Burk medium slants (5ml) prepared in (15×125mm) test tubes. For each culture, 12 tubes were inoculated and a set of four tubes was incubated at 30^{o}C and other two sets were incubated at 37^{o}C and 42^{o}C. After 48h of growth, the cotton plugs of the assay tubes were replaced with suba seals (alcohol washed and UV irradiated) and 1.0ml of air from each tube was withdrawn using 1ml disposable syringe (sterware Faridabad) and replaced with an equal volume of acetylene (C_2H_4) to create 10% C_2H_4 atmosphere (air: acetylene, 9:1). Acetylene was freshly prepared by treating calcium carbide with water [24].

The tubes were further incubated for 12h at respective temperature. The ethylene formed was analyzed chromatographically using a Nucon 5500 gas chromatograph fitted with a stainless steel column (2m × 5m) packed with TCD Porapak Column R (50-80 mesh) and flame ionization detector. The column temperature was maintained at 105^{o}C with N_2 as the carrier gas (25-30ml/min) and H_2 as the fuel gas (15-20ml/min). From each tube, 0.5ml gas sample was injected to determine the amount of produced ethylene. The extent of acetylene reduction was calculated by using 110vpm standard ethylene (EDT Research 65IVX Crescent, London).

Estimation of protein in whole cells

After the nitrogenase assay, the bacterial growth from each slant was collected in a total of 5ml sterilized distilled water. To a suitable aliquot (0.2-1.0ml) in triplicate, 0.5ml of 1N NaOH was added, and placed in a boiling water bath for 5mins to digest the cells, then neutralized with 0.5ml of 1N HCl. After cooling the tubes, 2.5ml of alkaline copper reagent was added, mixed thoroughly and allowed to stand for 10mins. This was followed by a rapid addition of 0.5ml of dilute Folin-Ciocalteu reagent with immediate mixing. After 30mins, the absorbance was read at 750nm against reagent blank using spectrophotometer.

Effect of A. inoculation on cotton biomass

Seed sterilization/germination: Cotton (*G. hirsutum*) seeds cultivar H777 were surface sterilized by immersing in 0.2% (w/w) mercuric chloride for 3mins followed by 95% ethanol for 1min. The seeds were washed repeatedly with sterilized distilled water. The seeds were treated with culture broth for 5mins and were germinated on 1% agar plates at 28^{o}C for 3-4 days. No seed treatment was done in the case of control.

Soil analysis: Sandy loam field soil from CCS Haryana Agricultural University (Hisar) farm with pH 8.5, organic carbon at 0.35%, total nitrogen at 0.034% and available phosphate at 4.2ppm were used for pot house experiment. Soil was sterilized (102 kPa, 3h) for three consecutive days and later pots (4kg sterilized soil per pot) were filled.

Soil temperature: Soil temperature data recorded at different depths was obtained from department of Meteorology, CCS Haryana Agricultural University, Hisar, India.

Fertilizers: Recommended dose of N (80kg/ha) and P (60kg/ha) was applied. The

source of N and P were urea and single superphosphate respectively.

Pot experiment: To study the effect of *Azotobacter* inoculation on cotton growth an experiment under pot house conditions was set up during summer months when the temperature of Hisar region touches to 50°C. The cotton seeds (H777) were sown in upper 5cm soil layer in pot containing 1kg of sterilized soil and recommended dose of N and P. Five pots were kept for each treatment including control. After germination, four plants per pot were maintained and irrigated with sterile water twice a day.

Sampling/observations recording: Observations were made after 15th day, 30th day and 40th day of sowing for: *Plant height*: The height of plant was measured in cm from the base of the plant to axil of the last leaf open at the time of final harvest. The average plant height was calculated. *Plant dry weight:* After harvesting, plant material was dried in an oven at 80°C to a constant weight and average dry root weight and average dry shoot weight was calculated.

Viable count in rhizosphere: To determine the viable count of *Azotobacter* in rhizosphere, rhizospheric soil closely adhering to the roots was removed. Ten gram of soil was shaken in 90ml of sterilized distilled water and serially diluted. Appropriate dilutions were plated on Jensen's agar plates [25] for viable count.

Nitrogen estimation: Finely ground dried plant material (500mg) was taken in 100ml Kjeldahl's flask. One gram of digestion mixture (potassium sulphate, selenium dioxide and copper sulphate were mixed in the ratio of 10:5:1 (w/w)) and 10ml of conc. H_2SO_4 were added and heated for one hour when solution turned perfectly transparent giving a bluish green color.

After cooling to room temperature the contents were transferred to distillation flask. Using Parnas Wagner distillation apparatus, ammonia was steam distilled by adding 10ml of 40% NaOH. The amount of ammonia absorbed by 2% boric acid was determined by titrating it with 0.02N H_2SO_4 using mixed indicator (0.2% solutions each of methyl red and bromocresol green in ethanol in a ratio of 1:5 (v/v)). The nitrogen content was calculated from the volume of 0.02N H_2SO_4 required:

1ml of 0.02N H_2SO_4 contains = 0.00028g of nitrogen

Amount of total N was calculated as given below: Total N = %N × shoot dry weight

%N = V × 14/1000 × 0.02 × 100/wt of material in gram; V = Volume of 0.02 N H_2SO_4 used

Results

Growth characteristics

The growth pattern of different strains suggested that 30°C is the optimum temperature for growth of thermotolerant mutants and mesophilic strains. In thermotolerant mutants growth is affected marginally by 10% at high temperature of 42°C, while in mesophilic strain viable count decreased drastically by 60% at the same temperature (Fig. 1). Growth pattern of different strains indicates that log phase remained up to 8th day and after that cells undergo stationary phase up to 12-16 days.

Ammonia excretion

All the isolates/mutants were capable of excreting ammonia at 30°C under stationary conditions of growth. Maximum ammonia excretion was recorded on the 12th day of growth and then decreased abruptly on the 16th day of incubation in all the four thermotolerant mutants. The maximum ammonia excretion in all the four thermotolerant strains was at 37°C indicating that the optimum temperature for ammonia excretion in thermotolerant mutants is 37°C.

Maximum ammonia excretion (24.1µg/ml) was observed in HTR71 at 37°C. However, with the increase in temperature from 30°C to 42°C, ammonia excretion was drastically decreased from 23.9µgml^{-1} to 4.0µg/ml and 35.0µg/ml to 5.0µg/ml in case of Mac27 and Mac68 respectively (Table 1). Growth as determined by viable count increased steadily and reached a stationary phase by about the 8th day while ammonia excretion reached the maximum around the 12th day. The ammonia excretion in all of the four thermotolerant mutants at 30°C was observed less than the parent (Mac68) from which they were derived. However, at high temperature (42°C), NH_3 excretion was more in thermotolerant mutants compared to mesophilic strains.

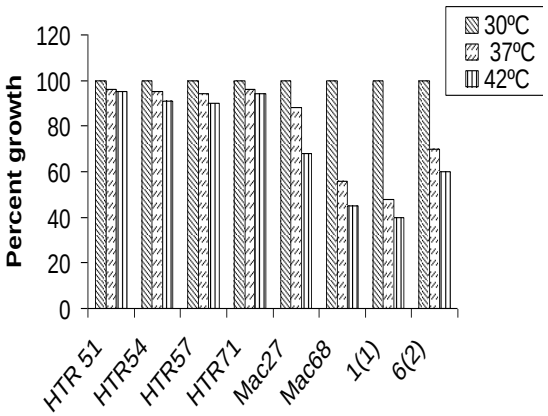

Fig. 1: Effect of temperature on growth of various thermotolerant and mesophilic strains of *A. chroococcum* under stationary conditions of growth

Table 1: Effect of temperature on ammonia excretion* by various thermotolerant and mesophilic strains of *A. chroococcum* under stationary conditions of growth

Days	HTR 54			HTR 71			Mac 27			Mac 68		
	30ºC	37ºC	42ºC	30ºC	37ºC	42ºC	30ºC	37ºC	42ºC	30ºC	37ºC	42ºC
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	5.6	5.5	5.2	5.5	5.5	5.5	5.5	4.0	2.0	5.5	5.0	2.0
8	5.6	5.5	5.1	5.7	5.6	5.6	6.0	4.5	2.0	6.2	5.7	4.2
12	22.0	24.0	20.0	23.9	24.1	21.0	23.9	18.0	4.0	35.0	16.0	5.0
16	5.8	5.8	5.8	5.9	5.8	5.8	5.9	4.0	2.0	10.9	4.2	1.0
20	5.3	4.0	4.0	3.0	2.0	-	5.1	1.0	1.0	5.6	1.0	1.0

* ug of ammonia released per ml of culture supernatant

Azotobacter species are aerobic organisms and need oxygen for the growth and nitrogen fixation. Study of ammonia excretion at shake culture conditions has shown no ammonia release although culture grew normally, while under stationary conditions of growth ammonia excretion began during stationary phase i.e. about the 12th day. Therefore, it appears that for ammonia excretion by these strains, vigorous aeration is not essential, a feature that has much importance in fermentative production of ammonia.

Effect of temperature on acetylene reduction activity (ARA)

The ARA by thermotolerant mutants increased at 37°C as compared to 30°C, but activity decreased at 42°C. This suggests that optimum temperature for nitrogenase activity in thermotolerant mutant is 37°C. At 37°C, HTR54 expressed maximum nitrogenase activity of 46.13 nmoles C_2H_4/h/mg protein among various thermotolerant mutants. Acetylene reduction activity (ARA) in mesophilic strains was decreased at 37°C as compared to 30°C and was completely lost at 42°C except in Mac68 which showed very little

activity of 2.75 nmoles C_2H_4/h/mg protein at 42°C (Table 2).

This suggests that optimum temperature for nitrogenase activity in these strains is 30°C. This might be due to the inactivation of enzyme at high temperature in case of mesophilic strains whereas nitrogenase remain stable at high temperature in thermotolerant mutants.

Table 2: Effect of temperature on nitrogenase activity by various thermotolerant and mesophilic strains of *A. chroococcum* under stationary conditions of growth

Isolate/ Mutant	ARA (n moles C_2H_4/h/mg protein)		
	30ºC	37 ºC	42 ºC
HTR 54	37.80	46.13	34.02
HTR 71	32.69	42.99	28.11
Mac 27	37.69	25.91	-
Mac 68	42.07	35.07	2.75

Performance of thermotolerant mutants of A. chroococcum on plant growth

Performance of thermotolerant mutants of *A. chroococcum* on plant growth was carried out in summer months under pot house conditions. The maximum increase in plant height was 5.2cm over control when inoculated with HTR71. However, both HTR54 and mesophilic strains behaved in the similar manner and showed 3.0cm increase in height over control. The maximum increase in shoot dry weight was 1040 mg over control when inoculated with HTR71. The root dry weight pattern was similar to that of shoot dry weight (Table 3).

Overall biomass accumulation in cotton was more when inoculated with thermotolerant mutants as compared to mesophilic strains. Total nitrogen incorporation in the plant with thermotolerant mutants was found to be three times over control (Fig. 2). The overall percent increase in plant height, plant dry weight and plant nitrogen after 40 days of growth was 13%, 13% and 62%, respectively by thermotolerant mutants over standard mesophilic strains (Fig. 3).

To see whether, the overall best performance of thermotolerant mutants in plant growth was due to their persistence in rhizosphere, studies were undertaken to determine the *Azotobacter* population in rhizosphere. The population of thermotolerant mutants in the rhizosphere of cotton increased from 1.5-7.0×10^5cfu/g soil to 1.5-20.0×10^5cfu/g soil after 40 days of plant growth. However, the population of mesophilic strains in the rhizosphere of cotton decreased from 1.3-11.0×10^5cfu/g soil to 1.0-5.7×10^5cfu/g soil after 40 days of plant growth (Fig. 4).

Table 3: Effect of *A. chroococcum* inoculation on growth of cotton plants (after 40 days of growth)

Character	Control	Thermotolerant mutants		Mesophilic standard strains	
		HTR54	HTR71	Mac27	Mac68
Plant Height (cm)	6.6	9.8	11.8	9.5	9.6
Dry root weight (mg)	330	490	590	475	480
Dry shoot weight	1320	1940	2360	1900	1920
% N of shoot	0.66	0.76	1.14	0.63	0.68
Total nitrogen of shoot (mg)	8.71	13.80	26.90	12.04	13.05

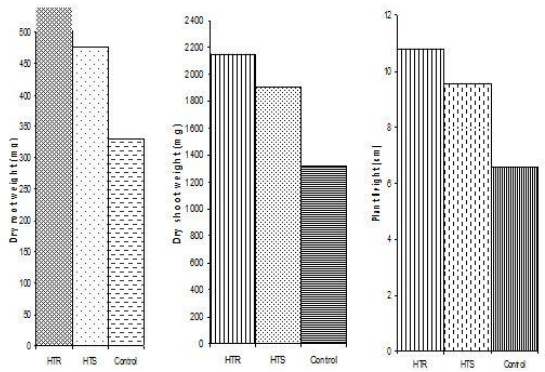

Fig. 2: Comparative performance of thermotolerant and thermosensitive strains of *A. chroococcum* on cotton biomass

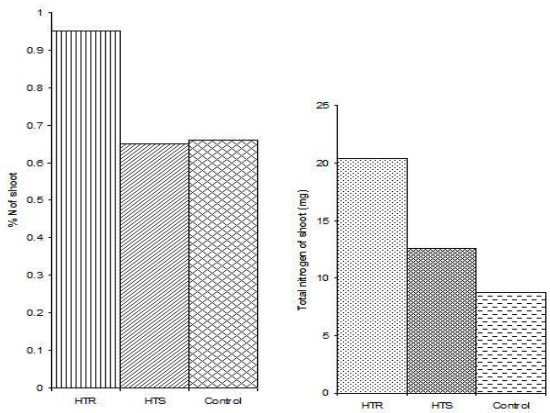

Fig. 3: Comparative performance of thermotolerant and thermosensitive strains of *A. chroococcum* on nitrogen content of cotton

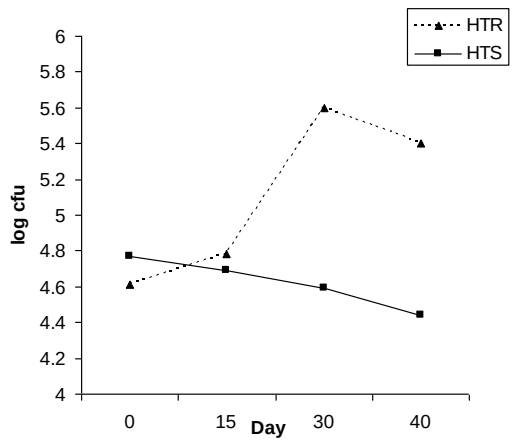

Fig. 4: Comparative survival of various thermotolerant (HTR) and mesophilic strains (HTS) of *A. chroococcum* in rhizosphere of cotton under pot house conditions of growth

Discussion

Azotobacter being a diazotroph, the beneficial response on crops is due to its nitrogen fixing ability and its subsequent mineralization. But this is contested by many workers, as the bacteria are poor colonizers with low survival rate [26]. In the present investigation, the rhizospheric count of *Azotobacter* after 40 days of plant growth showed considerable difference in colonization of different *Azotobacter* strains. Thermotolerant mutants particularly HTR54 and HTR71 showed a better establishment than mesophilic strains as the population of mesophilic strains decreased to a greater extent.

This differential behavior might be due to difference in the rate of root exudation and their composition, along with number of soil, ecological and environmental factors such as soil temperature, moisture, pH and humidity. This was most probable because of high ambient temperature of soil (Meteorological data) affecting the population of mesophilic strains in rhizospheric soil. Beneficial response of phosphate solubilising *A. chroococcum* on yield and quality of sunflower was also reported [27]. Better performance and persistence of phosphate solubilising *A. chroococcum* in wheat rhizosphere was also reported [28].

In addition, more ammonia excretion, high nitrogenase activity, better viability and establishment of thermotolerant mutants in rhizosphere at high temperature leads to overall increase in cotton biomass. There are reports also that strains of *A. chroococcum* isolated from temperate region have optimum temperature of 20-25^{o}C for growth whereas strains isolated from tropical region have optimum temperature of 30-35^{o}C [4]. The results showed that ammonia release occurs after the cells have reached the stationary phase

which is similar to the results of Narula *et al.* [25]. They found that ammonia excretion occurs only under non growing conditions.

The high ammonia excretion seems to be the results of nitrogenase activity and decrease in ammonia excretion at high temperature might be either due to repression of nitrogenase enzyme which stop reduction of nitrogen further, or excreted ammonia might have activated or induced the ammonia uptake [29]. As thermotolerant mutants are excreting ammonia more than the parent strain (Mac68) at 42^{o}C, it indicates that increase in ammonia release is not reciprocated by the simultaneous increase in glutamine synthetase (GS) enzyme. Small increase in the ammonia level can be adjusted with activation of GS by association and dissociation mechanism as reported [30] in *Candida utilis* thermotolerant mutants. But when there is some regulatory defect in Glutamate Synthase / Glutamine Oxogluta rate Aminotransferase (GS/GOGAT) syste m, intracellular ammonia is maintained at constant level by excretion of ammonia.

It is, therefore, presumed that GS/GOGAT system of thermotolerant mutants get modified at high temperature. Under the present day energy crisis with the high production and distribution cost of nitrogenous fertilizers, there is an urgent need to harness the full potential of thermo tolerant mutants of *A. chroococcum* for better productivity in tropical soil. So from above results discussed, it is inferred that the thermotolerant mutants of *A. chroococcum* can be exploited as crop inoculant in semiarid regions by virtue of their high nitrogen fixing ability and better survival at elevated temperature.

Conclusion

In the present study, the strains HTR54 and HTR71 are ideal diazotrophs for harnessing the maximum benefits of nitrogen fixing ability of *A. chroococcum* in cotton crops in semi-arid tropical regions of the world.

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