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INTRA- AND INTERSPECIFIC VARIATION FOR NITROGEN FIXING POTENTIAL IN *TOLYPOTHRIX* GERMPLASM

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Twenty cyanobacterial strains of the genus *Tolypothrix*, including 15 strains of *T. tenuis*, three strains of *T. ceylonica*, and one each of *T. nodosa* and *T. bouteillei*, that were collected from various agro-ecological regions of India were evaluated for important parameters related to nitrogen fixing potential, such as acetylene reduction activity, total protein and chlorophyll content. Distinct differences were observed in nitrogen fixing potential at both inter- and intraspecific levels. The *T. tenuis* strains, in general, exhibited superior nitrogen fixing ability as compared to strains belonging to other species. A statistical procedure based on simultaneous consideration of performances of various strains with respect to different parameters under study aided in identification of three highly promising *T. tenuis* accessions, namely ARM586, ARM75 and ARM460, for potential exploitation as biofertilisers.

Key words: biofertilisers, germplasm, nitrogen fixation, species variation, *Tolypothrix*

INTRODUCTION

Cyanobacteria, popularly known as blue-green algae (BGA), are photosynthetic microorganisms that are exploited worldwide as nitrogen supplements or biofertilisers (Metting 1988), and in some countries as food/feed (Becker 1988). These microorganisms are also attracting considerable attention as potential sources of value-added products such as phycobiliproteins (MacColl and Guard-Friar 1987), besides as agents for bioremediation, particularly in wastewater treatment (Oswald 1988).

The National Centre for Conservation and Utilisation of Blue-Green Algae (NCCUBGA) at the Indian Agricultural Research Institute (IARI), New Delhi, holds a collection of more than 600 cyanobacterial strains, including heterocystous and non-heterocystous forms. Heterocystous forms, such as *Anabaena*, *Nostoc*, *Tolypothrix* and *Aulosira*, are being used as biofertilisers in India since several decades (Venkataraman 1972). In India, around 2 million hectares are estimated to be inoculated with mixtures of cyanobacterial strains

of *Anabaena*, *Aulosira*, *Nostoc* and *Tolypothrix*, under the algalisation program in paddy fields. The demand for BGA biofertilizers was projected to be around 268,000 tonnes, but the current production is only about 400 tonnes (Motsara *et al*. 1995). This highlights the need for identification of promising cyanobacterial germplasm for utilisation as biofertilisers and concerted efforts for large-scale production of BGA biofertilisers.

Among various heterocystous cyanobacteria used as biofertilisers, the genus *Tolypothrix* merits attention because of its wide adaptability to diverse and severe environments, and high rates of nitrogen fixation despite slow growth potential (Roychoudhury and Kaushik 1989). *Tolypothrix* was also reported as a rich source of pigments, especially phycobilins, which find use as phycoflour probes as well as cosmetics and food colouring agents (Glazer and Stryer 1984). Application of specific strains of different cyanobacteria, including *Tolypothrix ceylonica*, followed by incubation and irrigation, revealed potential for reclamation of saline soils and dissolution of calcareous nodules (Kaushik 1990).

Characterisation of cyanobacterial strains for various attributes related to nitrogen fixation is an important research component for utilisation as biofertilisers. In this context, there is an increasing need to ascertain the inter- and intraspecific variability in cyanobacterial germplasm for traits of agronomic importance. The present investigation, therefore, has been undertaken with the following objectives: (a) to evaluate a set of *Tolypothrix* strains belonging to four different species, in relation to specific parameters related to nitrogen fixation, for the purpose of identification of highly promising strains as biofertilisers; and (b) to assess the intra- and interspecific variation among these strains on the basis of these parameters.

MATERIALS AND METHODS

Strains and growth conditions

Details regarding the 20 *Tolypothrix* strains selected for this study, including their geographical sources, are provided in Table 1. Unialgal isolates of these strains, designated as "ARM" (Agricultural Research Myxophyceae) strains, are being maintained at the NCCUBGA, New Delhi. For the present study, the strains were cultured in BG-II medium (Stanier *et al*. 1971) at 29±1 °C and 2,000–2,500 lux (14 : 10 LD cycle) to obtain optimal growth.

Table 1
Details of various *Tolypothrix* strains used in the study

S. No.	Strain No.	Species	Origin of isolate in India
1	ARM74	*Tolypothrix tenuis*	Allahabad, Uttar Pradesh
2	ARM75	*T. tenuis*	Allahabad, Uttar Pradesh
3.	ARM76	*T. tenuis*	Allahabad, Uttar Pradesh
4	ARM91	*T. nodosa*	Rajasthan
5	ARM113	*T. tenuis*	Rajasthan
6	ARM172	*T. tenuis*	Kanyakumari, Tamil Nadu
7	ARM173	*T. tenuis*	Kanyakumari, Tamil Nadu
8	ARM397	*T. ceylonica*	Unknown
9	ARM424	*T. tenuis*	Rampur, Maharashtra
10	ARM425	*T. tenuis*	Gadichirdi, Maharashtra
11	ARM426	*T. tenuis*	Chandrapur, Maharashtra
12	ARM460	*T. tenuis*	Rajasthan
13	ARM485	*T. ceylonica*	Kalyani, West Bengal
14	ARM520	*T. tenuis*	Kota, Rajasthan
15	ARM528	*T. tenuis*	Kota, Rajasthan
16	ARM531	*T. tenuis*	Kota, Rajasthan
17	ARM543	*T. ceylonica*	Kota, Rajasthan
18	ARM586	*T. tenuis*	Unknown
19	ARM592	*T. bouteillei*	Unknown
20	ARM617	*T. tenuis*	Unknown

Evaluation of growth parameters and nitrogenase activity

Selected *Tolypothrix* strains were analysed for their chlorophyll content and protein content, following the spectrophotometry procedures suggested by MacKinney (1941) and Herbert *et al.* (1971), respectively. Observations on Acetylene Reduction Activity (ARA), that provides an index of nitrogen fixing potential, were recorded on 10-day-old cultures of various *Tolypothrix* strains, following the procedure outlined by Kaushik and Venkataraman (1983). ARA was measured using a gas chromatograph (Nucon Model 5500) with a Poro-pak R column after injection of acetylene equal to 10% air space into glass vials tightly sealed using rubber stoppers. Incubation was carried out at 29±1 °C under 2,000 lux for 90 min. The gas phase was then analysed for ethylene using a Flame Ionisation Detector fitted to the chromatograph.

Statistical analysis

The data recorded for the three parameters in various strains were subjected to ANOVA (Analysis of variance) in accordance with the experimental design (completely randomised block design), using MSTAT-C statistical package, to quantify and evaluate the sources of variation. The linear model used for ANOVA was $Y_{ij} = \mu + S_i + \varepsilon_{ij}$, where Y_{ij} denotes an observation for the i^{th} strain for j^{th} variable, μ denotes overall mean, and ε_{ij} denotes random error associated with i^{th} strain and jth variable. To further analyse the differences between strains belonging to different species (in pair-wise comparison of species), orthogonal contrast was performed.

Duncan's Multiple Range Test (DMRT) was carried out to compare the mean performances of strains for specific traits under study. DMRT uses several critical values for conducting the test, where the critical values for each subset of mean pairs are associated with the same number of steps, R = 2, 3, …, k. Duncan's procedure also applies a different error rate, $a_R = (1–a)^R$ for each subset associated with R steps, R = 2, 3, …, k, in calculating all critical values. This procedure, thus, facilitated identification of a subset of superior *Tolypothrix* strains for specific traits.

Grouping of strains (genotypes) on the basis of mean comparison methods such as DMRT gives information only for specific character(s), while the purpose of considering a number of characters in an experiment may be to obtain an overall idea about various strains (genotypes) with respect to all characters. When the differences in the mean values were tested by DMRT, it is possible to make simultaneous comparison for all characters under study. A simple method for such an analysis was provided by Arunachalam and Bandopadhyay (1984). If K_1 was the number of groups for character 1 in case of "n" characters, genotypes in the highest ranked group were given a score K_1/K_1, genotypes in the second ranked group were given a score $(K_1–1)/K_1$, and the genotypes in the last ranked group were given a score of $1/K_1$. For n^{th} character, group scoring will be K_n/K_n, $(K_n–1)/K_n$, …, $1/K_n$, respectively. It is possible that a genotype could be found in group 1 as well as group 2 for a specific character. When such overlapping occurs, the score for that genotype occurring in two or more than two groups, is taken to be the average score of those groups in which the genotype is found. The individual scores, thus obtained, for each character are added up to provide a total score or final rank value for each strain (genotype). The strains can thus be ranked in an ascending or descending order on the basis of their total score or final rank value, depending on the nature of the character.

RESULTS

Evaluation of 20 selected *Tolypothrix* accessions belonging to four different species (*T. tenuis*, *T. ceylonica*, *T. nodosa* and *T. bouteillei*) for specific parameters that are considered important for biological nitrogen fixation revealed considerable intra- and interspecific variation in *Tolypothrix* germplasm. ANOVA indicated significant differences among strains belonging to various *Tolypothrix* species for different traits under study. Orthogonal contrasts between strains belonging to different species within *Tolypothrix*, with respect to protein content, ARA levels and chlorophyll content, showed significant differences among the four *Tolypothrix* species under study, except in comparison of chlorophyll content in *T. tenuis* vs. *T. ceylonica*, *T. tenuis* vs. *T. bouteillei* and *T. ceylonica* vs. *T. bouteillei* (Table 2).

Comparison of the mean performances of various *Tolypothrix* strains using Duncan's Multiple Range Test (DMRT) for protein content, ARA and chlorophyll content indicated the distinct superiority of *T. tenuis* strains over strains belonging to other species (Table 3). *T. tenuis* ARM586 was found highly promising with respect to protein content, while *T. tenuis* ARM426 displayed the highest ARA level. *T. tenuis* ARM528 and ARM113 recorded significantly higher chlorophyll content than other strains analysed.

After comparison of mean performances of the strains for growth and nitrogen fixing ability using DMRT, the individual strains were ranked through simultaneous consideration of performances with respect to the three parameters under study (Table 3). This procedure, utilised for the first time in agronomic evaluation of cyanobacteria, clearly revealed the potential of three *T.*

Table 2
ANOVA for selected traits evaluated in different *Tolypothrix* strains

Sources of variation	d.f.	MS*		
		Protein	ARA	Chlorophyll
Strains	19	356117.44	169135.08	1.061
T. tenuis vs. *T. ceylonica*	1	110009.26	108836.42	0.015^{NS}
T. tenuis vs. *T. bouteillei*	1	427781.25	13114.63	0.083^{NS}
T. tenuis vs. *T. nodosa*	1	701251.25	167576.79	3.441
T. ceylonica vs. *T. bouteillei*	1	162677.78	6125.67	0.105^{NS}
T. ceylonica vs. *T. nodosa*	1	321867.11	34391.08	2.537
T. bouteillei vs. *T. nodosa*	1	17930.67	46363.73	2.451
Residual	13	386516.46	218245.25	0.887
Error	40	281.87	18.48	0.037

*All values were significant at $P = 0.05$, except those marked as NS (non-significant)

tenuis strains, ARM586, ARM75 and ARM460 for possible exploitation as biofertilisers. Interestingly, ARM75 and ARM460 were not among the top-ranking strains with respect to any of the three individual parameters, but their consistently high performance for all three parameters was reflected in their final rank values. In contrast, ARM426 which ranked highest for ARA, as

Table 3
DMRT of mean performances of various *Tolypothrix* strains for selected traits, and scoring of strains based on DMRT rankings#

Strain	Protein (µg/ml)			Chlorophyll (mg/ml)			ARA*			Final Rank Value
	Mean	Rank	Score	Mean	Rank	Score	Mean	Rank	Score	
Tolypothrix tenuis										
ARM74	1183.0	F	0.64	0.0027	GH	0.35	593.10	B	0.93	1.92
ARM75	1487.0	C	0.86	0.0030	EFGH	0.45	498.70	C	0.87	**2.18** II
ARM76$	1210.0	F	0.64	0.0045	B	0.90	27.76	O	0.07	1.61
ARM113	1028.0	G	0.57	**0.0054**	**A**	1.00	122.60	K	0.33	1.90
ARM172	1413.0	D	0.79	0.0024	HI	0.25	304.90	E	0.73	1.77
ARM173	1580.0	B	0.93	0.0036	DE	0.65	124.00	K	0.33	1.91
ARM424	966.0	I	0.43	0.0031	EFGH	0.45	354.50	D	0.80	1.68
ARM425	701.7	L	0.21	0.0028	FGH	0.40	152.80	J	0.40	1.01
ARM426	353.7	N	0.07	0.0013	J	0.10	**1028.00**	**A**	1.00	1.17
ARM460	1030.0	G	0.57	0.0045	BC	0.85	212.80	G	0.60	**2.02** III
ARM520	765.0	J	0.36	0.0033	EFG	0.50	85.37	M	0.20	1.06
ARM528	1313.0	E	0.71	**0.0057**	**A**	1.00	63.72	N	0.13	1.84
ARM531	1018.0	G	0.57	0.0034	EF	0.55	195.30	I	0.47	1.59
ARM586	**1790.0**	**A**	1.00	0.0042	BCD	0.80	293.40	F	0.67	**2.47** I
ARM617	986.0	HI	0.46	0.0021	I	0.20	67.62	N	0.13	0.79
Tolypothrix nodosa										
ARM91	622.3	M	0.14	0.0015	J	0.10	30.90	O	0.07	0.31
Tolypothrix ceylonica										
ARM397	1018.0	G	0.57	0.0028	FGH	0.40	147.10	J	0.40	1.37
ARM485	971.7	I	0.43	0.0028	FGH	0.40	113.80	L	0.27	1.10
ARM543	1012.0	GH	0.54	0.0042	BCD	0.80	202.70	H	0.53	1.87
Tolypothrix bouteillei										
ARM-592	731.7	K	0.29	0.0037	CDE	0.70	206.70	GH	0.57	1.56
Mean±SE	1059.0±77.0			0.003±0.0001			241.31±53.09			
Range	353.7–1790.0			0.001–0.006			27.76–1028.25			

DMRT ranks indicated by alphabetic notations for different traits; same alphabetic notation denotes no significant difference in mean values.
*ARA measured in ηmol C_2H_4 mg chlorophyll$^{-1}h^{-1}$
$ Control (*Tolypothrix* strain used for biofertiliser production at IARI, New Delhi)

well as ARM528 and ARM113 showing highest chlorophyll content, did not figure among the top three strains after final ranking due to their relatively lower performance for other traits. Significantly, ARM76, a *T. tenuis* strain that is currently used in BGA biofertiliser production in India, and thereby, serving as a "control" in the present investigation, was not among the top-ranked strains either for individual traits or on the basis of combined analysis, suggesting the availability of superior strains among the *T. tenuis* accessions maintained in the germplasm collection.

DISCUSSION

The genus *Tolypothrix* was reported to be among the most preponderant cyanobacteria in the rice fields in India and Southeast Asia, particularly Japan (Watanabe *et al*. 1951). Researchers, particularly in Japan, have earlier demonstrated the utility of *T. tenuis* as a biofertiliser by carrying out field experiments (Watanabe 1959a, b, 1962). Also, the quantity of nitrogen fixed by *T. tenuis* was among the best in an analysis of strains tested in flask cultures (Watanabe and Yamamoto 1971). In India, mixed inocula of *Nostoc* and *Tolypothrix* have shown yield gain equivalent of 20 kg N/ha (Subrahmanyan 1972), and many *Tolypothrix* strains are known to be compatible with routine field level applications of biocides (Goyal 1990).

The present investigation is the first detailed analysis of nitrogen fixing potential in strains belonging to four different *Tolypothrix* species isolated in India. The study clearly indicated the potential utility of *T. tenuis* as biofertiliser and also highlighted the existence of considerable inter- and intraspecific variation in *Tolypothrix* germplasm with respect to nitrogen fixing potential. Although the number of strains belonging to *T. nodosa*, *T. bouteillei* and *T. ceylonica* were considerably lesser in comparison with the number of *T. tenuis* strains evaluated, the study nevertheless indicates the possible level of variation for different attributes analysed. With regard to all the three parameters evaluated (ARA, protein content and chlorophyll content), the top-three ranked strains belonged invariably to *T. tenuis*. A wide range of variation (27.76 to 1,028 ηmol C_2H_4 mg chlorophyll^{-1} h^{-1}) was particularly recorded for ARA among the *Tolypothrix* strains studied. Such a wide range of ARA in *Tolypothrix* germplasm was also recorded earlier by Roychoudhury and Kaushik (1989). Although *T. tenuis* ARM426 recorded the highest ARA value in the present study, there might be superior *Tolypothrix* strains in the germplasm collection with regard to ARA; for instance, Roychoudhury and Kaushik (1989) recorded an ARA value of 2,210 ηmol C_2H_4 mg chlorophyll^{-1} h^{-1} in *Tolypothrix*, although details of specific ARM accession possessing such

high ARA was not mentioned. In the present study, the high ARA value of ARM426 is also reflective of its low chlorophyll content, as the ARA values are expressed in terms of chlorophyll content. It is relevant to note that researchers worldwide have been expressing ARA of cyanobacterial strains in diverse ways, that is, in terms of either protein content, culture volume, dry weight or even weight of oven-dried soil in case of soil-based studies (Williams and Burris 1952, Watanabe *et al*. 1978, Wilson *et al*. 1980). However, since the content and activity of chlorophyll, the primary photosynthetic pigment in cyanobacteria, regulates the supply of electron donors and energy for nitrogen fixation, some researchers consider chlorophyll content as a more reliable growth attribute for evaluating nitrogen fixing potential in the form of ARA (Kaushik and Venkataraman 1983).

Analysis of variation in nitrogen fixing potential between and within specific cyanobacterial genera in India was also carried out by some earlier researchers, including Venkataraman (1972) and Roychoudhury *et al*. (1986). However, so far, no concerted efforts have been made to evaluate the biofertiliser potential of *Tolypothrix* germplasm by simultaneously considering important attributes such as ARA, protein and chlorophyll content. Also, to our knowledge, the present study is the first to show significant differences among different *Tolypothrix* species for nitrogen fixing potential. DMRT of the mean performances of various *Tolypothrix* strains showed the superiority of some of the *T. tenuis* strains with respect to nitrogen-fixing potential over ARM76, a *T. tenuis* strain currently employed in biofertiliser production in India. Ranking of strains based on DMRT not only aided in identification of promising *Tolypothrix* strains with superior performance for individual parameters, but also facilitated an objective assessment of strains through simultaneous consideration of multifactorial variation. Although ANOVA for individual traits provides an univariate analysis, which is indispensable for statistical analysis of variation, simultaneous utilisation of results from DMRT for different traits using the statistical procedure recommended by Arunachalam and Bandopadhyay (1984) provides an opportunity for utilisation of data reflecting variation for all the characters under study. For instance, ARM426, ranked first for ARA, did not rank among the top three ranked strains based on simultaneous consideration of all attributes. But, ARM586 that showed considerably high ARA value and chlorophyll content, besides being top-ranked for protein content, was ranked as the overall first among various strains of *Tolypothrix*. There was no apparent relation between the performance of the strains and their geographical origin within the country.

Among the strains analysed in *T. ceylonica*, ARM543 showed a final rank score (1.87) that was comparable to many of the *T. tenuis* strains. Chlorophyll content and ARA of ARM543 was also found to be superior to ARM397, a *T.*

ceylonica strain that has been utilised for reclamation of saline-alkali soils in the Uttar Pradesh State of India (Kaushik 1990).

The strategy employed in the present study to ascertain inter- and intraspecific variation for nitrogen fixing potential in cyanobacterial germplasm, and for evaluation of strains based on a combined analysis of performance for relevant attributes, could also be possibly utilised for identification of promising strains in microbial germplasm collections for other agronomically or commercially relevant traits.

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