Plant Science Research 43 (1&2) : 1-12, 2021

Plant Science Research

ISSN 0972-8546

Analysis of genetic diversity in submergence introgression varieties of different duration rice (*Oryza sativa* L.) of Odisha through RAPD markers

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ARTICLE INFO

Article history:
Received : 12 October, 2021
Revised : 2 December, 2021
Accepted : 18 December, 2021

Keywords:

DNA marker,
genetic polymorphism,
genetic distance,
rice breeding,
phylogeny.

ABSTRACT

Genetic diversity of ten rice (*Oryza sativa* L.) varieties of different duration was analyzed through agronomic traits along with RAPD markers. Mid-Early (IR87439, Ciherang *Sub1*, Lalat), Medium (IR88228, Swarna *Sub1*, Pratikshya), and Late duration (IR85086, Savitri *Sub1*, Mahanadi) varieties were tested with a check variety (Swarna) which revealed variation in flowering duration (85 d in Lalat to 110 d in IR85086), plant height and panicle length. 100-grain weight was ranged from 1.92 g in Swarna) to 3.79 g in IR88228. A significant positive correlation of 0.812 was noticed between plant height and leaf area. Plant height, panicle length, flag leaf area have significant positive correlation with 100-grain weight. Out of the 60 RAPD primers, 31 primers produce 280 amplicons (150 to 1960 bp) with a mean 57.24 % polymorphism. Dendrogram obtained from RAPD markers revealed that all the Mid-Early and Medium duration varieties formed Cluster-I except Swarna *Sub1* and the rest of the Late duration varieties formed Cluster-II with check variety Swarna. The PCA analysis confirmed that IR88228, IR87439, Ciharang *Sub1*, and Pratikshya have close genetic similarities as compared to Lalat. Thus, RAPD markers could be used in the identification of varieties and seed certification in rice breeding programs.

1. Introduction

Oryza sativa L. commonly known as rice belongs to the family Poaceae is one of the most important commercial food grains in the world. High temperature and humidity with high rainfall during the monsoon season favor rain-fed crop cultivation of rice in India. Out of about 162.06 million hectares' total area of rice cultivated in the world, India covers an area of 43.79 million hectares (Anonymous, 2020) under the total irrigated and rain-fed area at present. India accounts for the second-highest production in world of 118.87 million metric tons of milled rice which follows 146.73 million metric tons of rice produced by China. A large number of rice landraces compatible with varied agro-ecological conditions have been evolved and a number of submergence introgressed varieties of rice have been developed in India are being cultivated in the country. A number of such varieties have been cultivated in Odisha conditions to check the yield potential of rice submergence introgressed rice varieties with the non-introgressed landraces of different duration of maturity. It is very much essential to explore the genetic variability of these indigenous rice varieties for better yield and adaptability without replacing indigenous cultivars and landraces. The selection of plant varieties based on morphological characters is sometimes biased with environmental interference and sometimes not reliable because major characters of interest possess low heritability and are genetically complex. Several attempts have been made to synthesize heterogeneous populations of rice taking into account different morphological and yield attributes to segregate the bulk population (Das, 2013, 2018). The influence of environmental factors and nearly similar morpho-characters are the serious concern to distinguish genetically close varieties. Thus, DNA markers are found to be more reliable (Hadrys *et al.*, 1992; Bowditch *et al.*, 1994; Raghunathachari *et al.*, 2000). RAPD, a PCR-based technique

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are used extensively in gene mapping of rice (Welsh and McClelland, 1990; Williams *et al.*, 1990; Mackill, 1995) besides the use of RFLP (Restriction fragment length polymorphism) markers (Nakano *et al.*, 1992; Zhang *et al.*, 1992; Ishii *et al.*, 1993) which have been used in DNA fingerprinting studies. The use of RAPDs has been applied besides restriction fragment pattern of chloroplast DNA (Dally and Second, 1990) in the classification of Indian and Japonica rice cultivars (Zheng *et al.*, 1991; Fukuoka *et al.*, 1992; Yu and Nguyen, 1994). Molecular markers provide information to estimate close relatives and the phylogenetic position of the varieties. DNA fingerprinting based on RAPD markers found varietal distinctiveness and relativeness unambiguously in rice from time to time for salt-tolerant local and inbred rice (Mazumder *et al.*, 1999), non-aromatic rice (Rahman *et al.*, 2007; Joshi *et al.*, 2012; Rajani *et al.*, 2013; Alam *et al.*, 2014; Singh and Sengar, 2015; Karande *et al.*, 2017), coarse and fine grain rice (Arshad *et al.*, 2011), traditional glutinous rice (Shaptadvipa and Sarma, 2009), and aromatic rice (Baishya, 2000; Hasan and Raihan, 2015; Zakiyah *et al.*, 2019). However, the study on genetic variation of submergence tolerant and landraces of different duration of rice varieties of Odisha is scanty. Thus, examination of genomic variation is especially useful for quick varietal genetic variability through RAPD markers accompanying yield attributes for analysis of inherent genetic differences among the individuals for use in breeding programs and introduction of submergence tolerant rice besides the traditional landraces. Keeping all these in purview the present work was undertaken to estimate genetic variation in the germplasm of Mid-Early, Medium and Late duration rice varieties using yield attributes and RAPD markers for future use in selection, hybridization, biodiversity assessment, evaluation and conservation of diverse gene pools of Odisha.

2. Materials and methods

2.1 Plant materials

Ten rice varieties of Odisha were grown in an on-field trial for the study of adaptive variations during Kharif season in the year 2020 in the experimental field of Orissa University of Agriculture and Technology, Odisha, India. The materials were grown in randomized block design, each accession grown in the plant to plant spacing 15 × 15 cm and between-row spacing of 20 cm. General agronomic procedures were practiced at various stages of crop growth and development. Well-rotted Farm Yard Manure (4.0 tones ac^{-1}) was applied 4 to 6 weeks before seed sowing and 10 kg ZnSO4 ac^{-1} during the last puddling stage. Basal fertilization of NPK (0.5:0.5:0.5) kg^{-1} 100 sq. m was applied for robust seedling growth and NPK (100:60:60) was applied in three splits (at 30 days after transplantation, at active tillering stage, and at panicle initiation stage) in field condition. Observations of all the quantitative characters were recorded (Table 1).

2.2 Agronomic parameters

The morphological and yield attributing characters of ten varieties of rice were recorded by selecting randomly three plants in each replication for each variety. Observation on days to 50% flowering and days to maturity was recorded on a pilot basis. The agronomic characters collected have been presented in Table 2. Plant height was measured in cm from the ground level to the tip of the longest panicle of all tillers of the main stem at the time of the harvest. Flag leaf length and breadth were obtained from the mature plants during harvest in cm and the area was calculated. Six randomly selected plants from each plot were harvested and numbers of panicles and panicle lengths were measured. The mean length of all the panicles of a plant was measured and the average was taken. The number of fertile seeds per panicle of each variety was recorded and the fertility percentage was also obtained. The weight of 100 grains of individually selected plants was recorded randomly from each replicated plot and weighed separately and the average was taken.

2.3 DNA extraction and quantification

Nuclear DNA was isolated for each variety from young leaves following the CTAB method (Saghai-Maroof *et al.*, 1984). Leaves were ground to a fine powder in liquid nitrogen using mortar and pestle and suspended in six-volume of CTAB extraction buffer [100 mM Tris HCl (pH 8.2), 20mM EDTA, 0.5M NaCl, 2% CTAB, and 2% beta-mercaptoethanol]. The suspended rice leaf powder in CTAB buffer was incubated in a water bath at 60 ^{o}C for 2 h. The solution was cooled down to room temperature and an equal volume of chloroform: isoamyl alcohol was added to it. The mixture was emulsified for 15 min and centrifuged at 10, 000 rpm for 30 min. The aqueous layer was transferred into a sterilized centrifuge tube and DNA was precipitated with equal volume of pre-chilled isopropanol (kept at 4 ^{o}C for overnight). The thread of DNA was collected with a sterile plastic loop or precipitate was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. DNA was washed in 70% ethanol and was dried in a vacuum concentrator. Dried DNA samples were dissolved in $T_{10}E_1$ (10 mM Tris HCl, 1 mM disodium EDTA, pH 8.0) and treated with RNase A (10 mg ml^{-1}) and proteinase K solution. The DNA was re-precipitated with an equal volume of ethanol after mixing 3 mM sodium acetate, air-dried, and dissolved in $T_{10}E_1$ buffer. The concentration of DNA was checked in 0.8% agarose gel with lambda DNA as standard in the Gel Doc system. The DNA was diluted into a final concentration of 25 ng μl^{-1} using $T_{10}E_1$ buffer for use as a template during PCR amplification with different primers (Table 5).

2.4 PCR reaction and RAPD analysis

RAPD profiles were generated by using 60 ten base Operon Primers of series A, B, and C having 20 primers each in a polymerase chain reaction (PCR) following the standard protocol. Each polymerase chain reaction (PCR) mixture (25 µl) was prepared with 25 ng of template DNA, master mix having 200 µM of each dNTPs, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India), 25 ng of primer, and 10 × PCR assay buffer (50 mM KCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$, pH 9.0). The reaction was performed in a thermal cycler (GENEAMP-9700; Applied Biosystems, USA) with the program having initial denaturation at 95 °C for 5 min, followed by 44 cycles of denaturation (94 °C for 1 min), annealing (42 °C for 1 min), and extension (72 °C for 2 min) followed by a final extension at 72 °C for 8 min. The amplification products after completion of PCR reaction were stored at 4 °C. The amplified products were separated in 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide in TAE buffer (40 mM Tris-acetate, pH 8.0; 2 mM EDTA) at a constant 55 V for 60 to 80 min. A gel loading buffer [20 % (w/v) sucrose; 0.1 M EDTA, 1.0 % (w/v) SDS; 0.25 % (w/v) bromophenol blue; 0.25 % (w/v) xylene cyanol] was used as a tracking dye. Amplified fragments of DNA were observed on a 1.2% agarose gel under a gel documentation system (Geldoc XR system, BioRad, USA) after staining with ethidium bromide solution and photographed. Gene ruler 100 bp DNA ladder was used as marker DNA to determine size of the amplified DNA fragments. Constantly appeared amplification products were used for scoring and further analysis.

2.5 Statistical analysis

Mean data, standard deviation and standard error were calculated for all the replicated agronomic parameters. ANOVA analysis was done among different agronomic parameters. The mean data of yield attributing characters collected against all the varieties of rice were used as a set of variables for the similarity matrix. The correlation coefficient was calculated using Excel Program. The presence or absence of the bands was scored as present (1) or absent (0) in each variety for each set of primers for RAPD analysis. RAPD profile was scored in a binary matrix which was used for the analysis of Jaccard's coefficient matrix (Jaccard 1908). A dendrogram was obtained, from this matrix, by cluster analysis following UPGMA using NTSYSpc version 2.11s software (Rohlf 2008).

3. Results

3.1 Variation in agronomic characters

The agronomical characters of ten studied rice varieties have been given in Table 1. Duration of all varieties of rice was classified as Mid-Early (IR87439, Ciherang *Sub1*, Lalat), Medium(IR88228, Swarna *Sub1*, Pratikshya), and Late duration[IR85086, Savitri *Sub1*, Mahanadi] and Swarna (the check variety). The range of mean plant height varied from 89.53 cm in Swarna *Sub1* to 120.25 cm in IR85086 (Table 2). The duration of the flowering differed in the ten studied varieties of rice recorded 85 days in Lalat to 94 days in IR87439 among Mid-Early duration varieties. Medium duration varieties showed flowering time from 95 days in IR88228 to 105 days in Swarna *Sub1* and Late duration varieties had flowering time variation from 96 days in Pratikshya to 110 days in IR85086 (Tables 1 and 2). The mean panicle length showed a minimum of 22.10 cm in Mahanadi with a standard error value of 0.25 to a maximum of 26.60 cm in IR87439 with a standard error value of 0.22. The flag leaf area varied from 25.62 cm^2 in Swarna to 56.08 cm^2 in IR85086. Fertility percentage showed a range from 79.36 % in Pratikshya to 93.93 % in Savitri *Sub1*. Moreover, fertility percentage ranged from 80.10 % (IR87439) to 88.06 % (Lalat) among Mid-Early duration varieties, 79.36% (Pratikshya) to 88.50 % (IR88228) among Medium duration varieties, and 85.60 % (IR85086) to 93.93 % (Savitri *Sub1*)among Late duration varieties. 100-grain weight varied significantly among the 10 varieties of rice. Swarna showed a minimum of 1.92 g and IR88228 showed a maximum of 3.79 g per 100-grain weight which indicates the size variation of the seeds among the varieties (Table 3). Correlation coefficient analysis revealed a significant correlation between plant height and flag leaf area (r=0.81) followed by 100 grain weight versus plant height (r=0.62), panicle length (r=0.56), flag leaf area (r=0.52), flag leaf area versus panicle length (r=0.48), duration of flowering (r=0.38) and panicle length versus plant height (r=0.38). The rest of the characters showed no significant or negative correlation among the agronomic traits (Table 3). ANOVA showed significant variations among all yield attributing characters (Table 4).

3.2 Estimation of genetic variation

Amplification with 31 RAPD primers out of the 60 tested primers generated 280 scorable DNA amplicons with a range size of 150 bp to 1960 bp (Table 5, Figs. 1a,b). The maximum mean polymorphic percentage obtained was 57.24%. A maximum 14 locus was amplified with the primer OPA04 followed by 13 loci in OPA14 and 12 loci in OPA15, OPB04, OPC01, and OPC08 (Table 5). The RAPD bands of highly significant varietal characteristics were observed in $OPA04_{750bp}$ in Swarna *Sub1*, Lalat and Swarna, whereas, $OPC08_{860bp}$ was found absent in Mahanadi. Swarna and Lalat showed a unique band of $OPC08_{700bp}$. IR87439, Swarna *Sub1*, Savitri *Sub1*, and Mahanadi produce $OPC08_{680bp}$ which was not found in Pratikshya and Lalat (Figs. 1a,b). No single primer was able to distinguish all the genotypes of rice. The polymorphic percentage varied from 19.36 % between IR8828 versus IR87439 to 45.59 % between Ciherang

Table 1

Different varieties of rice (*O. sativa*) with some important agronomic characters used for RAPD marker analysis.

Variety	Duration	Days to 50% flowering	Plant height (cm)±SE	Panicle length (cm)±SE	Flag leaf area (cm^2)±SE	Fertility % ±SE	100 grain wt. (g.) ±SE
IR87439	Mid-Early	93-94	104.33±0.69	26.60±0.22	33.88±0.07	80.10±0.76	2.48±0.022
Ciherang *Sub1*	Mid-Early	88-90	90.35±1.12	24.85±0.42	26.26±1.85	85.34±1.25	2.76±0.015
Lalat	Mid-Early	85-88	97.12±1.39	26.56±0.49	30.65±1.82	88.06±0.77	2.73±0.022
IR88228	Medium	95-96	110.15±0.80	26.55±0.47	42.56±0.90	88.50±0.29	3.79±0.029
Swarna *Sub1*	Medium	102-105	89.53±1.04	23.83±0.20	27.06±1.71	86.24±0.51	2.56±0.078
Pratikshya	Medium	96-98	91.70±1.15	25.30±0.23	28.88±1.56	79.36±0.35	2.22±0.07
IR85086	Late	108-110	120.25±1.98	26.48±0.14	56.08±2.53	85.60±0.96	2.77±0.055
Savitri *Sub1*	Late	100-102	105.83±1.78	23.09±5.14	30.86±1.28	93.93±0.45	2.18±0.50
Mahanadi	Late	104-106	102.60±1.29	22.10±0.25	38.70±2.93	88.41±0.71	2.15±0.016
Swarna	Late (Check variety)	101-103	89.95±0.75	22.70±0.59	25.62±0.099	87.92±2.67	1.92±0.052

Table 2

Variation of quantitative characters of 10 rice varieties of Odisha.

Characters	Range	Mean±SE	CV%
Plant height (cm)	87.73-115.93	100.13±2.80	08.85
Panicle length (cm)	22.09-27.40	24.69±0.57	07.35
Panicle number	09.32-18.10	13.67±0.82	19.02
Days to 50% flowering	89.00-105.00	98.13±2.16	06.98
Flag leaf length (cm)	23.98 – 45.54	32.19±1.82	17.95
Flag leaf area (cm^2)	22.54 – 56.13	32.51±2.96	28.88
Fertility %	78.98 – 93.08	86.57±1.07	03.92
100 grain weight	1.63 – 3.16	2.37±0.14	18.89

Table 3

Correlation coefficient of yield attributes of ten varieties of rice tested in the experiments.

	Duration to 50% flowering	Plant height	Panicle length	Flag leaf area	Fertility %
Plant height	0.226^{NS}				
Panicle length	-0.402^{*}	0.375^{*}			
Flag leaf area	0.380^{*}	0.812^{**}	0.480^{**}		
Fertility %	-0.012^{NS}	0.224^{NS}	-0.192^{NS}	-0.023^{NS}	
100 grain weight	-0.481^{**}	0.622^{**}	0.595^{**}	0.516^{**}	0.168^{NS}

NS=Not significant, *=Significant at ≥0.05, **=Significant at ≥0.01

Table 4

Analysis of variance (ANOVA) for different yield attributes of ten varieties of rice with *Sub1* gene introgression.

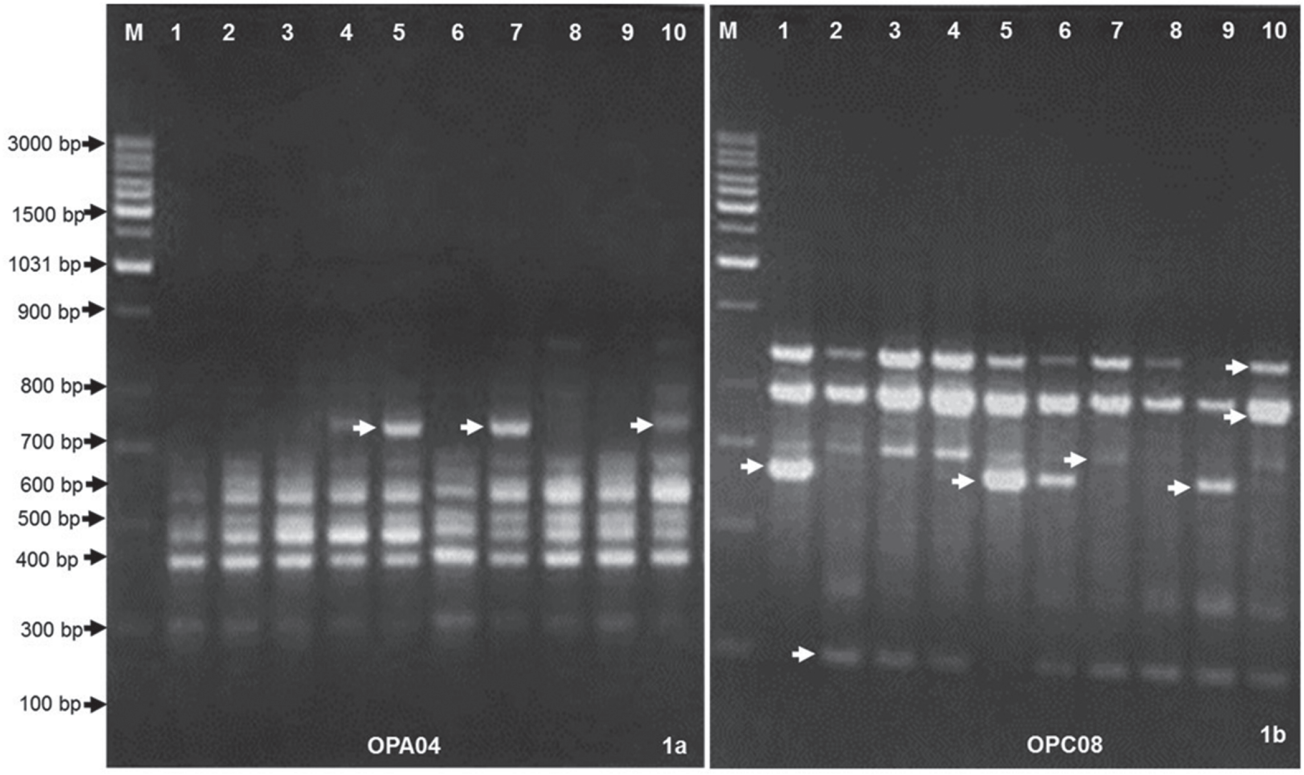
Source	DF	SS	MS	F
Plant height (cm)				
Between Groups	9	2362.981	262.553	26.846*
Within Groups	20	195.593	9.779	
Total	29	2558.574		
Panicle length (cm)				
Between Groups	9	99.137	11.015	24.494*
Within Groups	20	8.994	0.449	
Total	29	108.131		
Panicle number				
Between Groups	9	127.205	14.134	4.506*
Within Groups	20	62.726	3.136	
Total	29	189.932		
Flag leaf length (cm)				
Between Groups	9	1002.405	111.378	32.896*
Within Groups	20	67.713	3.385	
Total	29	1070.119		
Flag leaf area (cm^2)				
Between Groups	9	2645.289	293.932	33.006*
Within Groups	20	178.106	8.905	
Total	29	2823.495		
Fertile seed number				
Between Groups	9	263923.006	29324.778	25.076*
Within Groups	20	23387.993	2269.399	
Total	29	287310.994		
Fertility %				
Between Groups	9	346.455	38.495	6.726*
Within Groups	20	114.466	5.723	
Total	29	460.922		
100 seed weight (g)				
Between Groups	9	6.013	0.668	104.390*
Within Groups	20	0.128	0.006	
Total	29	6.141		

*Significant at ≥ 0.001 level. DF, degrees of freedom; SS, sum of squares; MS, mean squares; F,variance ratio.

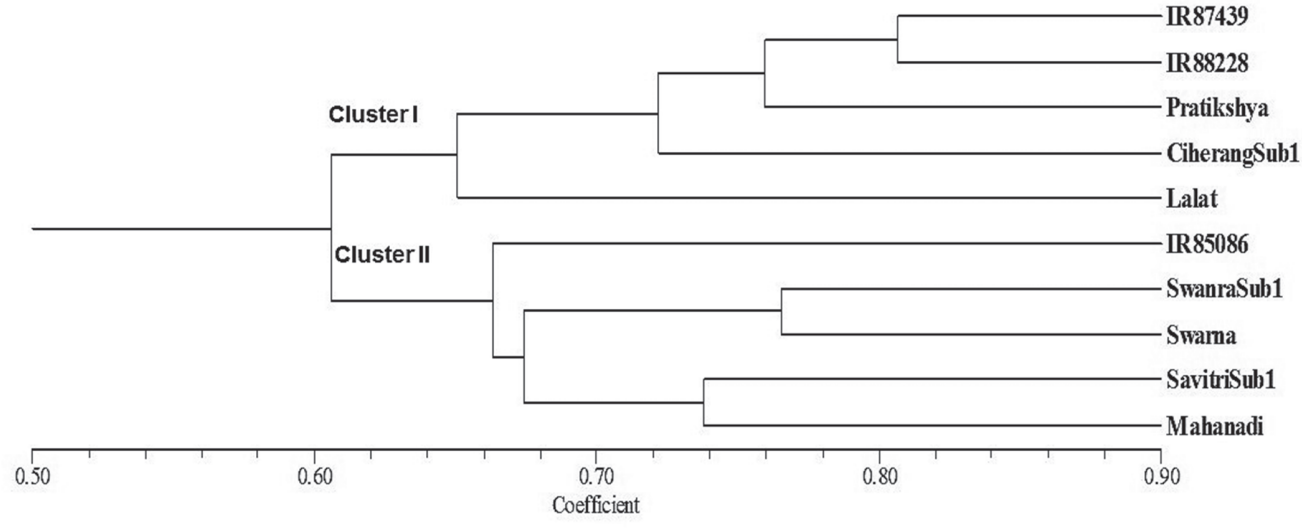
Table 5

Amplification pattern of RAPD bands of the 10 varieties of rice (*O. sativa*).

Sl. No.	Primer Name	Sequence 5'….....3'	No. of allele	Number of Polymorphic bands	Number of monomorphic bands	Polymorphic %	Size range (bp)
1	OPA01	CAGGCCCTTC	8	5	3	62.50	300-1450
2	OPA02	TGCCGAGCTG	11	10	1	90.90	200-1200
3	OPA03	AGTCAGCCAC	8	5	3	62.50	280-1150
4	OPA04	AATCGGGCTG	14	8	6	57.14	150-1430
5	OPA05	AGGGGTCTTG	5	2	3	40.00	320-1180
6	OPA07	GAAACGGGTG	6	4	2	66.66	450-1450
7	OPA08	GTGACGTAGG	11	5	6	45.45	250-1600
8	OPA10	GTGATCGCAG	8	5	3	62.50	200-1180
9	OPA12	TCGGCGATAG	9	4	5	44.44	250-1250
10	OPA14	TCTGTGCTGG	13	8	5	61.53	150-1600
11	OPA15	TTCCGAACCC	12	5	7	41.66	250-1450
12	OPA17	GACCGCTTGT	9	4	5	44.44	200-1650
13	OPA19	CAAACGTCGG	11	4	7	36.36	300-1800
14	OPA20	GTTGCGATCC	8	6	2	75.00	240-900
15	OPB01	GTTTCGCTCC	9	7	2	77.77	350-1420
16	OPB03	CATCCCCCTG	5	2	3	40.00	245-1630
17	OPB04	GGACTGGAGT	12	8	4	66.66	190-1450
18	OPB06	TGCTCTGCCC	8	3	5	37.50	300-1650
19	OPB07	GGTGACGCAG	9	5	4	55.55	250-980
20	OPB08	GTCCACACGG	8	5	3	62.50	350-1650
21	OPB09	TGGGGGACTC	8	3	5	37.50	450-1480
22	OPB10	CTGCTGGGAC	9	5	4	55.55	200-1200
23	OPB11	GTAGACCCGT	9	6	3	66.66	180-1550
24	OPB12	CCTTGACGCA	8	6	2	75.00	250-1680
25	OPC01	CTCACCGTCC	12	8	4	66.66	200-1530
26	OPC03	GGGGGTCTTT	10	5	5	50.00	560-1700
27	OPC04	CCGCATCTAC	6	4	2	66.66	600-1875
28	OPC07	GTCCCGACGA	10	7	3	70.00	200-1220
29	OPC08	TGGACCGGTG	12	7	5	58.33	290-1680
30	OPC09	CTCACCGTCC	5	2	3	40.00	250-1960
31	OPC15	GACGGATCAG	7	4	3	57.14	155-900
		Total	280	162	118	1774.66	
		Mean	9.03	5.22	3.80	57.24	



Figs. 1. RAPD profiles of 10 varieties of rice amplified with different primers. (a)OPA04 (b) OPC08. M=DNA molecular weight marker, 1-10 lanes represent different variety of rice. 1=IR87439, 2=IR88228, 3=IR85086, 4=Ciherang *Sub1*, 5=Swarna *Sub1*, 6=Savitri *Sub1*, 7=Lalat, 8=Pratikshya, 9=Mahanadi, 10=Swarna.



Figs. 2. Dendrogram of 10 varieties of constructed using UPGMA based on Jaccard's correlation similarity coefficient of the RAPD characters.

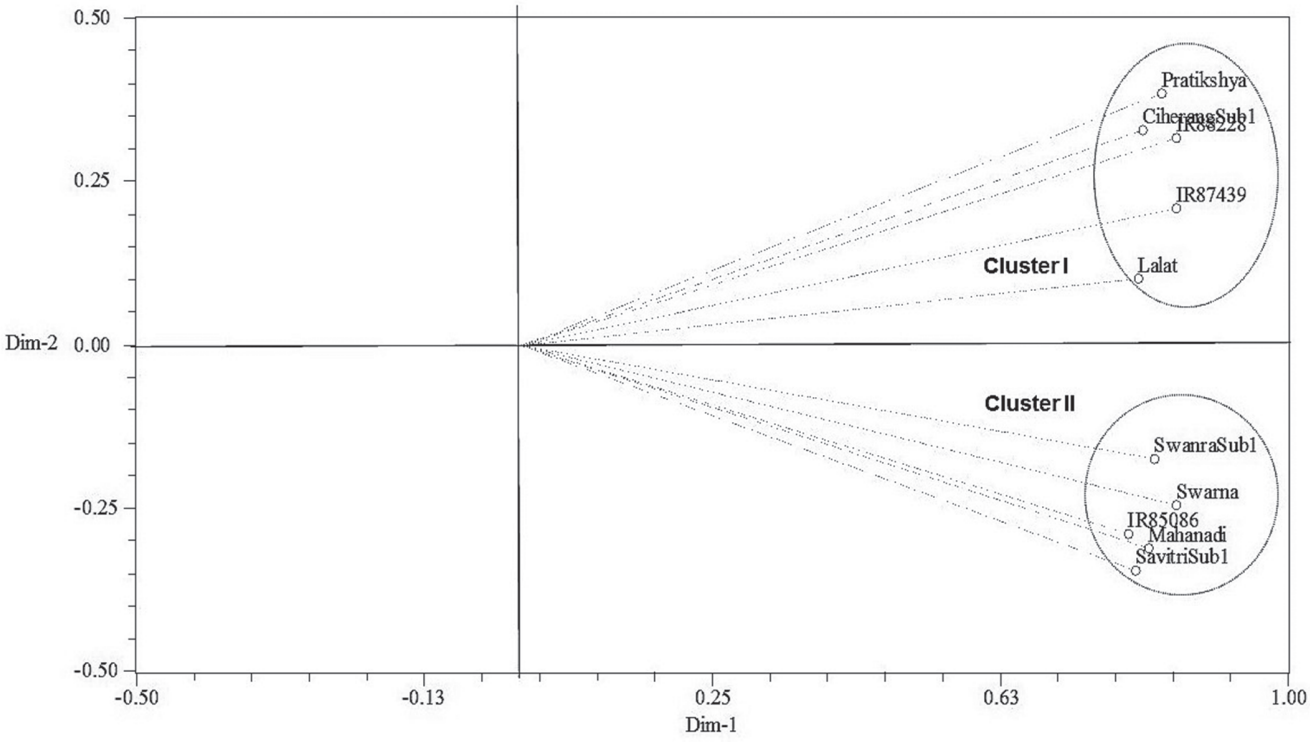


Fig. 3. Three-dimentional principal component analysis of 10 varieties of rice constructed using UPGMA based on Jaccard's similarity coefficients for the RAPD data set.

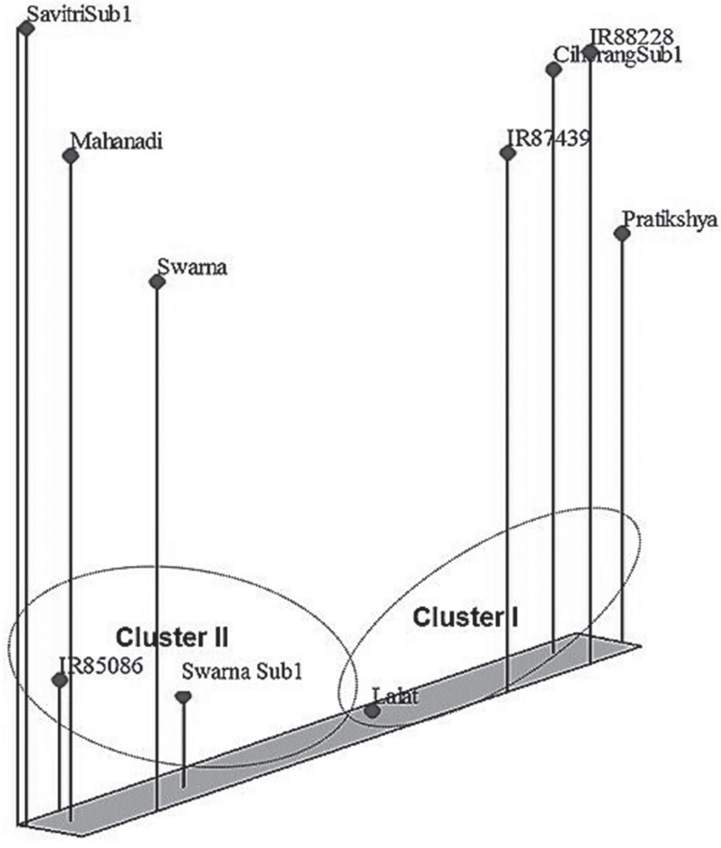


Fig. 4. Three-dimensional plot of principal component analysis of 10 varieties of rice using RAPD data showing genetic diversity.

Sub1 versus IR85086 which also showed a moderate genetic variation among these varieties at the DNA level (Table 5). The number of bands produced varied with each primer with an average mean of ~9.0 bands per primer.

3.3 Cluster analysis

Cluster analysis of RAPD bands showed two main clusters, i.e. Cluster-I having five Mid-Early and Medium duration varieties except for Swarna *Sub1* and Cluster-II having Late duration varieties with check variety Swarna (Fig. 2). Two-dimensional principal component analysis (PCA) showed also two clusters similar to cluster analysis dendrogram. The Cluster-II with Late duration varieties showed less genetic variability besides Swarna *Sub1* as a distinct variety in this group (Fig. 3). Cluster-I showed comparatively greater genetic variability where Lalat showed a distinct variation from the others. Three-dimensional PCA analysis clearly indicates that Lalat has higher genetic variability among all the studied varieties whereas the rest Mid-Early, Medium and Late duration varieties showed greater genetic variation between Cluster-I and Cluster-II (Fig. 4).

4. Discussion

4.1 Agronomic characteristics

The morphological variation was found significant among different characters as depicted in ANOVA (Table 4). The height of the plants showed significant variations along with leaf area and grain weight. Correlation coefficient analysis revealed a significant correlation between plant height and flag leaf area (r=0.81) followed by 100-grain weight versus plant height (r=0.62), panicle length (r=0.56), flag leaf area (r=0.52). Duration of flowering showed significant variation among the varieties. The maximum leaf area of 42.56 cm^2 was found in IR88228, a Medium duration variety, which also has a larger grain size having a maximum 100-grain weight of 3.79 g. The second highest leaf area 56.08 cm^2 was found in Late duration variety, IR85086, having 100 gain weight of 2.77 g. However, all the varieties showed more leaf area and 100-grain weight as compared to a check variety Swarna, having 25.62 cm^2 leaf area and 1.92g grain weight. Seed fertility had a maximum of 93.93 % in Savitri *Sub1* followed by 86.24 % in Swarna *Sub1* and 85.34 % in Ciherang *Sub1*. Thus, introgression of submergence gene (*Sub1*) into these varieties has not much effect on seed fertility percentage. The morphological variation of rice agronomic characters was also reported earlier in different landraces of India (Dikshit *et al.*, 2013). Although, considerable crop genetic diversity continues to be maintained in farms in traditional varieties (Jarvis *et al.*, 2008). The high dominance occurred of all the valuable alleles, with low frequency of the variety richness suggested that diversity may be maintained as insurance to meet future environmental changes. The proportion of genetic diversity in autogamous species, such as rice, is expected to be greater amongst those within each landrace.

4.2 Phylogenetic and PCA analysis

Dendrogram constituted with RAPD markers showed a single common ancestry that basically formed Cluster-I (five Mid-Early and Medium duration varieties including Ciherang *Sub1*) and -II (four Late duration varieties including check variety Swarna and a Medium duration variety Swarna *Sub1*). Principal component analysis (PCA) in 2-D form could distinguish four varieties (IR87239, IR88228, Pratikshya, Ciherang *Sub1*) forming minor clusters from Lalat with a separate branch forming Cluster-I. Similarly, among the Late duration varieties, IR85086 formed a separate branch while Swarna and Swarna *Sub1* formed a single minor cluster showing their genetic affinity and Savitri *Sub1* and Mahanadi formed a single minor cluster with little higher genetic variability forming Cluster-II (Fig. 3). Similar types of RAPD derived clustering were also reported in ten Indian rice varieties by Karande *et al.,* (2017). The average sizes of amplicons were between 150 bp to 1900 bp which is in accordance with the other reports of traditional lowland rice varieties of Assam (Bhuyan *et al.*, 2007), Pakistan (Ukoskit, 2004), wild rice of Thailand (Arif *et al.*, 2005). It is expected that the same length of DNA fragments is from the appropriate locus, and represent the dominant single locus with two possible alleles (Nagy *et al.*, 2012) in RAPD markers. The RAPD profiles in the present study displayed a moderate degree of polymorphism which confirms the suitability of RAPD markers for discrimination of different varieties of rice plants. Our study yielded highly reproducible RAPD fingerprints which might be a useful tool for discrimination of genetic variation in ten varieties of rice specially submergence introgression varieties in the normal environment. A high level of genetic diversity among the non-basmati groups holds a promise in the quest of conserving crop diversity and broadening the gene pool for breeding (Mathure *et al.*, 2010). The average polymorphism of 57.24 % in our study found the contrast to a high polymorphic percentage (80 to 95%) in rice genotypes (Yu and Nguyen, 1994; Raghunathachari *et al.*, 2000; Davierwala *et al.*, 2000; Ravi *et al.*, 2003; Rabbani *et al.*, 2008; Ray *et al.*, 2012; Roopa and Chikkaswamy, 2016; Tahmina *et al.*, 2017. However, our result could be in accordance with the result of Beverley *et al.,* 1997; Choudhury *et al.,* 2001 and Kanawapee *et al.*, 2011 having 50% to 68.94% polymorphism in rice using RAPD markers. In contrast, hybrid rice parental lines of rice in Iran revealed only 35% polymorphism using 15 RAPD primers (Kiani and Katalani, 2018). However, very low polymorphism was recorded in PCA clearly distinguished all the clusters of Mid-Early and Medium

duration varieties and Late duration varieties. It is evident that Lalat, a Mid-Early duration variety, of Cluster-I found distinct forming a separate branch showing high genetic variability as compared to the rest of the varieties. Cluster-II with all the Late duration varieties found a high variation of genetic makeup with IR85086 having distinct genetic distance from the rest of the varieties. It was evident from the fact that rice varieties belonging to different maturity time were grouped into the same phylogenetic cluster. Partially salt-tolerant local and inbred rice of Bangladesh was reported to distinguish with the use of Operon primer using RAPD (Mazumder *et al.*, 2020).

The dendrogram analysis provides clusters on the basis of similarity which has high efficiency and the ability for variety identification (Aliyu *et al.*, 2000). Overall, this study reveals that the rice germplasms of Odisha have moderate genetic diversity. Nowadays simple sequence repetitive DNA markers (SSR) are used for genetic diversity evaluation of rice rather than RAPD markers. A variety of studies with SSR markers have been reported to distinguish rice varieties which could be useful for co-dominant maker analysis (Shahriar *et al.*, 2014; Shakil *et al.*, 2015; Siddique *et al.*, 2016; Rashid *et al.*, 2018; Syed *et al.*, 2019; Verma *et al.*, 2019) as compared to dominant alleles in RAPD marker. Thus, SSR marker analysis in different duration of rice variety could be more useful for genetic variation study in the future among rice germplasms of Odisha.

5. Conclusion

The present study resulted in the development of RAPD markers that can be efficiently used for the genetic diversity assessment of different varieties of rice. On the basis of morphological traits and RAPD markers, Mid-Early and Medium duration varieties of Odisha formed separate clusters (IR87439, Ciherang *Sub1*, Lalat, and IR88228) with the highest grain weight in IR88228 in Odisha condition compared to check varieties. Thus, the putative yield attributing polymorphic RAPD loci found in this study can be used in mapping and linkage analysis. The polymorphic markers could be potential for developing a decision support system for creating crosses based on RAPD-based genetic distance matrices. The introgression of the *Sub1* gene has not much effect on fertility % and grain weight attributing characters. The genetic diversity knowledge could be a useful tool for the identification of duplicates in maintaining genetic stock to make a core collection of pre-breeding genotypes. The assessment of genotypes based on yield attributing phenotypic characters along with RAPD alleles seems to be a more reliable strategy for the selection of parents in hybridization.

Acknowledgments

AD acknowledge the field and laboratory facility used in the Orissa University and Technology, Bhubaneswar and Department of Botany, Utkal University respectively. ABD acknowledges the Emeritus Professorship received from Human Resource Development Group, Council of Industrial Research (CSIR), Scheme No. 21 (1107) / 20/EMR-II, Ministry of Science and Technology, Govt. of India.

Conflict of interest

All author declares any conflict of interest in the paper.

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Plant Science Research

ISSN 0972-8546

Protochlorophyllide oxidoreductase protects the oil seed crop plant mustard (*Brassica juncea*) from water-stress

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ARTICLE INFO

Article history:
Received : 24 October, 2021
Revised : 12 November, 2021
Accepted : 18 December, 2021

Keywords:

Brassica juncea,
PAM2100,
Protochlorophyllide
oxidoreductase C,
Drought stress

ABSTRACT

Plant cells lack antioxidative enzyme-mediated reactions for quenching singlet oxygen ($^1O_2$) making it the major cause of damage to plants during daytime. A chlorophyll biosynthesis intermediate protochlorophyllide is a photosensitizer that absorbs light and transfers the energy to $O_2$to generate $^1O_2$.Higher the $^1O_2$ production, greater is the oxidative damage to the plants. Protochlorophyllide oxidoreductase (POR) is a light-dependent enzyme that photo-transforms protochlorophyllide to chlorophyllide using light energy as the substrate. Water-stress severely down regulates the gene and protein expression of POR leading to reduced synthesis of POR enzyme. Therefore, the oil seed crop *Brassica juncea* over expressing the C isoform of POR i.e., PORC along with wildtype (WT) plants were exposed to water stress to ascertain the role of PORC in the protection of plants from drought. The stress treatment was applied to mustard WT and PORC over-expressers (PORCx) plants by withholding water supply up to 8 days. Recovery from stress was monitored up to 48 h after re-watering the drought-treated plants. WT plants wilted after 8th day of drought stress and had lower PSII-dependent electron transport rate (ETR) and initial chlorophyll a fluorescence (Fo)during stress and recovery phase than the PORC over-expressers. Reduced $^1O_2$ produced in *Bj*PORCx plants minimized damage to the photosynthetic machinery allowing for a faster recovery from water stress than the WT plants. Therefore, PORC could be genetically modulated in crop plants to protect them from water-stress.

1. Introduction

Brassica juncea, an amphidiploid originated due to the natural hybridization between black mustard (*Brassica nigra* (L.) Koch) and turnip mustard (*Brassica rapa* L.) (Szõllõsi, 2020), is a major oilseed crop and is grown in approximately six million hectares in India during the winter season (Singh *et al.*, 2009). With the increase in world population and a steady decline of arable land, enhancing the tolerance of crops to various stress, thereby enabling its maximal productivity is the need of the hour.

Biologically stress for plants is defined as any alteration to the normal physiology, development and functioning that can cause an irreversible damage to the biological machinery. Various environmental factors are responsible for the overall growth and development of plants, any change in these leads to a stress- induced response by the plants.Various abiotic stress, such as drought, flood, extreme temperatures, etc., are serious threats to agricultural practices. Drought spans across continents and has affected several crop plants and the farmers worldwide and it is expected to increase in frequency and intensity due to climate change (Rojas, 2021).

Plant cells have tightly regulated metabolic reactions to minimize production of reactive oxygen species. Under drought stress many metabolic processes including photosynthesis, are negatively affected. Water stress is known to affect thetransfer of electrons from water to NADP by damaging the oxygen evolving complex of PSII (Dalal & Tripathy, 2018). Physiologically, water deficit in plants affects leaf area expansion, absorption of photosynthetically active radiation and subsequently the efficiency of utilization of

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absorbed radiation to carry out carbon fixation in leaves (Flexas *et al.*, 2004). The activity of Rubisco is also impaired by water stress. This ultimately results in complete breakdown of the photosynthetic process, which in turn affects the overall productivity of crop plants(Fariduddin *et al.*, 2009). Several plant species have evolved mechanisms that allow them to adapt and survive periods of water deficit (Cruz de Carvalho, 2008). Mature plants have mechanisms in place to counter reactive oxygen species (ROS) induced damage to the photosynthetic apparatus. ROS can be extremely reactive, singlet oxygen ($^1O_2$) superoxide (O_2^-) and the hydroxyl radical ($OH^·$), unlike atmospheric oxygen can oxidize multiple cellular components like proteins and lipids, DNA and RNA. Unrestricted oxidation of the cellular components will ultimately lead to cell death (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992; Mittler, 2002). To cope with continuous ROS production, plants have numerous enzymatic and nonenzymatic antioxidants, that function as the defense system. The major scavenging mechanisms include superoxide dismutase (SOD), enzymes and metabolites from the ascorbate-glutathione cycle (Foyer-Halliwell-Asada pathway), and catalase (CAT). They are located throughout the different compartments of the plant cell, except for catalase that is exclusively located in peroxisomes (Cruz de Carvalho, 2008).

Chlorophyll (Chl) biosynthetic process yields several tetrapyrrolic intermediates and their degradation products which are photodynamic in nature. These photodynamic molecules absorb light and transfer energy to oxygen molecule that results in production of the ROS i.e., singlet oxygen ($^1O_2$) and subsequent cell death (Chakraborty and Tripathy, 1992; Tripathy *et al.*, 2007). The metabolism of Chl is highly regulated during plant development to prevent ROS production. Previous work in our laboratory on *Arabidopsis thaliana* revealed that overexpression of *AtPORC* resulted in enhanced Chl biosynthesis and conferred tolerance to ALA-mediated oxidative stress (Pattanayak & Tripathy, 2011). The work on overexpressing *AtPORC* gene in *Brassica* genome (Pandey, Tripathy unpublished) have shown similar tolerance to various oxidative stresses (ALA-mediated and salinity). *Arabidopsis thaliana* has three isoforms of POR, namely POR A, POR B, and POR C (Armstrong *et al.*, 1995; Masuda *et al.*, 2003; Oosawa *et al.*, 2000; Pattanayak and Tripathy, 2002; Reinbothe *et al.*, 1996). Among them, POR C is highly expressed in response to light and is found in photosynthesizing tissues (Masuda *et al.*, 2003; Oosawa *et al.*, 2000; Pattanayak and Tripathy, 2002; Vedalankar and Tripathy, 2019). The over expression of POR C enables the conversion of accumulated Pchlideinto Chlide, reducing the possibility of Pchlide derived $^1O_2$ production (Pattanayak& Tripathy, 2011).

POR gene expression and POR protein abundance is severely downregulated in plants leading to impairment of Shibata shift (Dalal and Tripathy, 2012). Therefore, POR C was overexpressed in *Brassica juncea* under the control of a 35S constitutive promoter to ascertain if it could protect plants from water deficit. The impact of drought was monitored on the photosynthetic efficiency of WT and transgenic *Brassica* plants overexpressing POR C (*BjPORCx*) monitoring chlorophyll a fluorescence as its non-invasive signature. It is shown that POR C could protect plants from drought.

2. Materials and Methods

Brassica juncea cv. VarunaWT and *BjPORCx* (Pandey, Tripathy unpublished data) plants were grown in transgenic greenhouse during the *Brassica* growing season i.e., October to February under a natural photoperiod. The plants were watered at regular intervals and grown till 6 weeks. The watering was then withheld to measure the effect of water stress on various chlorophyll a fluorescence parameters. Measurements were carried out in 2-day interval till 8th day. The plants were then rewatered to study the recovery process of WT and transgenic plants.

Chl a fluorescence measurements were performed in dark-adapted leaves (20 min) (Demmig *et al.*, 1987) at 25°C using portable chlorophyll fluorometer-PAM-2100 (Walz, Effeltrich, Germany); all measurements were repeated 5 times. The minimal (Fo) and maximal (Fm) fluorescence, were measured on leaves that were dark-adapted for 20 min. The instrument uses a low intensity (<0.1µmol photons m^{-2} s^{-1}) red measuring beam (650 nm), with a frequency of 0.6KHz for Fo followed by a 0.8 s saturation light pulse of approximately 8,000 µmol photons m^{-2} s^{-1} (20kHz) to measure the maximum fluorescence (Fm). The Fo and the Fm values were used to calculate the PSII quantum yield (Fv/Fm), where Fv is the variable fluorescence (Fv=Fm–Fo).

The light response curves of the electron transport were obtained by measuring fluorescence as a function of increasing actinic light intensity (0 to 1200µmol photons m^{-2} s^{-1}). The electron transport rate of photosystem II was calculated from the equation ETR = φPS II x PAR x 0.5 x 0.84, where φPSII is effective PSII quantum yield (calculated by (*Fm' –Ft)/ Fm'=ΔF/ Fm'*) where*Fm'*is referred as the maximum fluorescence yield when the samples are illuminated, and *Ft* is the fluorescence yield at any given time (t). PAR is the photonflux density of incident photosynthetically active radiation, measured in µmol photons $m^{-2}s^{-1}$, 0.5 is the factor of the ratio of PS II and PS

I (1:1), 0.84 is the value thatcorrelates with the percentage of incident photons absorbed by the leaf to drive photosynthesis (Schreiber, 2004).

3. Results and Discussion

Overexpression of PORC enabled the plants to recover faster from the water-deficit.

The WT and BjPORCx plants were grown in transgenic greenhouse and water was withheld from 6-week-old plants. Wilting of WT plants was observed on 8th day of water withholding. Chlorophyll a fluorescence parameters were used as a non-invasive signature of photosynthesis (Govindjee, 2004; Schreiber *et al.*, 1995) in control and drought-affected WT and *Bj*PORCx plants. Drought stress primarily causes damage to PSII (White and Critchley, 1999; Dalal and Tripathy, 2012, 2018). PSII actively regulates the electron transport rate and the photochemical efficiency, it prevents or relieves the damage caused by excessive lightenergy to other systems via heat dissipation (Bu *et al.*, 2010). In drought stress, the damage of PSII and antioxidant enzyme system is a non-stomatal limiting factor for the decrease in photosynthetic rate (Li *et al.*, 2017). The energy absorbed by Chl molecule can be used in either one of the three processes, primarily, the energy absorbed by the Chl molecule is directed to initiate the photochemistry; secondly, the absorbed energy could be dissipated in the form of heat; and third, the excited Chl molecules return to the ground state by fluorescence. These are three competing processes. Analysis of modulated chlorophyll fluorescence in response to different light pulses provide valuable information regarding the PSII activity in a quick and non-invasive manner (Blankenship, 2008).

The Fo was determined in dark-adapted leaves of control and water-stressed WT and *Bj*PORCx plants. The Fo of WT and transgenic plants was similar in control conditions. Due to drought treatment the Fo of both plants declined. However, on 8th day of drought, the Fo of WT plants was 14% lower than *BjPORCx* plants. The WT plants substantially wilted on the 8th day of water withholding.Upon rewatering, the Fo of both WT and *Bj*PORCx plants recovered from stress to a large extent (Fig. 1). The Fm decreased both in WT and *Bj*PORCx plants in response to drought. However, on the 8th day the Fm of WT was 15% lower than the transgenics. Upon rewatering the Fm recovered, although it had lower recovery in WT than *Bj*PORCx plants (Fig. 2).

The Fv/Fm declined in WT plants as the stress progressed. The *Bj*PORCx plants always had higher Fv/Fm ratio than that of the WT plants. On day 8 of stress treatment *Bj*PORCx plants had ~20% higher Fv/Fm than water-stressed WT plants. Upon re-watering the *Bj*PORCx plants recovered faster than WT (Fig 3). There was no complete recovery of Fv/Fm from water stress when stress treatment continued

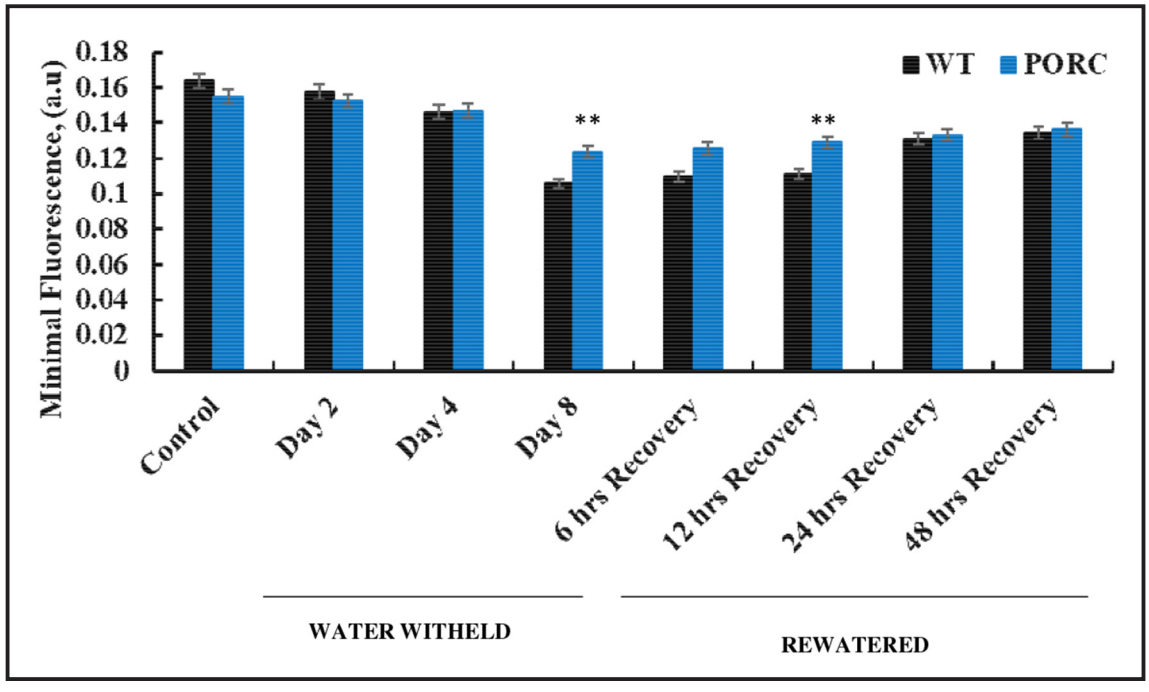

Fig 1. Minimal fluorescence (Fo) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

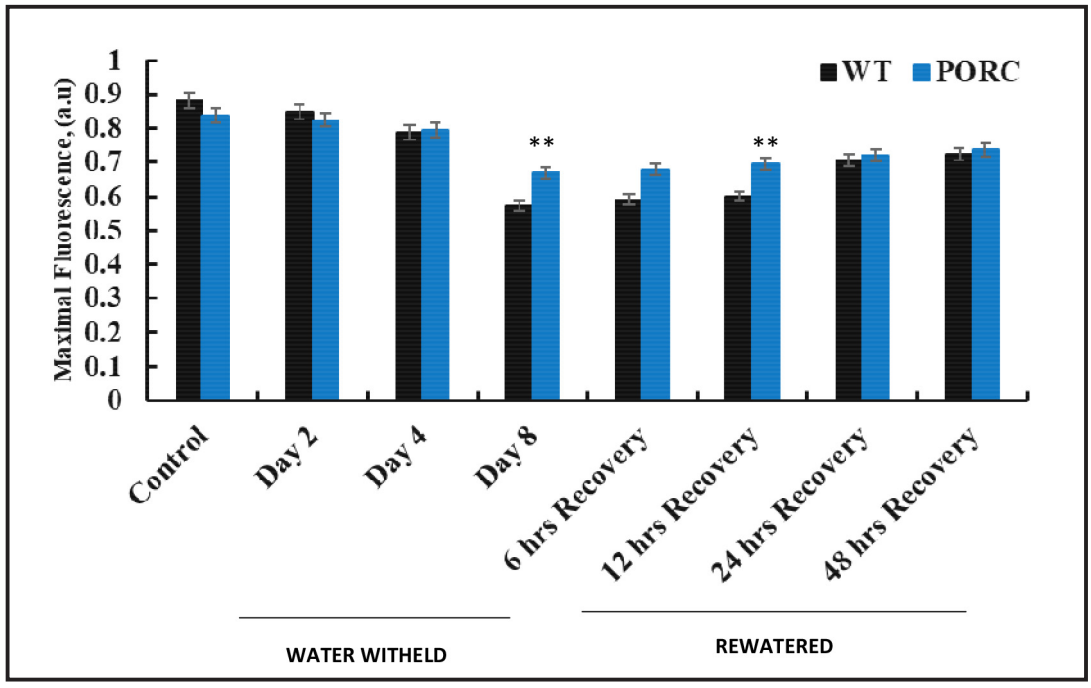

Fig 2. Maximal fluorescence (Fm) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

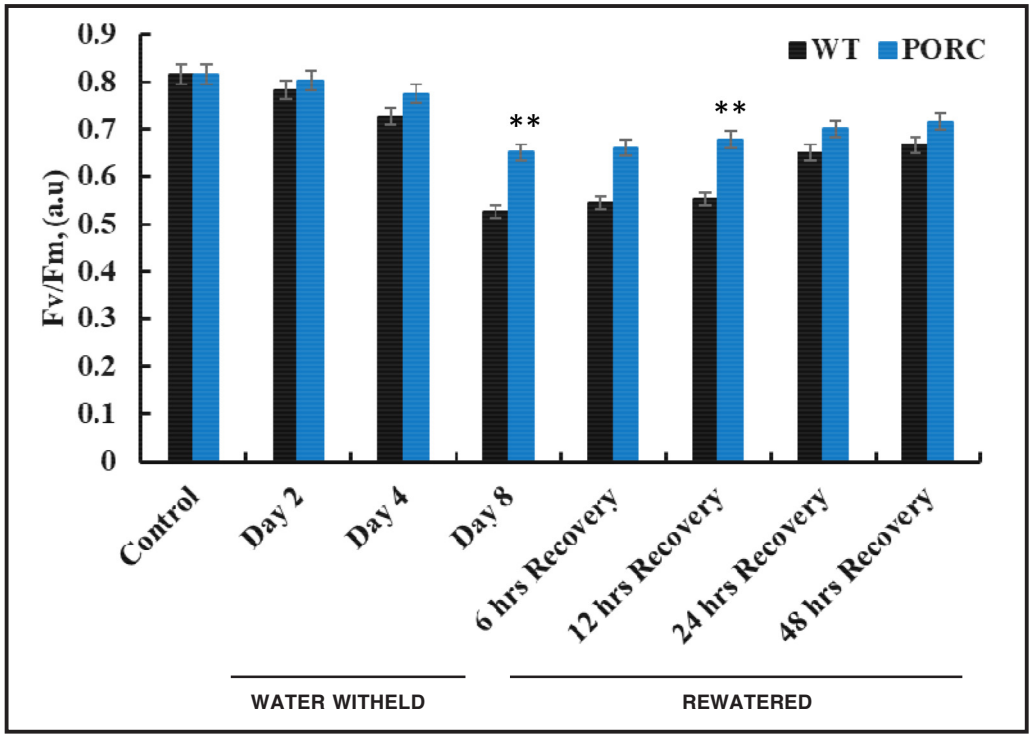

Fig 3. Ratio of variable to maximal fluorescence (Fv/Fm) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

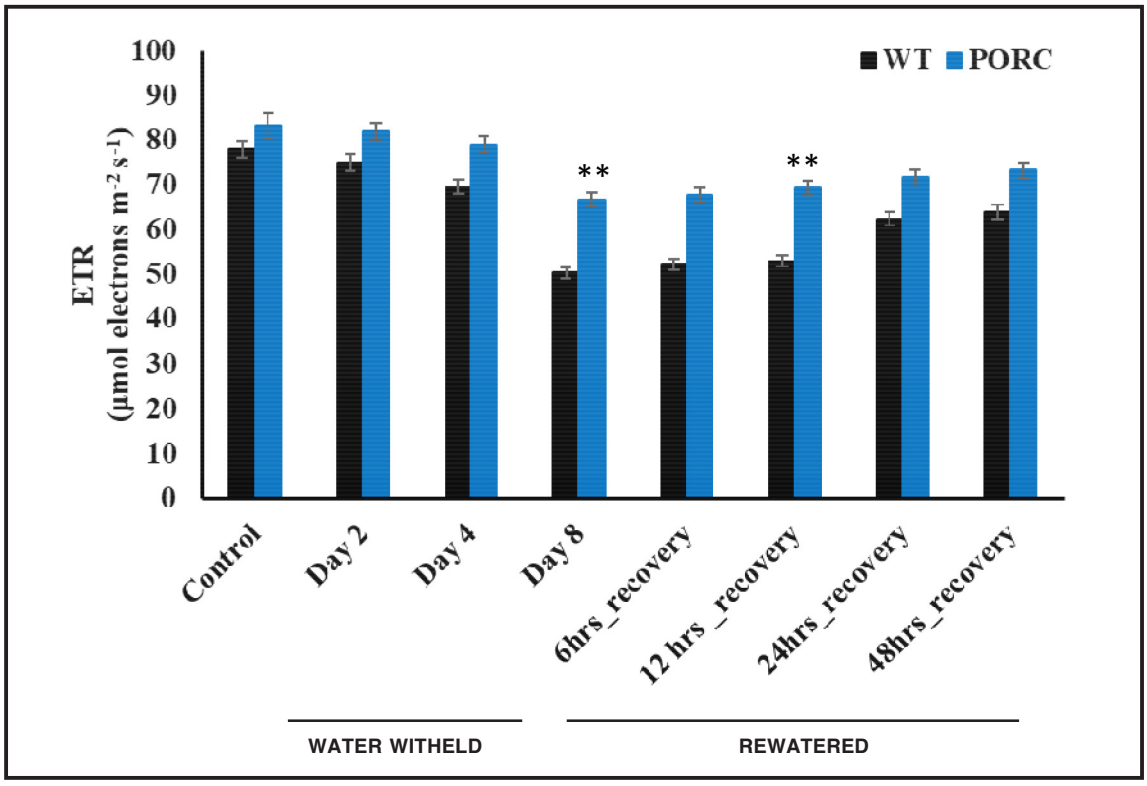

Fig 4. Electron transport rate (ETR) of PSII measured in response to photosynthetic active radiation at 1200 µmol photons observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

till 8th day. However, the maximum photochemical efficiency of PSII of *Bj*PORCx plants were always higher than the WT both during stress treatment and recovery phase.

ETR represents the relative photosynthetic electron transport rate expressed as µmol electrons $m^{-2}s^{-1}$, which is calculated based on the measured values of yield of PSII and of PAR (see materials and method). Due to stress treatment, the PSII-dependent ETR decreased both in WT and *Bj*PORCx plants. The ETR of WT plants declined by 36%. Under identical conditions due to 8 days of drought the ETR decreased by 20% in *Bj*PORCx plants. Upon rewatering, although there was not complete recovery, the transgenic plants recovered faster than WT plants, as evident from the ETR data (Fig 4).

Our results demonstrate that *Bj*PORCx plants are more tolerant to drought than the WT. We have shown that Chl biosynthesis is severely impaired due to water-stress. Photo-transformation of Pchlide to Chlide is severely affected by water stress and the Shibata shift is grossly altered (Dalal & Tripathy, 2012). In the PORCx plants, due to generation of abundant POR enzymes, Pchlide is efficiently phototransfomed to Chlide (Pattanayak and Tripathy, 2011). The Chlide produced due to photo transformation is immediately converted to chlorophyll molecules which then associate with the binding proteins present in thylakoid membrane subsequently transferring the absorbed energy to the reaction centers to drive photosynthetic reactions (Pattanayak & Tripathy, 2002, 2011; Tripathy *et al.*, 2004). Non-photo-transformable Pchlide acts as a photosensitizer to generate excess $^1O_2$ in chloroplasts (Ambastha *et al.*, 2020; Chauhan & Tripathy, 2019) and executes programmed cell death in the nucleus by retrograde signaling. The ROS especially $^1O_2$is known for inactivation of PSII and degradation of PSII reaction center D1 protein (Chakraborty and Tripathy, 1991; Graßes *et al.*, 2001a; Trebst *et al.*, 2002; Tripathy and Chakraborty, 1992).

*Bj*PORCx plants with efficient photo-transformation of Pchlide to Chlide due to abundant POR enzyme resulting in reduced accumulation of the photosensitizer Pchlide generated minimal reactive oxygen species (ROS) more specifically $^1O_2$. This resulted in smaller damage to PSII and its faster recovery upon re-hydration than the WT plants..

4. Conclusion

POR expression and activity is substantially decreased due to Water stress. This causes Pchlide accumulation and $^1O_2$ generation in light. Therefore, it is important to overexpress PORC to photo-transform accumulated Pchlide

to Chlide to minimize the accumulation of non-phototransformable Pchlide generated during stress condition. Due to efficient photo-conversion of Chl biosynthesis intermediate Pchlide by the abundant POR enzyme to Chlide non-photo transformable Pchlide does not accumulate, and this results in reduced generation of $^1O_2$ and consequently smaller damage to the photosynthetic apparatus.

Acknowledgment

The work was supported by a research grant from the Science and Engineering Board, Department of Science and Technology, Government of India (No EMR/2016/002732).

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Plant Science Research 43 (1&2) : 20-26, 2021

Plant Science Research

ISSN 0972-8546

Documentation of Chromium tolerant species for mitigation of mining pollution: A mini-review

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ARTICLE INFO

Article history:
Received : 28 October 2021
Revised : 20 November 2021
Accepted : 21 December 2021

Keywords:

Chromium (Cr);
Mining;
Toxic;
Phytoremediation;
Accumulation;
hyperaccumulators.

ABSTRACT

Extensive mining activities increased the chromium (Cr) contamination in the environment. Cr, a potentially toxic metal arising from various natural and anthropogenic activities such as the electroplating, steel and leather industries, is carcinogen to living organisms as well as a risk to ecology. Hence, the remediation of Chromium pollution has gained widespread attention. The toxic nature of Cr severely affects plant growth and development. Naturally Cr exists in various oxidation states, including Cr(III) and Cr(VI). The hexavalent Cr is the most toxic and persistent form in soil. Plants uptake Cr through various transporters such as sulfate and phosphate transporters. In recent years, Cr accumulating plants has been recognized as one of the promising Phytoremediation methods for the Cr contaminated region. This review summarized the Phytoremediation techniques of Cr and uptake mechanisms of different plants.

1. Introduction

Environmental contamination of Chromium in soil arises from numerous natural and anthropogenic activities have gained substantial consideration worldwide due to its high level in the soil. (Ashraf *et al.*, 2017). Chromium accumulates in food crops from the contaminated soils and possesses severe health risks in humans via food chain (Ahmed *et al.*, 2016). Plant physiology (plant type, rate and type of root secretions, root surface area and transpiration) and soil conditions (texture, pH, cation exchange capacity) all influence Cr transport from the soil to the plant (Banks *et al.*, 2006; Zeng et al., 2011b; Santos and Rodriguez, 2012). Cr is translocated to aerial regions in the majority of plant species with slow process and mostly reserved in root tissues (Jaison and Muthukumar, 2016). Cr-hyperaccumulators like atlantic cord grass (*Spartina argentinensis*), jelutong (*Dyeracostulata*) and spleen amaranth (*Amaranthus dubius*), on the other hand, can ingest and translocate high Cr levels in shoot tissues (de Oliveira *et al.*, 2016).

Indian mineral sector is playing a vital role not only to generate employment opportunities with improved livelihoods but also responsible for environmental degradation. Moreover, the major impact of mining is depletion of natural resources, decrease in rainfall, loss of cultivable land and pollution in soil, water and air etc. As per NMI database based on UNFC system, the total reserves/ resources of chromite in the country as on 2020 has been estimated at 344 million tonnes with 102 million tonnes as "Reserves" (30%) and 241 million tonnes as "Remaining Resources" (70%). More than 96% resources of chromite are located in Odisha, mostly in Jajpur, Kendujhar and Dhenkanal districts. The Principal producers in Odisha are OMC Ltd., TATA (TISCO), Balasore Alloys Ltd. IMFA and Facor Ltd. Minor deposits are scattered over Manipur, Nagaland, Karnataka, Jharkhand, Maharashtra, Tamil Nadu, Telangana and Andhra Pradesh (Figure 1). The Sukinda valley in Odisha contains 96% of India's chromite reserves. The pollution in and around the area made this place as Odisha's Chernobyl and identified one of the world's top 10 most polluted regions.

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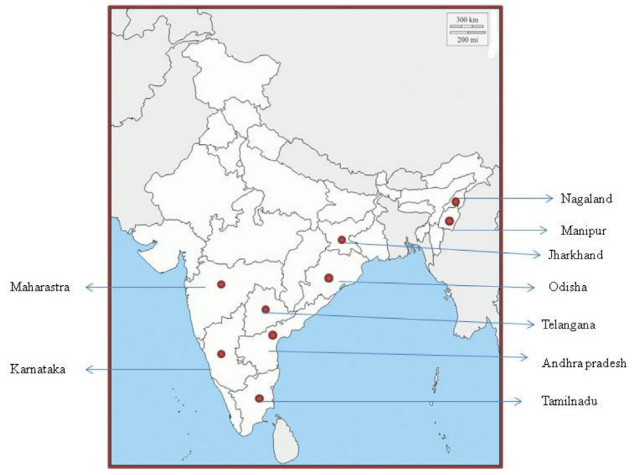

Source: India Minerals Year Book, 2018,57th edition, November 2019.

Figure 1- Distribution of Chromite deposits across different states of India.

According to the Orissa Voluntary Health Association (OVHA), chromium extraction is responsible for 84% of deaths in Sukinda's mining districts and 86% of deaths in neighboring industrial villages.

The Supreme Court of India has emphasized the need of Environmental Impact Assessments in light of the negative environmental and social effects of mining (Goa Foundation Case, April 2014). When "the macro effect of such wide-scale land and environmental degradation caused by the absence of remedial actions (including a rehabilitation plan)" is not taken into account, it has negative repercussions, according to the report (Karnataka mining case, Supreme Court of India, April 2013). The Supreme Court has also stated that mining operations must be conducted within the parameters of Article 21 of the Constitution, which includes the right to a clean environment and pollution-free air, the precautionary principle, and the principles of sustainable development and inter-generational equity. It makes financial sense to protect the environment. Natural resource degradation places a tremendous strain on the economy.

According to the World Bank, India's total yearly cost of environmental degradation is around Rs. 3.75 trillion (US$80 billion), or 5.7 percent of GDP in 2009, the base year for most damage estimates (World Bank, 2013). This is based on the total cost of air and water pollution, as well as the deterioration of cropland, pasture land, and forest area. Greenpeace's latest analysis (February 2020) backs this up, estimating that the yearly cost of air pollution in India is US$ 150 billion. After China and the United States, India has the third greatest cost of fossil fuel air pollution in the world, according to the analysis.

2. Environmental and chemical aspect of chromium

Chromium is naturally present in mineral form as crocoites and is usually a silvery hard metal with atomic number 24 and molecular weight 51.1u with density of 7.19 gram per cubic centimeter. It is the 7th most prevalent element in the earth's crust and the 21st most abundant metal. It is among 18 core hazardous air pollutants, 33 urban air toxicants and registered as 7th among top 20 dangerous substances by Agency for toxic compounds and disease registry (ASTDR). Chromium usually is found in the form of chemical compounds such as chromate and dichromate with high oxidizing potential, solubility and mobility across membranes in living organisms and the environment.

Different chemical speciation of chromium makes it unique among hazardous metals. Cr(VI) being the most toxic than Cr(III) has many toxic implications such as carcinogenic property. Similarly, both species differ significantly in terms of their absorption, bioavailability, and transport to the aerial parts (Choppala *et al.*, 2016). It can move to the animal system if a plant accumulated with chromium is ingested by the animals. Chromium toxicity has been shown to interfere with plant development, create ultrastructural changes in the cell membrane and chloroplast, cause chlorosis in leaves, harm root cells, diminish plant pigment content, disrupt water and mineral uptake as well as affect many enzymatic activities (Ali *et al.*, 2015; Farooq *et al.*, 2016; Reale *et al.*, 2016).

3. Phytoremediation of Cr by hyperaccumulating plants

Over the last few decades, Researchers have identified tolerant and hyperaccumulator plants in order to investigate their mechanisms and applications in the phytoremediation process. Nearly 500 plant species from more than 45 families have been recognised to date. The harmful metals were mostly converted into less toxic and immobile forms by the tolerant hyper-accumulator plants (Cervantes *et al.*, 2001). The function of high-affinity ligands like as amino acids, peptides, and organic acids, which chelates the metal ions and sequesters them within the vacuole, is central to the mechanism of Cr hyper-accumulators. Increased rhizospheric metal mobilisation by organic acids; absorption using different families of transporters and then translocating it into the shoot via xylem loading, finally detoxifying it via chelation and compartmentalization within the vacuoles are all important factors governing the hyper-accumulation of Cr and other heavy metals (Shahid *et al.*, 2013).

In mining areas, some naturally growing plant species can be found as these have high ability for uptake of contaminants as well as have innate mechanism to tolerate

Table-1

Accumulation of Cr at different parts of some hyperaccumulative Plants

Sl No.	Plant Name	Plant parts	Cr uptake (mg/Kg)
1.	*Alternanthera sessilis*	Roots Leaves	1,017201
2.	*Azolla caroliniana*	Whole plant	356
3.	*Brassica juncea*	Roots Shoots	1,6404,100
4.	*Callitriche cophocarpa*	shoots	1,000
5.	*Convolvulus arvensis*	leaves	2,800
6.	*Eichhorniacrassipes*	roots	3,951
7.	*Helianthus annuus*	Shoot root	1,356556
8.	*Leersia hexandra*	Leaves Stem Root	2,1643,4753,299
9.	*Leptospermum scoparium*	Foliage ash	20,000
10.	*Marsilea drummondii*	Roots	1,300
11.	*Nopalea cochenillifera*	Roots Shoots	25,263705
12.	*Nymphaea spontanea*	Plant	2,200
13.	*Polygonum hydropiperoides*	Roots	2,980
14.	*Pteris vittata*	Roots Shoots	5,7171,145
15.	*Thlaspi caerulescens*	Roots	3,400-3,500
16.	*Laguncularia racemosa*	Roots	560,000
17.	*Aerobryopsis longissima*	Moss ash	7,500
18.	*Rinorea niccolifera*	Leaves	30,000
19.	*Pearsonia metallifera*	Foliage ash	20,000
20.	*Typha angustifolia*	Roots	20,120
21.	*Prosopis juliflora*	Whole plant	372
22.	*Salix matsudana*	Roots	746
23.	*Salsola kali*	Roots Stems Leaves	2900790600
24.	*Salvinia natans*	Roots Leaves	52007400
25.	*Spartina argentinensis*	Whole plant	15,100
26.	*Vallisneria spiralis*	Roots Leaves	11271378
27.	*Lemna minor*	Plant tissue	2870
28.	*Marsilea drummondii*	Roots	1300
29.	*Phragmites australis*	Rhizome Shoot Leaves	4825883627

[Source: Singh *et. al.*, Environ Chem Lett (2013) 11:229-254]

their toxicity. These plants are called hyperaccumulator plants (Table 1) and with respect to this, plants with tendency to accumulate about 1000mg/kg (0.1 % of dry weight) have been categorized as chromium hyperaccumulators (Reeves and Baker 2000). Most of the chromium uptaken by the plant is retained inside the root and a little is translocated to the shoot. In fact, plants also show a great degree of difference in tolerance, uptake and accumulation of chromium (Shahandeh and Hossner 2000).

Hyperaccumulators are such plants which are capable of concentrating higher amount of heavy metal in their above-ground tissues which is far more than those present in the soil or in the nearby growing non-accumulating plants Memon *et al.*, 2001; Memon and Schröder, 2009). Two factors play important role while estimating the hyperaccumulation capacity of a specific plant. One is bioaccumulation factor and the other is translocation factor. Bioaccumulation factor

Table-2

Tolerance mechanism of Plants towards Chromium

Sl. No.	Plant	Family	Tolerance Mechanism
1	*Mesembryanthemum crystallinum* L.	*Aizoaceae*	Phyto-extraction
2	*Gomphrena celosoides* Mart.	*Amaranthaceae*	Increased proline and antioxidant enzyme activities
3	*Allium griffifithianum* Boiss.	*Amaryllidaceae*	Hyper-accumulation
4	*Calotropis procera* (Aiton) W.T. Aiton	*Apocynaceae*	Increased activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR)
5	*Colocasia esculenta* (L.) Schott	*Araceae*	High accumulation of Cr(VI)
6	*Lemna minor* L.	*Araceae*	Increased anti-oxidant activity, Phyto-extraction
7	*Lemna minuta* Kunth	*Araceae*	Increased anti-oxidant activity
8	*Pistia stratiotes* L.	*Araceae*	Anti-oxidant activity and hyper-accumulation
9	*Arundodonax* L.	*Poaceae*	Hyper-accumulation
10	*Brachiaria mutica* (Forssk.) Stapf	*Poaceae*	Phyto-stabilizer
11	*Chrysopogon zizanioides* (L.) Roberty	*Poaceae*	Hyper-accumulation
12	*Diectomis fastigiata* (Sw.) P. Beauv.	*Poaceae*	Hyper-accumulation
13	*Pennisetum americanus* L. *X*	*Poaceae*	Hyper-accumulation
14	*Pennisetum purpureum* (Schumach)	*Poaceae*	Hyper-accumulation
15	*Leersia hexandra* Sw.	*Poaceae*	Hyper-accumulation
16	*Miscanthus sinensis* Andersson	*Poaceae*	Hyper-accumulator
17	*Oryza sativa* L.	*Poaceae*	Hyper-accumulation
18	*Phragmites australis* (Cav.) Trin.	*Poaceae*	Phyto-reduction
19	*Phragmites communis* (Trin.)	*Poaceae*	Phyto-reduction
20	*Spartina argentinensis* (Trin.) Merr.	*Poaceae*	Hyper-accumulation
21	*Cymbopogon flexuosus*	*Poaceae*	Hyper-accumulation
22	*Eichhornia crassipes* Mart.	*Pontederiaceae*	Hyper-accumulation
23	*Pteris vittata* L.	*Pteridaceae*	Hyper-accumulation
24	*Genipa americana* L.	*Rubiaceae*	Hyper-accumulation
25	*Salvinia minima*	*Salviniaceae*	Increased anti-oxidant activity and hyper-accumulation
26	*Solanum viarum* Dunal	*Solanaceae*	Hyper-accumulation
27	*Origanum vulgare* L. Mediterranean,	*Lamiaceae*	Hyper-accumulation
28	*Salvia moorcroftiana* Wall. ex Benth.	*Lamiaceae*	Biosorptivedetoxifification
29	*Callitriche cophocarpa* Sendtn.	*Plantaginaceae*	Hyper-accumulation
30	*Euphorbia helioscopia* L. Desert	*Euphorbiaceae*	Hyper-accumulation
31	*Rumex dentatus* L.	*Euphorbiaceae*	Hyper-accumulation
32	*Arachis hypogea* L.	*Fabaceae*	Hyper-accumulation
33	*Cassia tora* L.	*Fabaceae*	Hyper-accumulation
34	*Medicago sativa* L.	*Fabaceae*	High proline and GST accumulation

35	*Medicago truncatula* Gaertn.	*Fabaceae*	Regulating the sulphur transport and metabolism
36	*Sesbania sesban* (L.) Merr.	*Fabaceae*	Phyto-stabilizer
37	*Vigna unguiculata* (L.) Walp.	*Fabaceae*	Hyper-accumulation
38	*Cirsium vulgare* (Savi) Ten.	*Asteraceae*	Hyper-accumulation
39	*Dicoma niccolifera*	*Asteraceae*	Hyper-accumulation
40	*Gynura pseudochina* (L.) DC.	*Asteraceae*	Cr (VI) reduction
41	*Helianthus annuus* L.	*Asteraceae*	Hyper-accumulation
42	*Parthenium hysterophorus* L.	*Asteraceae*	Hyper-accumulation
43	*Vernonia cinerea* (L.) Less.	*Asteraceae*	Hyper-accumulation
44	*Origanum vulgare* L. Mediterranean	*Lamiaceae*	Hyper-accumulation
45	*Salvia moorcroftiana* Wall. ex Benth.	*Lamiaceae*	Biosorptivedetoxification
46	*Callitriche cophocarpa* Sendtn.	*Plantaginaceae*	Hyper-accumulation
47	*Phyllostachys pubescens*	*Poaceae*	Phyto-extraction

[Source: Srivastava *et. al.,* Sustainability, 2021, 13, 4629.]

is the concentration of metal accumulated in the aboveground part to that of concentration of metal in soil.

Some plants are having natural ability of hyperaccumulation for specific heavy metal and are known as natural hyperaccumulators. On the other hand, genetic modifications can also be performed to facilitate higher uptake capacity of plants for heavy metals and such plants are regarded as transgenic plants.

3.1 Techniques of phytoremediation

Phytoremediation in recent times has been exploited in several ways including phytoextraction, phytostabilisation, phytofilteration, phytvolatilisation, phytodegradation etc. (Table 2).

3.1.1 Phytoextraction

Also known as phytoaccumulation, phytoabsorption or phytosequestraction is the uptake of contaminants from soil or water by plant roots and their translocation to and accumulation in aboveground biomass (shoots). Metal translocation is crucial for effective phytoextraction and can be determined by using translocation factor i.e, concentration of metal in aboveground plant parts to that of soil concentration.

3.1.2 Phytofiltration

It is removal of pollutants from contaminated surface waters and waste waters by plants. It may be rhizofiltration as roots are used or blastofiltration if seedlings are used, when excised plant shoots are used it is regarded as caulofiltration. As the contaminants are absorbed or adsorbed, their movement to underground water is minimised.

3.1.3 Phytostabilisation

Use of certain plants for stabilisation of contaminants in polluted soils, mostly used to reduce the mobility and bioavailability of pollutants in the environment. Plants can immobilise the heavy metals in soils through absorption by roots, precipitation, complexation, or metal valence reduction in rhizosphere. In turn, the metals vary in toxicity owing to their special redox mechanisms; these are converted into less toxic forms thus decreasing the deleterious effects. For this purpose, the reduction of Cr^{6+} to Cr^{3+} is widely studied, as the latter is comparatively less toxic and less mobile.

3.1.4 Phytovolatilisation

It is the uptake of pollutants from soil by plants, thus facilitating their conversion to volatile form and subsequently release into the atmosphere. This technique is usually applied for organic pollutants and some heavy metals like Hg and Se. However, it is limited by the fact that it doesn't actually remove the pollutant completely rather transfers it from soil to the atmosphere from where it can be re-deposited.

3.1.5 Phytodegradation

This involves degradation of organic pollutants by plants with the help of enzymes such as dehalogenase and oxygenase and not dependent on rhizospheric microorganisms. Plants have the ability to accumulate organic pollutants from the soil and detoxify them through their

metabolic activities. Recently, major focus is being given to synthetic herbicide and insecticides, and their degradation using efficient plant species.

3.1.6 Rhizodegradation

It refers to breakdown of organic pollutants in the soils by microorganisms in the rhizosphere, which extends about 1mm around the root and is under the influence of the plant. The higher metabolic activities of the microbes in this zone are responsible for enhanced degradation of pollutants. In turn plants can stimulate microbial activity by 10-100 times higher by secreting exudates containing carbohydrates, amino acids, flavonoids. These provide carbon and nitrogen sources to the soil microbes and creates a nutrient rich environment in which microbial activity is stimulated (Kuiper et al., 2004; Yadav et al., 2010).

4. Conclusion and Future Prospective

Mining of Chromium affects various life forms by altering the physiological and metabolic pathways. With the exposure to Cr, plant remodulates its genetic and transcriptional regulation for better adaptation. However, bioremediation techniques still have some disadvantages because they are time consuming, limited to moderately contaminated sites and readily disturbed by the external environment. Thus, in order to fully exploit bioremediation for Cr contamination, we must first understand the complete Cr remediation processes and mechanisms for biologically-derived materials, living organisms, and their coupled effects because there is still some uncertainty about the processes and mechanisms involved. Thus, great efforts and practices are required to study the processes and mechanisms of Cr(VI) remediation, and to promote efficient field applications of bioremediation techniques for site-specific Cr(VI) pollution.

Therefore, we suggest the following areas for future remediation research.

- Specific research is required to evaluate various transporters involved in chromium uptake and associated metabolic pathways.
- Tolerant and native hyperaccumulative plants need to be screened/evaluated for the better adaptation and can bioremediate Cr from the Cr-contaminated regions.
- The mechanisms related to the remediation with microbes need to be elucidated in order to identify more Cr-resistant microbial strains and applications of microbial strains in the contaminated area should be taken care especially protecting the surrounding water in an ecological environment.
- Molecular biology and genetic engineering techniques should be employed to develop transgenic plants with hyperaccumulating capability with rapid reproduction and growth, high resistance to Cr.
- Further studies are required of the complex functions of plant roots, microbial strains and carrier molecules, including the characteristics of different plant species and rhizosphere microbes.

Acknowledgments

The Authors are highly thankful to the Director of CSIR- IMMT Prof. Suddhaswata Basu for providing necessary lab facilities to carry out the work.

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Plant Science Research

ISSN 0972-8546

Hexavalent chromium alters the antioxidative efficiency and increases Lipid peroxidation in germinating wheat seeds

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ARTICLE INFO

Article history:
Received : 13 November, 2021
Revised : 7 December, 2021
Accepted : 30 December, 2021

Keywords:

ascorbic acid content,
catalase,
germination percentage,
oxidative stress,
peroxidase,
superoxide dismutase

ABSTRACT

Wheat (*Triticum aestivum* L. cv. Sonalika) seeds were germinated in presence of hexavalent Cr (0.5, 1.0, 2.0, and 4.0 mM) for 24 h and germination percentage, ascorbic acid content, proline content, soluble protein content, activities of antioxidative enzymes like superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7) along with lipid peroxidation level were determined in embryonic tissues. It was found that the activities of SOD, CAT and POX increased with increase of metal in the medium and it was significant ($p < 0.05$) even at lowest concentration of the metal tested. The level of lipid peroxidation increased significantly ($p < 0.05$) in the embryonic tissues. Ascorbic acid content decreased whereas proline content increased in the embryonic tissues in response to increase in Cr concentration in the medium. The results indicated the imposition of oxidative stress situations during germination stage by Cr (VI) stress which might be one of the probable reasons behind Cr induced toxicity in germinating seeds.

1. Introduction

Chromium (Cr) pollution has now become a serious environmental problem throughout the world (Shahid *et al.,* 2017). This is mainly because of the multiple industrial uses of this metal such as leather tanning, mining and electroplating. Along with its other affects, Cr pollution poses a serious threat to crop growth and human health (Ertani *et al.,* 2017; Sharma *et al.,* 2020). Differently from other heavy metals like cadmium and copper, Cr mainly exists in two valence states i.e. Cr (VI) and Cr (III) (Ashraf *et al.*, 2017). Other valance states are unstable and short lived in biological systems. Hexavalent Cr usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions and is therefore, highly soluble in water. It is highly mobile and considered as the most toxic form of Cr. On the other hand, Cr (III) is less mobile, less toxic and is mainly found bound to organic substances in the environment (Becquer *et al.*, 2003). It's worth noting that Cr (VI) has stronger stability, mobility and toxicity than Cr (III) (Choppala *et al.*, 2018). Germination is a phase in plant growth and development during which the radicle and plumule emerge out of the seed upon absorption of water. Since Cr does not have any physiological role in plants and the embryonic tissues are tender during germination, exposure to chromium during this stage is expected to cause toxicity. Studies on the rice (*Oryza sativa* L.) (Sharma *et al.*, 2016) and corn (*Zea mays* L.) (Hou *et al.*, 2014), revealed that the seed germination of plants was inhibited to varying degrees under Cr stress.

The phytotoxic effects of Cr includes inhibition of seed germination, loss of photosynthetic pigments, reduction in growth and yield along with several other physiological anomalies. Heavy metals like Cd and Pb are known to impose oxidative stress situations in plants by altering the natural antioxidative efficiency of the cells (Dey *et al.*, 2007). Oxidative stress situations are generally created when there is generation of higher amounts of reactive oxygen species (ROS) like superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2),

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hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) beyond the cell's capacity of endogenous antioxidative protective system to scavenge them off. Thus oxidative stress is essentially a regulated process and the equilibrium between oxidative and antioxidative capacities determines the fate of plants. Assessment of antioxidative efficiency of plants subjected to heavy metal stress is very vital to understand the toxicity mechanism and perhaps this is why lots of works have been done in this aspect for different heavy metals. But works on detail antioxidative efficiency of germinating seeds exposed to Cr are rare. Even though some studies like germination percentage and lipid peroxidation level in embryonic tissues of germinating wheat seeds exposed to Cr (VI) were reported from this laboratory earlier (Dey *et al.* 2009) but other analyses related to antioxidative efficiency were not done to ascertain a probable mechanism of Cr induced toxicity. Therefore, in this work a detail study on the activities of antioxidative enzymes like superoxide dismutase (SOD, EC 1. 15. 1. 1), catalase (CAT, EC 1. 11. 1. 6) and peroxidase (POX, EC 1. 11. 1. 7) along with lipid peroxidation level have been determined in germinating embryonic tissues of wheat under exposure to chromium. At the same time assessment of some metabolies like soluble protein, ascorbic acid and proline has been done in order to understand their probable role during Cr stress in germinating seeds.

2. Materials and Methods

2.1 Plant material, growth conditions and imposition of Cr stress:

Wheat (*Triticum aestivum* L. cv. Sonalika) seeds were selected for uniform size and surface sterilized with freshly prepared filtered 3% solution of commercial bleaching powder (calcium oxychloride) for 30 min, followed by washings with distilled water for several times. In different Petri dishes 50 uniform size surface sterilized wheat seeds were spread over filter paper, moistened with 15 ml of 0.5, 1.0, 2.0 and 4.0 mM Cr (VI) as $K_2Cr_2O_7$, prepared with half strength Hoagland's solution. In another Petri dish 15 ml of half strength Hoagland's solution, without Cr, was taken as control. The Petri dishes were covered and kept in an incubator at 30°C for 24 hr. Number of seeds germinated in each Petri dish was counted to determine the percentage of germination. The germinated embryonic tissues, leaving the endosperm portion, were excised from the seeds and taken for different biochemical analyses.

2.2 Extraction and estimation of soluble protein:

For soluble protein extraction, small volumes of respective enzyme extracts (the preparations of which are given below) utilised for enzyme assays, were used separately. For protein precipitation, an equal volume of 20% (w/v) trichloroacetic acetic acid (TCA) was added to the enzyme supernatants and were kept overnight in a refrigerator. The pellets were then washed successively with 10% cold TCA, ethyl alcohol, ethyl alcohol: chloroform (3:1, v/v), ethyl alcohol: ether (3:1, v/v) and finally with ether. The pellets were evaporated to dryness and solubilized with 0.3 N NaOH for overnight. The supernatants were collected for protein estimation using bovine serum albumin as standard, as described by Lowry *et al.* (1951).

2.3 Extraction and estimation of malondialdehyde (MDA):

In this study, the level of lipid peroxidation was measured by estimating MDA which is a decomposition product of peroxidized polyunsaturated fatty acid components of membrane lipid. Thiobarbituric acid (TBA) was used as the reactive material and the extraction and estimation was done following the method of Heath and Packer (1968).

2.4 Extraction and estimation of proline content:

Poline content was estimated following the method of Bates *et al.* (1973). The embryonic tissues were homogenised in 3% aqueous solution of sulphosalicylic acid and centrifused at 5000 rpm for 10 min. Then, 2 ml of supernatant was taken in a test tube to which 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added. The contents were mixed and boiled in a water bath at 100°C for 1 hr. The test tubes were cooled and 4.0 ml of toluene was added to each, mixed vigorously and then allowed to separate the phases for few min. The upper chromophore phase was collected carefully and absorbance was measured at 520 nm in a spectrophotometer. The proline content was determined by comparing the absorbance with that of a standard curve drawn with known concentrations of proline.

2.5 Extraction and estimation of ascorbic acid content:

For the estimation of ascirbic acid, the method of Mitsui and Ohta (1961) was followed. The embryonic tissues were collected and washed thoroughly with distilled water and then air-dried. The tissues were homogenized with 6% metaphosphoric acid. The homogenates were centrifuged at 5,000 rpm for 10 min. The supernatants were used for spectrophotometric analysis of ascorbic acid.The assay mixture was composed of 2 ml of 2% sodium molybdate and 2 ml of 0.15 N sulphuric acid. After this, they were mixed and chilled. Then 1 ml of 1.5 mM monobasic sodium phosphate buffer was added. Finally 1 ml of tissue extract or standard ascorbic acid solution was added to it. Then, it was incubated at 60°C in a water bath for 40 min, then cooled and centrifuged. The absorbance was taken at 660 nm in a

spectrophotometer. The ascorbic acid content of the tissue extract was calculated by comparing the absorbance of the samples with the standard curve drawn with 0 to 176 µg per ml of ascorbic acid.

2.6 Extraction and estimation of antioxidative enzymes:

The activities of three antioxidative enzymes, viz, superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POX) were assayed in this study. For enzyme extraction, embryonic tissues were homogenized under ice-cold conditions in extraction buffers containing 10% (w/v) insoluble polyvinylpolypyrrolidone. The buffers used were: 50 mM sodium phosphate buffer, pH 7.4 for SOD, and 50 mM sodium phosphate buffer, pH 7.5 for CAT and POX. Homogenates were centrifuged at 17, 000 *g* for 10 min at -4^0 C and the resulting supernatants were desalted by passing through gel filtration columns, packed with presoaked Sephadex G-25 (fine). The eluted fractions were tested for protein and the fractions responding to protein test were collected and used for the assay of the enzyme. The activity of SOD (U/ gFW) was assayed by measuring the inhibition of superoxide driven nitrite formation from hydroxylamine hydrochloride, following the method of Das *et al.* (2000). SOD activity was calculated using the formula Vo/V-1, where Vo is the absorbance at 543 nm of the control (without enzyme) and V is the absorbance of sample (with enzyme) at the same wavelength. Catalase activity (nkatal/ gFW) was assayed by measuring the decreasing concentration of H_2O_2 at 240 nm due to CAT activity (Aebi, 1983) and the activity was calculated by using the extinction coefficient of 40.0 mM^{-1} cm^{-1} for H_2O_2 at 240 nm. For assaying POX, guaiacol and H_2O_2 were used as substrates. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm, as described by Kar and Feierabend (1984) and the peroxidase activity (µ katal/ gFW) was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} due to tetraguaiacol formation under assay conditions.

2.7 Statistical analysis:

All the experiments were performed at least for three times with three replicates in each time. The mean values are presented in Figures and standard deviations are indicated. For analyzing the level of significance among the means, ANOVA test using Sigmaplot 11.0 was performed.

3. Results and Discussion

Germination of seed is an important phase in the life of the plants where upon imbibition of water the radicle and plumule emerge out of the seeds rupturing the seed coat. This is a crucial initial stage that ensures the healthy growth and development of plants. Like other phases of plant growth, the germination phase is also affected by different biotic and abiotic factors including heavy metals. In this study, wheat seeds were exposed to Cr (VI) and after 24 h the number of seeds germinated was counted to determine the % of germination and the results are presented in Fig. 1. It has been found that with increase in the Cr in the medium, there was decrease in the germination % of the seeds and it was significant even at the lowest concentration of the metal tested (0.5 mM). The process of seed germination is regulated by the mobilization of reserve food, such as starch, protein, and phytate etc. and the transfer of digested derivatives to the growing embryonic axis. The inhibitory effect of Cr (VI) might have occurred by decreasing the activities of key enzymes involved in carbohydrates, amino acid and peptides metabolism in germinating seeds (Zeid, 2001; Kuriakose and Prasad, 2008; Sethy and Ghosh, 2013). Similar suppression in seed germination due to exposure to Cr (VI) has also been reported in *Vigna radiata* (Samantaray, 2002); *Triticum aestivum* (Dey *et al.,* 2009; Dotaniya *et al.*, 2014); *Beta vulgaris*, *Daucus carota, Solanum melongena,* and *Raphanus sativus* (Lakshmi and Sundermoorthy, 2010); and in tomato (Khan *et al*., 2021). In general, the process of germination require high energy which is taken from rapid oxygen uptake and oxidative phosphorylation (Hourmant and Pradet, 1981). It is a fact that the mobilization of reserve food and oxidative phosphorylation causes the formation of reactive oxygen species (ROS) resulting in oxidative damage and consequent several metabolic alterations (Tommasi *et al*., 2001). Therefore, analysis of antioxidative efficiency in seeds germinating in presence of Cr (VI) is essential in order to understand the mechanism of toxicity and to manipulate the conditions to restore the seed germination in contaminated sites.

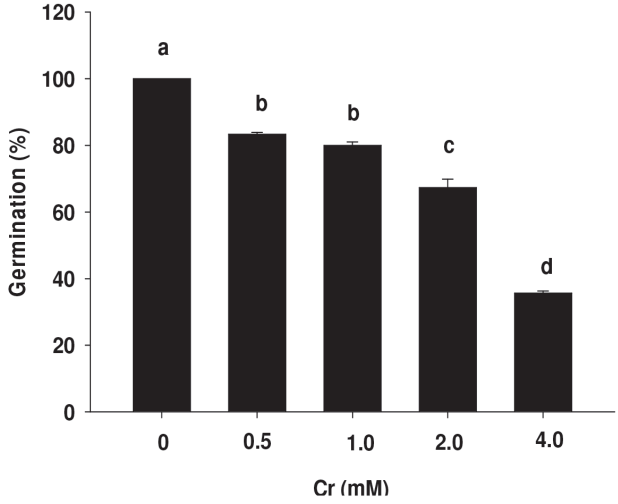

Fig. 1: Effect of Cr (VI) on the germination percentage of wheat seeds after 1 day of incubation. The values are the mean ± SD of three independent experiments. The mean values followed by the same letters are not significantly different ($p<0.05$; ANOVA test).

Proteins are considered to be the building blocks of life and have many functions in living organisms. The soluble protein of a cell mostly represents the total enzymes involved in the metabolic processes. In this study the soluble protein content of the embryonic tissues was estimated and the results are presented in Fig. 2. The results showed that there was increase in the soluble protein content in the tissues with increase in Cr (VI) concentration, except for the concentration of 0.5 mM at which protein content decreased than the control sample. This indicated that probably there was induction in the synthesis of some enzymes in response to Cr exposure as an adaptive cellular strategy to alleviate the stress injury.

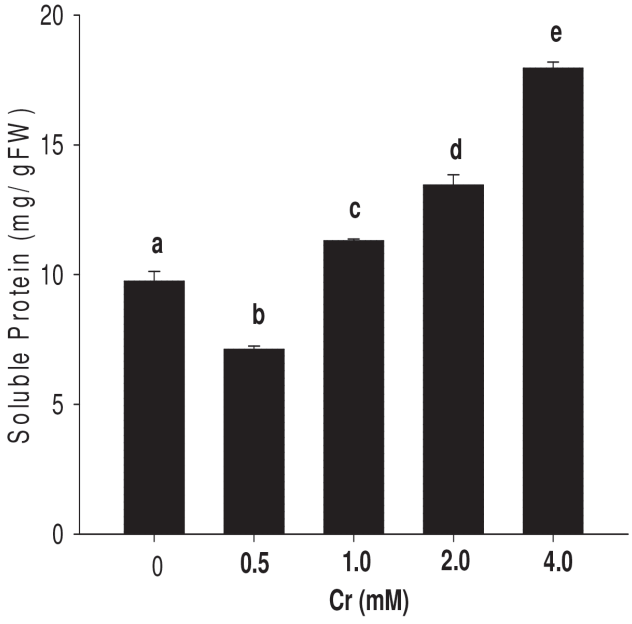

Fig. 2: Changes in the soluble protein content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

In aerobic organisms, generation of reactive oxygen species (ROS) takes place as a consequence of oxygen metabolism. These ROS include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Singlet oxygen ($^1O_2$) is another form of ROS which is formed when ground state molecular oxygen receives some extra energy as a result of which one of its unpaired electron in the outermost orbital gets excited and jumps to a higher state. The superoxide radical and hydroxyl radical are oxygen free radicals whereas hydrogen peroxide and singlet oxygen are non-radicals. These ROS are highly reactive which can oxidatively damage the cellular macromolecules at the sites of their generation causing various toxic effects and even cell death (Halliwell and Gutteridge, 2007; Pourrut *et al*., 2011). However, under normal conditions the toxicity of ROS are not realized due to the neutralizing effects of endogenous antioxidative defense systems which include the antioxidative enzymes and the low molecular weight antioxidative compounds (Elstner, 1982; Elstner *et al.*,1988; Halliwell and Gutteridge, 2007; Caverzan *et al*., 2016; Wu *et al.,* 2017). But under certain developmental stages and when the organisms are exposed to biotic and abiotic stresses the generation of ROS takes place beyond the scavenging capacity of the endogenous antioxidative protective systems and under such situations, the organisms are said to be exposed to oxidative stress (Elstner *et al.*, 1988; Halliwell and Gutteridge, 2007; Yu *et al*., 2019). Thus, there exists a delicate balance between the ROS generation and their scavenging by antioxidative protective systems and hence, the ability of plants to cope with oxidative stress is characterized by the degree of antioxidant activities (Shahzad *et al.,* 2018; Anjum *et al.,* 2015). Therefore, it is highly essential to assess the level of antioxidants under metal stress in order to determine the mechanism of toxicity. In this study the activities of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) have been assayed along with determination of ascorbic acid content in the germinating embryonic tissues and the results are presented in Figures 3, 4, 5 and 6 respectively.

The SOD is responsible for dismutation of superoxide radical to O_2 and H_2O_2 and thereby decreases the chances of buildup of this toxic oxygen species (Halliwell and Gutteridge, 2007; Yu *et al*., 2019). Here in this study, the SOD activity increased in the embryonic tissues with increase in the Cr (VI) in the medium (Fig. 3) which indicated that there was protection against the superoxide radical. But protection against the deleterious effects of H_2O_2 is equally important since it is the product of the SOD catalysed superoxide dismutation reaction along with being formed through other metabolic routes. Catalase and peroxidases are the principal antioxidative enzymes that decompose H_2O_2 and thereby reduce its buildup and further toxicity in the cell (Elstner, 1982; Halliwell and Gutteridge, 2007; Foyer and Noctor, 2009). In this study the activities of both CAT and POX were found to increase with increase in the Cr (VI) in the medium (Fig. 4 and 5). This indicated that protection against H_2O_2 was also maintained in the germinating wheat seeds under Cr stress. This increase in the activities of SOD, CAT and POX might be due to *de novo* synthesis of these enzymes in response to Cr stress which has been corroborated by the increase in soluble protein content in the tissues mentioned earlier (Fig. 2).

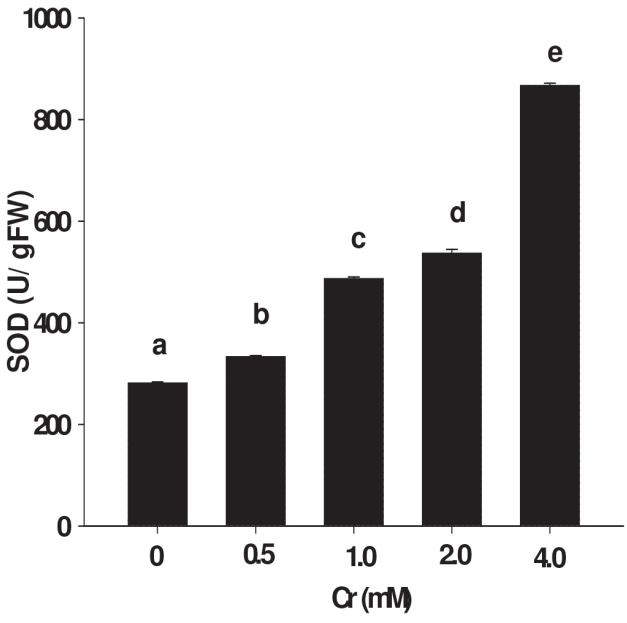

Fig. 3: Changes in the superoxide dismutase (SOD) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

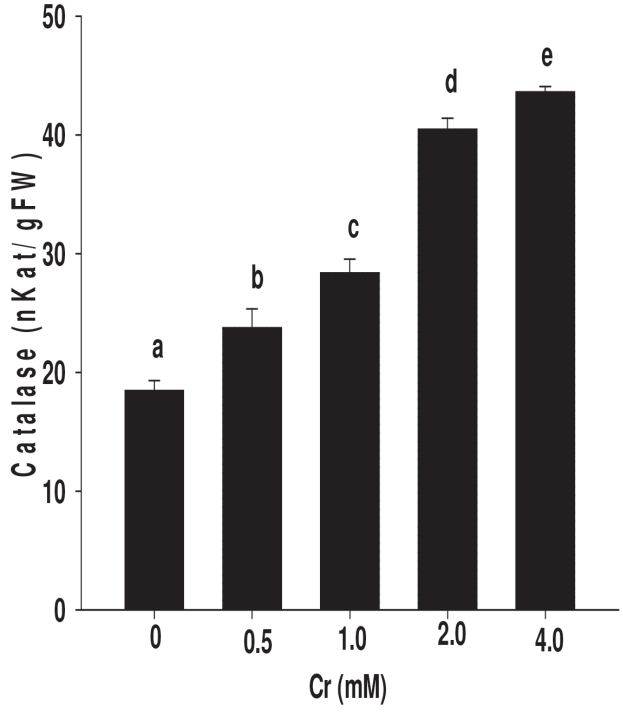

Fig. 4: Changes in the catalase (CAT) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

Alterations in the activities of SOD, CAT and POX have been reported in various systems under exposure to different heavy metals (Verma and Dubey, 2003; Dey *et al*., 2007; Dey *et al*., 2009; Pati *et al*., 2014) and the metal induced toxicity have been ascribed to the imposition of oxidative stress in respective cases. Even though the increase in SOD and CAT activities in the embryonic tissues under Cr stress, as reported in this study, may be attributed to enhanced efficiency of the cells to protect against superoxide radical and H_2O_2, augmentation in POX activity may be correlated to some other reason. The enhanced POX activity may be due to increased release of cell wall bound peroxidases in the cells and such enhancement in POX activity has been found in plants exposed to different metals (Verma and Dubey, 2003; Srivastava *et al*., 2006; Dey *et al*., 2007; Dey *et al*., 2009; Pati *et al*., 2014). However, decrease in POX activity has also been observed when the metal concentration was high (Srivastava *et al*., 2006; Pati *et al*., 2014). According to Zhang *et al*. (2007) enhancement in POX activity is a biomarker of heavy metal stress and hence imposition of stress due to Cr (VI) can also be presumed in this study. In this situation the elucidation of the role of ascorbic acid, the low molecular weight antioxidant, in the seeds germinating in presence of Cr seems very logical.

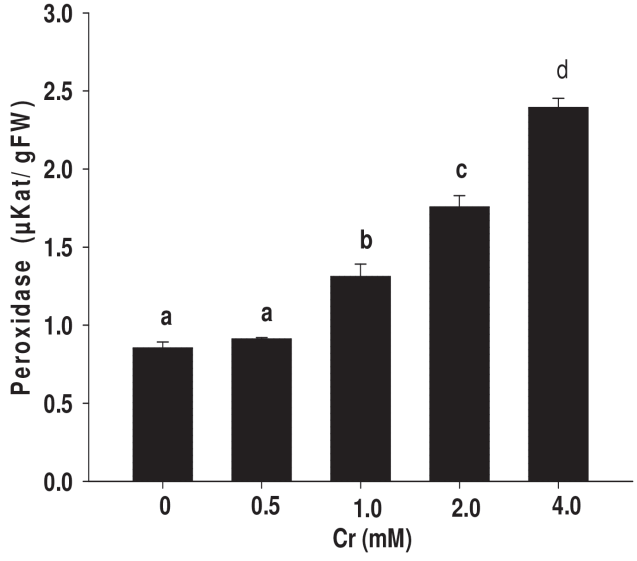

Fig. 5: Changes in the peroxidase (POX) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

The ascorbic acid content of the embryonic tissues has been found to be significantly affected due to Cr (VI) stress. The results presented in Fig. 6 show that there was decline in ascorbic acid content with increase in the Cr in the medium. Ascorbic acid is an important low molecular weight antioxidant of the cell which is known to scavenge the ROS like superoxide radical, hydroxyl radical and singlet oxygen (Bodannes and Chan, 1979). Among the different low molecular weight antioxidants ascorbate plays an important role in chloroplasts. In plant cells about 30-40% of the total ascorbate is localized in chloroplasts and its concentration in the stroma is about 50 mM (Foyer and Noctor, 2005). High levels of reduced ascorbic acid and glutathione are necessary in order to remove H_2O_2 permanently. Therefore, several enzymes involved in the ascorbate-glutathione pathway operate together in the chloroplasts to ensure the neutralization of H_2O_2. The pathway includes interrelated redox reactions involving ascorbate, glutathione, and NADPH (Asada, 1999).

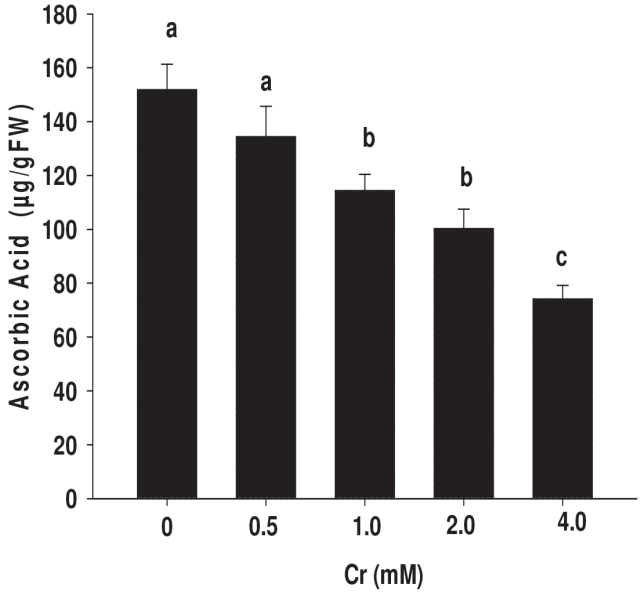

Fig. 6: Changes in the ascorbic acid content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

Even though neither the glutathione content nor the activities of enzymes of ascorbate-glutathione pathway have been analysed in this study, the decrease in the ascorbate content in the tissues indicated the low level of antioxidant status which might favour the pro-oxidation process. Therefore, measurement of lipid peroxidation level was highly essential since the ROS are a well-known cause of damage to membranes through lipid peroxidation process (Su *et al.*, 2019). Malondialdehyde (MDA), a decomposition product of peroxidised polyunsaturated fatty acid component of membrane lipid taking thiobarbituric acid as the reactive substance, content was measured to determine the level of lipid peroxidation. The results presented in Fig. 7 show that lipid peroxidation level increased with increase in the Cr (VI) in the medium.

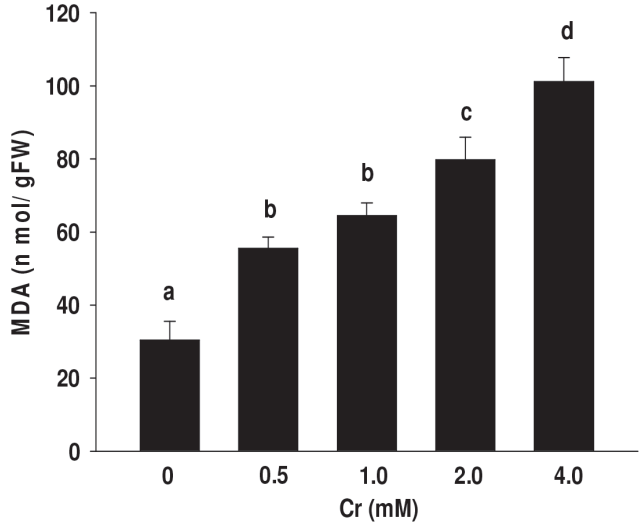

Fig. 7: Changes in the lipid peroxidation level in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

The hydroxyl radical is known to be the most potentially toxic species in aerobic cells. The polyunsaturated fatty acid components of membrane lipids are highly susceptible to hydroxyl radical attack which are peroxidized in its presence. Therefore, lipid peroxidation is the consequence of free radical mediated reactions in aerobic cells and is a good indicator of prevalence of oxidative stress (Kappus, 1985). The increase in lipid peroxidation level (Fig. 7) indicated that there was imposition of oxidative stress due to Cr (VI) in the embryonic tissues of germinating wheat seeds. The increase in the SOD and CAT activities indicated that the defense against superoxide radical and H_2O_2 was high in the seeds during germination but probably it was not enough to maintain the required antioxidant pool in the tissues. The decrease in the ascorbic acid content due to Cr stress (Fig. 6) further corroborates this assumption. Under these circumstances, further studies involving the determination of glutathione content and the activities of enzymes involved in the ascorbate-glutathione pathway are essential to elucidate the mechanisms of Cr -induced oxidative stress in germinating wheat seeds.

As an osmolyte and compatible solute, proline protects the plants from various stresses and helps the plants to recover from stress more rapidly. It accumulates in many plants in response to environmental stresses which has been positively correlated with the stress tolerance (Serraj and Sinclair, 2002; Ashraf and Foolad, 2007; Hayat *et al*., 2012). Besides acting as an osmolyte, proline plays three important roles in plants during stress situations, i.e., as a metal chelator, a signaling molecule and an antioxidative defense molecule (Smirnoff and Cumbes 1989; Bohnert *et al*., 1995). The proline content of the germinating embryonic tissues was also determined in this study and the results are presented in Fig. 8.

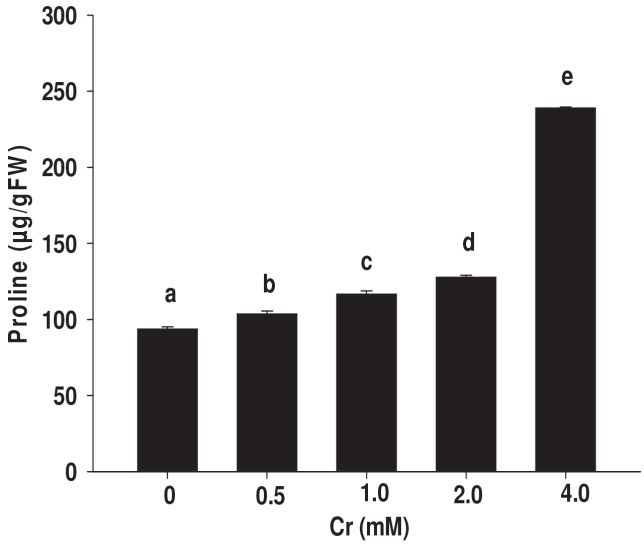

Fig. 8: Changes in the proline content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

The increase in the proline content was initially slow up to 2.0 mM of Cr (VI) in the medium which increased rapidly further at 4.0 mM. The accumulation of free proline in plants in response to heavy metal stress has been attributed as a consequence, rather than as a cause of metal tolerance (Schat *et al*., 1997). In this study the increased proline content in the tissues can be viewed as an indicator of stress imposition rather than emphasizing its protective roles, as mentioned above, which is because of the fact that despite enhancement in proline content toxic effects of Cr (VI) on the germinating wheat seeds have been observed in the forms of decrease in germination percentage as well as increase in the lipid peroxidation level.

4. Conclusion

Thus, the findings of this study indicate that Cr (VI) is toxic to the germinating wheat seeds which was observed initially in the form of decrease in germination percentage. In order to elucidate the mechanism of toxicity the activities of antioxidative enzymes like SOD, CAT and POX were analysed. Even though the activities of these enzymes were found to increase, it was perhaps not enough in giving antioxidative protection since pro-oxidative effect was noticed in the form of increase in lipid peroxidation level. The increase in proline content indicated the stress imposition due to Cr (VI). The decrease in the ascorbic acid content can be assumed to be the cause behind the Cr induced toxicity in germinating wheat seeds and for this, further studies involving the determination of glutathione content and enzymes involved in the ascorbate-glutathione pathway are highly essential to validate such proposition and to understand the mechanism of toxicity. The toxicity imposed in the embryonic tissues during seed germination in the form of oxidative injury may persist which may affect the plant during its further growth and development. It is also a fact that maintaining metabolic functions under stress conditions is crucial for plants to survive. Therefore, remediation of soil contaminated with hexavalent chromium should be done before sowing seeds.

Acknowledgements

The authors are thankful to the Head, Department of Environmental Science, Fakir Mohan University, Vyasa Vihar, Balasore-756089 for providing the facilities to carry out this work.

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Plant Science Research 43 (1&2) : 36-49, 2021

Plant Science Research

ISSN 0972-8546

Morphological, molecular and biochemical characterization of cyanobacteria from rice field cultivated for last 75 years

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ARTICLE INFO

Article history:
Received : 8 October, 2021
Revised : 21 November, 2021
Accepted : 20 December, 2021

Keywords:

Cyanobacteria,
Rice fields,
Biofertilizer,
Growth,
Pigments

ABSTRACT

The present study investigated the diversity of cyanobacteria population existent in the rice fields of National Rice Research Institute, Cuttack, India. Rice is being continuously grown in two seasons annually for last 75 years with different crop management practices. A total of 20 different isolates of cyanobacteria were collected based on morphological and molecular markers and were characterised for growth pattern, primary metabolites, different pigments and nitrogen fixing enzymes. *Anabaena variabilis, Nostoc* sp. (1) and *Scytonema* sp. (2) had significantly higher content of cell dry weight, protein and carbohydrate as compared to others. Similarly, different pigments such as chlorophyll *a*, carotenoids and phycobiliproteins were maximum in *Nostoc* sp. (1), *Westiellopsis* sp. (2) and *Niveispirillum cyanobacteriorum,* respectively. The nitrogen fixing ability was checked by studying nitrate reductase and glutamine synthatase and they were significantly higher in *Nostoc* sp. (1) and *Anabaena variabilis,* respectively. Based on principal component analysis and heat map study, *Anabaena variabilis* and *Nostoc* sp. (1) could be used as biofuel producer and biofertilizer; *Westiellopsis* sp. (2), *Fischerella* sp., *Synechocystis* sp. and *Anabaena variabilis* could be promoted in cosmetic/commercial industries. There is a great scope for further utilisation of these characterised isolates of cyanobacteria in different industries.

1. Introduction

Cyanobacteria (Blue Green Algae) are the largest group of gram-negative, oxygen-evolving photoautotrophic prokaryotes, which belong to the kingdom Eubacteria. This group is highly diverse based on ecological, biological and morphological characters (Flores and Herrero, 2010; Komárek, 2010). Morphologically, they are differentiated as filamentous, non-filamentous, unicellular, planktonic or benthic and colonial (coccoid) forms (Burja *et al.,* 2001). Ecologically, they are widely distributed in almost every habitats of the world (Sao and Kritika, 2015). They can easily be found in the diverse habitats such as terrestrial areas, desert, freshwater and hypersaline environments because of their specialized features. They are present as both free-living form as well as in syntrophic/symbiotic association with algae, fungi, bryophytes, pteridophytes, gymnosperms, angiosperms, and animals like ascidians (Adams, 2000).

Cyanobacteria play a crucial role in different ecosystem functions. They can fix nitrogen, mobilize phosphorus and can metabolise CO_2, H_2 and O_2 (Wilson, 2006). They can act as a bio-resource group for different industries such as biofuels, biofertilizers, vitamins, bioremediants, natural colouring agents, pharmaceutical drugs, biopolymers, neutraceuticals, cosmetics and feed, etc. (Gupta *et al.,* 2013; Singh *et al.,* 2016). Cyanobacteria are also helpful in enhancing the plant growth, crop yields, crop weight, microbial biomass carbon, soil fertility, water holding capacity, the availability of nutrients and provides oxygen to the rhizosphere (Wilson, 2006; Rana *et al.,* 2015).

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The environment of rice fields is most suitable for the growth of cyanobacteria as it provides convenient temperature, nutrient facility with continuous water supply and high level of CO_2. Comprehensive work on cyanobacterial diversity of paddy fields have been carried out in several parts of the world i.e., Odisha (Singh, 1973; Adhikary, 2002; Dash *et al.,* 2011; Dash *et al.,* 2020), Vietnam (Pham *et al.,* 2017), Srilanka (Amarawansa *et al.,* 2018), Indonesia (Purbani, 2019), Assam (Thajamanbi *et al.,* 2016), Kerala (Vijayan and Ray, 2015), Karnataka (Basavaraja and Naik, 2018). Abiotic attributes viz., light, pH, temperature, water content, nutrient availability, cropping pattern and different types of management practices are able to influence the diversity of cyanobacteria (Kirrolia *et al.,* 2012). For example, the alkaline soil contains more Cyanobacterial sp. (Prasanna and Nayak, 2007) and they are more abundant in rainy season. The soil of the rice field, confronts with both anoxic and aerobic conditions, gives a diversified microbial community (Mohanty *et al.,* 2017).

Indian Council of Agricultural Research-National Rice Research Institute (20° 252 N, 85° 552 E) is doing research on different aspects of rice with an experimental farmland of 60 hectares. Rice is cultivated for last 75 years with different management practices in two different seasons, i). *Kharif* (rainy season, June to November) and ii). *Rabi* (Dry season, December to May). Various management practices (such as organic field, long term pesticide applied field, short term pesticide applied fields) are being practiced here. In the current scenario, pesticides are an integral part of agriculture. They protect crops from various pests, weeds, and diseases by securing high yield. On the other hand, they are adversely affecting the growth of non-target soil microbes including cyanobacteria (Meena *et al.,* 2020). Herbicides and insecticides affects soil microbes and cyanobacteria by reducing their growth and diversity whereas some insecticides have hermetic effects on cyanobacterial populations of rice fields (Das *et al.,* 2015; Dash and Mohapatra, 2018). One-time pesticide application may not have substantial effect on the soil microbiology but continuous application of different pesticides (organophosphorous, organochlorine etc.) in the same field may alter the soil micro-biota (Kumar *et al.,* 2017). Here, the long term pesticide field trial was initiated around 10 years ago with pretilachlor, cartap hydrochloride, chlorpyriphos and carbendazim as treatments. The organic fields (maintained for more than 10 years) were supplemented with FYM, biofertilizers, green manure, etc. Therefore, the above stated management conditions may affect the microbial and cyanobacteria population in the field.

The main objective of this study was to isolate and identify the paddy field cyanobacteria having different physiological characteristics. This investigation was carried out to find out the morphological and biochemical variations of cyanobacteria present in paddy soil. Based on different growth attributes and biochemical parameters of cyanobacteria, they are grouped as potential biofertilizer, biofuel as well as different other industrial applications.

2. Materials and Methods

2.1 Collection of soil samples

The soil and cyanobacteria samples were collected from the ICAR-NRRI paddy fields during both the seasons. They were randomly collected from the top surface of soil up to a depth of 1 cm. The places were selected on the basis of their different management practices i.e., long term and short term pesticide applied fields, organic fields and other experimented fields, so that the diversity of cyanobacteria can be ascertained in ICAR-NRRI paddy fields. Collections were made from the sites of the fields where there were visible growth of cyanobacterial colonies. No specific comparison of the cyanobacteria diversity among different management practices was made.

2.2 Physiochemical properties of soil samples

The physiological properties of soil such as, pH and Electric Conductivity (EC) were measured as per standard protocol. The pH meter (CyberScanpH 510, Eutech Instruments, Oakton, Singapore) electrode contained KCl was calibrated by using pH buffer 4.0 and 9.2. The 10 g of soil samples were dissolved in 25 ml of distilled water and the content was shaken for 30 minutes. Measurement was done in three replications. The electric conductance of different soil samples was measured by using EC meter (PCSTestr™35, Eutech Instruments, Oakton, Singapore).

2.3 Purification and maintenance of culture

The purification of cyanobacterial strains was done by serial dilution and pour plate spreading technique (Andersen and Kawachi, 2005). The isolated and purified strains were maintained in nitrogen free BG11 liquid medium (Rippka *et al.,* 1979) at pH 7.2, 28±2°C and light intensity of 50±5.83 µE/m^2 s with a 14/10 h light/dark cycle. The cultures were hand homogenized (to break the clumps and obtain uniform suspension) for further analysis.

2.4 Microscopic characterisation of isolates

The morphological examination was conducted by viewing under a compound microscope (Zeiss, Germany) equipped with digital camera. Isolates were identified based on the shape and size of vegetative cells, heterocysts and akinetes, using morphological keys of Rippka *et al.,* (1979).

2.5 Genomic characterisation

The genomic DNA was isolated from cyanobacteria using Valerio *et al.* (2009) method. Cyanobacteria-specific primers such as CYA106F, CYA359F, CYA781R (a), CYA781R (b) and CYA1281R were used to amplify the cyanobacteria specific site and evident the presence of cyanobacteria community. The primer sequences used for PCR are listed in Table 1. The PCR conditions were as follows: 1 cycle at 95^0C for 10 min; 35 cycles of 94^0C for 45 secs, 55^0C for 45 secs, and 72^0C for 1 min, 1 cycle at 72^0C for 5 min, and a final step at 4^0C. For the ITS primers, the PCR cycling program was the same, except that the initial denaturation temperature was optimized at 95^0C for 6 min. PCR were performed in 50 µl containing 1X PCR buffer (Invitrogen, Thermo fisher scientific, USA), 0.4 mM of each of the four dNTPs (Invitrogen, Thermo fisher scientific, USA), 0.5 mM of each primer, 10–15 ng genomic DNA, 2.5 mM $MgCl_2$, 0.5 mg BSA ml^{-1}, 1% triton X, 0.1% gelatin and 1 U Taq DNA polymerase (Invitrogen, Thermo fisher scientific, USA). The amplifications were performed in a PCR Thermal cycler (BIORAD, T100 Thermal Cycler). The PCR products were verified by observing the PCR bands in a 1.4% (w/v) agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and electrophoresis at 90 V for about 1.5 h using a gel electrophoresis system (HU25 Maxi-plus standard horizontal, SCIE-PLAS LTD). DNA ladder (1Kb plus) was assigned to determine the band size. The gels were documented by gel documentation unit. PCR reactions were repeated twice for each primer to check the reproducibility of the banding patterns. The purified PCR products were further used for Sanger sequencing. Only good quality DNA sequences based on the chromatogram data were used for further phylogenetic analysis.

The sequence of twenty isolates were manually edited using BioEdit software (Hall, 1999). The phylogentic tree was inferred using the UPGMA method in MEGA X (Sneath and Sokal, 1973). The tree has been drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Table 1

Primers and their sequences used for genetic characterisation

Sl No.	Name Of the Primer	Sequences(5'-3')
1	CYA106F	CGG ACG GGT GAG TAA CGC GTG A
2	CYA359F	GGG GAA TYT TCC GCA ATG GG
3	CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T
4	CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T
5	CYAN1281R	GCA ATT ACT AGC GAT TCC TCC
6	ITSCYA236F	CTG GTT CRA GTC CAG GAT
7	ITSCYA225R	TGC AGT TKT CAA GGT TCT

2.6 Growth attributes and biochemical characteristics

The growth (as cell dry weight) of cyanobacteria isolates was determined as per the standard procedure (Sorokin, 1973). The pigments of cyanobacteria were extracted using ice cold methanol (98%) from 2 mL of culture. The chlorophyll *a* (Ritchie, 2006), carotenoids (Jensen, 1978), Phycobiliprotein contents (Bennett and Bogoard, 1973), proteins (Lowry *et al.,* 1951), total carbohydrates (Herbert *et al.,* 1971), nitrate reductase (NR) (Lowe and Evans, 1964) and glutamine synthetase (GS) (Shapiro and Stadtman, 1970) were measured as per the standard protocols.

2.7 Statistical Analysis

The data were taken in triplicates for each characteristic. Statistical analyses were performed by using the Statistical Analysis Software (SAS) of Indian Agricultural Statistics Research Institute (IASRI), New Delhi through the portal www.iasri.res.in/sscnars/-. All the data were subjected to one-way classified analysis of variance (ANOVA) and means of treatments were compared based on Tukey's honestly significant difference test (HSD) at 0.05 probability level using SAS.

To check the potential of different isolates, we considered for principal component analysis (PCA) and heat map analysis using *Clustvis*: a web tool for visualizing clustering of multivariate data (Metsalu and Vilo, 2015). Nitrate reductase (NR) and glutamine synthatase data from different isolates were used to identify potential biofertilzer.

Similarly, dry weight and carbohydrate content were used to understand the potential biofuel producers. Parameters namely; chlorophyll *a*, carotenoids, phycobiliprotein (phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC)) were taken to identify the potential pigment producer isolates. In *Clustvis* tool, data were transformed to unit variance scaling, and singular value decomposition (SVD) with imputation was used to calculate principal components. In heat maps, rows and columns were clustered using correlation distance and average linkage.

3. Results

3.1 Characteristics of field soil

The abiotic environment of the sample collection sites varied in their physicochemical characters in terms of pH and Electrical Conductivity (EC) (Table 2). The soil pH of sampling sites ranged from 6.31-7.25. The isolates, *Scytonema* sp. (3) was obtained from soil with the highest pH value (7.25); whereas *Scytonema* sp. (2) was isolated from soil with the lowest pH value (6.31). The EC values of different sampling sites varied from 141.61 µS/cm (*Aphanizomenon* sp.) to 296.31 µS/cm (*Fischerella* sp.) (Table 2).

3.2 Morphological characterization

The heterocystous isolates were grown in nitrogen free BG11 liquid medium, whereas the non heterocystous isolates were grown in BG11 (N+) medium. They were planktonic and having coloured thalli i.e., pale green, bluish green, dark green, olive green and brownish violet (Fig. 1). The non-filamentous isolates were found in homogenized form whereas the filamentous isolates were having thick masses. *Scytonema* spp. (isolates 1-3), *Brasilonema* sp., and *Westiellopsis* sp. (1) were dark green in colour with mucilaginous balls and they were distributed either randomly or settled at the bottom. *Hapalosiphon* spp. (isolates 1&2) were pale greenish brown with mucilage balls. The non-filamentous isolates, *Synechocystis* sp., *Aphanothece* sp. and *Gloeothece* sp. were found as colonial form. The filaments of *Scytonema* sp. showed false branching, while

Table 2

Molecular identification of collected isolates and physiochemical properties of the collected sites

Isolate code	Species	Genetic Similarity (%)	Location (GPS)	pH	EC (µS/cm)
PP1	*Anabaena variabilis*	100%	20°26'56.0"N 85°56'24.4"E	6.45 ±1.31	154.66±2.31
PP2	*Scytonema* sp.(1)	98.97%	20°26'58.2"N 85°56'11.1"E	6.65±0.25	279.27±11.81
PP3	*Scytonema* sp. (2)	98.99%	20°26'57.7"N 85°56'20.2"E	6.31±1.21	251.07±0.97
PP4	*Hapalosiphon* sp. (1)	98.90%	20°27'10.8"N 85°56'20.4"E	6.74±0.72	158.94±2.14
PP5	*Brasilonema* sp.	98.99%	20°27'11.1"N 85°56'17.9"E	7.19±0.28	214.18±23.11
PP6	*Nostoc* sp. (1)	99.47%	20°27'08.1"N 85°55'59.6"E	7.24±0.17	242.21±11.21
PP7	*Synechocystis* sp.	98.09%	20°27'10.6"N 85°55'57.6"E	6.32±1.84	208.94±11.43
PP8	Uncultured cyanobacterium (1)	98.00%	20°27'09.5"N 85°55'58.3"E	6.58±1.13	244.70±12.1
PP9	*Aphanothece* sp.	98.27%	20°27'03.5"N 85°56'01.3"E	7.21±0.43	249.49±25.12
PP10	*Gloeothece* sp.	98.03%	20°26'55.4"N 85°56'01.4"E	6.33±1.65	203.11±2.31
PP11	*Westiellopsis* sp. (1)	99.00%	20°27'04.8"N 85°56'24.0"E	6.85±1.39	244.82±12.08
PP12	*Hapalosiphon* sp. (2)	96.90%	20°27'08.4"N 85°56'27.6"E	7.22±0.59	248.67±11.3
PP13	*Westiellopsis prolifica*	100%	20°27'12.5"N 85°56'15.0"E	6.44±1.31	146.32±6.56
PP14	Uncultured cyanobacterium (2)	95.05%	20°27'09.4"N 85°56'17.9"E	7.24±0.19	189.12±8.25
PP15	*Niveispirillum cyanobacteriorum*	98.91%	20°26'56.7"N 85°56'21.7"E	7.16±0.18	217.54±9.59
PP16	*Nostoc* sp. (2)	98.99%	20°26'49.4"N 85°56'25.2"E	6.46±1.81	155.63±1.18
PP17	*Fischerella* sp.	98.89%	20°26'57.3"N 85°56'28.0"E	6.38±1.52	296.31±48.39
PP18	*Aphanizomenon* sp.	99.12%	20°27'13.0"N 85°56'14.9"E	6.85±1.28	141.61±1.31
PP19	*Westiellopsis* sp. *(2)*	93.88%	20°27'10.7"N 85°56'20.3"E	7.21±0.33	255.02±11.24
PP20	*Scytonema* sp. *(3)*	99.19%	20°26'58.2"N 85°56'09.7"E	7.25±0.12	181.59±2.06

Remarks: The species mentioned are the most abundantly distributed species of that particular site.

other (*Hapalosiphon* sp. (1), *Westiellopsis* sp. (1&2), *Westiellopsis prolifica* and *Fischerella* sp.) showed true branching. The main and lateral branches of *Hapalosiphon* sp. (1&2) could not be distinguished morphologically. Shape of vegetative cells was different among the isolates (Table-3).

The shape and size of heterocysts varied significantly among the isolates (Table 3). Among the heterocystous cyanobacteria, the minimum size of vegetative cell was noted in *Scytonema* sp. (1) and the maximum one was found in *Aphanizomenon* sp. whereas, in non-filamentous and non-heterocystous strains the minimum size of vegetative cells was observed in *Synechocystis* sp. and the maximum size was observed in *Aphanothece* sp. (Table 3). The maximum size of akinetes was observed in *Aphanizomenon* sp. and minimum size was observed in uncultured cyanobacterium (1).

The akinetes help the species to survive under unfavorable conditions and maintain its fundamental metabolic activity. The length and width of akinetes were 1.5 and 1.2 times more than that of the vegetative cells as they were produced by enlarging of the vegetative cells (Table 3). *Nostoc* sp. (2) and *Aphanizomenon* sp. exhibited distinctly large sized oval and barrel shaped akinetes, respectively as compared to other isolates in the present case, while the akinetes of uncultured cyanobacterium and *Hapalosiphon* sp. (2) were much smaller in size.

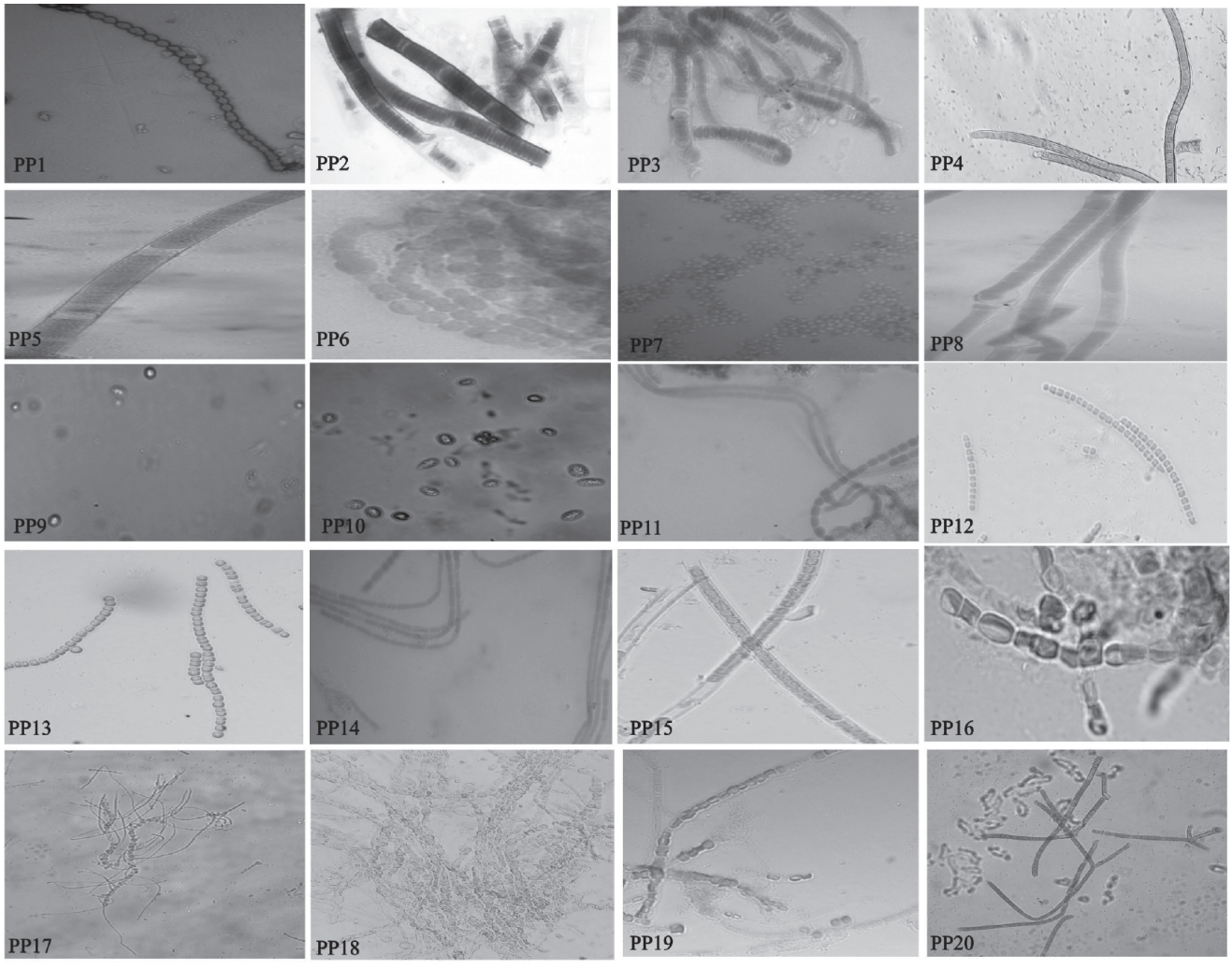


Fig 1. Images of 20 isolates of cyanobacteria (PP1:*Anabaena variabilis*, PP2:*Scytonema* sp.(1), PP3:*Scytonema* sp. (2), PP4:*Hapalosiphon* sp. (1), PP5:*Brasilonema* sp., PP6:*Nostoc* sp. (1), PP7:*Synechocystis* sp., PP8:Uncultured cyanobacterium (1), PP9:*Aphanothece* sp., PP10:*Gloeothece* sp., PP11:*Westiellopsis* sp. (1), PP12:*Hapalosiphon* sp. (2), PP13:*Westiellopsis prolifica*, PP14:Uncultured cyanobacterium (2), PP15:*Niveispirillum cyanobacteriorum*, PP16:*Nostoc* sp. (2), PP17:*Fischerella* sp., PP18:*Aphanizomenon* sp., PP19:*Westiellopsis* sp. (2), PP20:*Scytonema* sp. (3))

Table 3

The morphological characteristics of different isolates obtained from the study sites.

Isolate	Vegetative cell					Heterocyst				Akinetes		
	Shape	Size		P/A	Position	Shape	Size		P/A	Position	Shape	Size	
		Length (µm)	Width (µm)				Length (µm)	Width (µm)				Length (µm)	Width (µm)
PP1	S	4.26±0.15	4.21±0.15	P	I/T	S	5.51±0.36	5.51±0.36	P	I	O/B	7.91±0.32	6.00±0.55
PP2	E	3.51±0.31	4.96±0.40	P	I	B/C	9.03±0.50	6.16±0.55	P	I	B	7.76±0.55	7.00±0.36
PP3	B	3.86±0.55	4.23±0.41	P	I	B	6.16±0.35	8.83±0.51	P	I	C	6.76±0.47	7.73±0.15
PP4	B	6.33±0.55	8.91±0.52	P	I/T	B/S	7.56±1.07	10.56±0.32	P	I/T	Ss	9.11±0.88	10.93±0.37
PP5	C/R	5.66±0.40	6.16±0.35	P	I	C/D	8.23±0.30	5.93±0.56	P	I	S	6.33±0.15	6.33±0.15
PP6	R	5.32±0.43	4.96±0.49	P	I/T	R	10.16±0.45	9.73±0.40	P	I/T	O	10.66±0.56	10.56±0.20
PP7	S	3.93±0.15	3.93±0.15	A	NA	NA			A	NA	NA	NA	NA
PP8	O/C	7.46±1.25	5.52±1.17	p	I	C	8.76±0.45	6.71±1.34	P	I/T	Re	5.61±1.11	6.33±0.64
PP9	Ov	6.33±1.05	5.71±1.90	A	NA	NA	-	-	A	NA	NA	NA	NA
PP10	O	4.13±0.60	4.63±0.73	A	NA	NA	-	-	A	NA	NA	NA	NA
PP11	C	6.21±0.65	4.63±0.45	P	I/T	O	5.63±0.55	8.73±0.35	P	I/T	B	6.81±0.52	8.43±0.90
PP12	B	5.23±0.70	5.36±0.25	P	I/T	B	5.61±0.71	6.41±0.31	P	I/T	O	5.83±0.50	6.46±0.96
PP13	S	4.53±0.92	4.73±0.47	P	I/T	O	5.86±0.45	7.42±0.88	P	I/T	B	6.63±0.41	7.96±0.58
PP14	C	6.13±0.65	4.76±0.40	P	I/T	R/C	6.00±0.55	8.83±0.35	P	I/T	E	6.73±0.77	8.43±1.00
PP15	C	6.36±0.55	4.91±0.31	P	I/T	O	5.73±0.58	8.53±0.25	P	I/T	B	6.71±0.45	8.26±0.97
PP16	C	4.73±0.85	4.93±0.37	P	I	O	5.73±0.30	3.86±0.30	P	I/T	O	11.43±0.25	9.11±0.42
PP17	S/B	5.210±0.3	5.93±0.15	P	I/T	O	6.73±0.55	6.16±0.41	P	I	S	6.81±0.42	6.81±0.42
PP18		9.06±0.75	8.43±0.86	P	I	C	10.71±0.51	8.33±0.15	P	I/T	B	11.31±0.22	8.26±0.35
PP19	B	5.33±0.40	5.63±0.37	P	I/T	C	6.63±0.30	6.23±0.45	P	I/T	O	7.86±0.30	8.13±0.20
PP20		6.30±0.15	6.63±0.20	P	I/T		8.93±0.45	7.51±0.36	P	I/T	O	9.26±0.55	9.26±0.32

Note: Shape: B-Barrel, C- Cylindrical, D-discoid, E- Elliptical, Ob-Oblong, O-Oval, Ov-Ovoid, Re-Rectangular, R-Rounded, S-Spherical, Ss-Sub-spherical, P/A- Present/ Absent; I-Intercalary; T-Terminal

3.3 Genetic characterization

Gel pictures of 20 cyanobacterial isolates were presented in Fig 2. Based on the genetic analysis, different isolates were identified on percent similarity from NCBI database and is given in (Table 2). The dendrogram divided the whole 20 cyanobacteria isolates into six main groups (I-VI) (Fig. 3). The Uncultured cyanobacterium (1) might own enough similarity to *Nostoc* sp. isolate. *Niveispirillum cyanobacteriorum,* which was found to be distinct from the rest of the cyanobacteria, was grouped the major group VI. The results of this dendrogram concluded that the isolates which had genetically kindred but they might be differed in their other parameters.

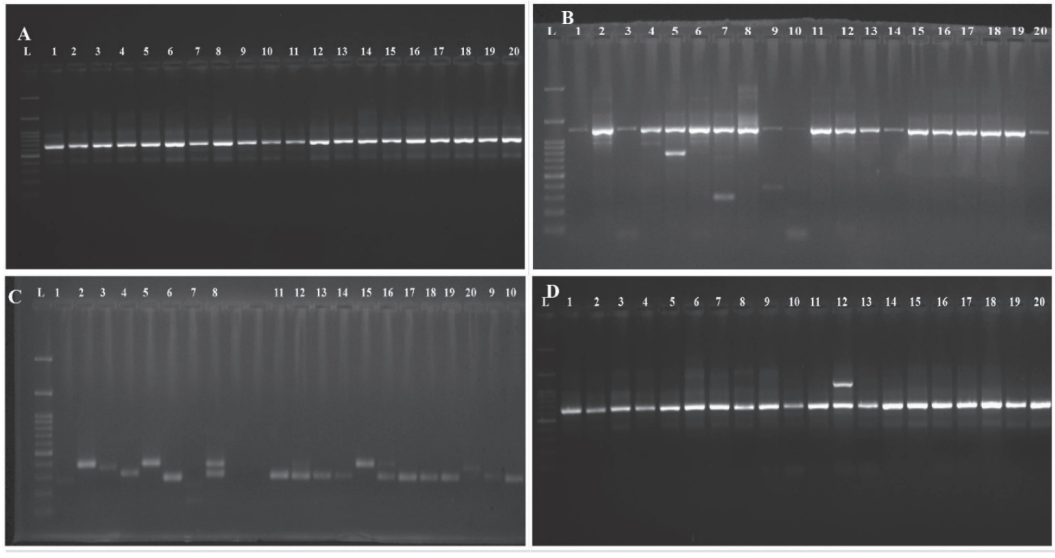


Fig. 2. Documented gel picture of 20 Cyanobacterial isolates (A. CYA106F + CYA781R(a) + CYA781R(b), B. CYA359F + CYA781R(a) + CYA781R(b), C. CYA106F + CYAN1281R, D. ITSCYA236F + ITSCYA225R) (Left to Right)

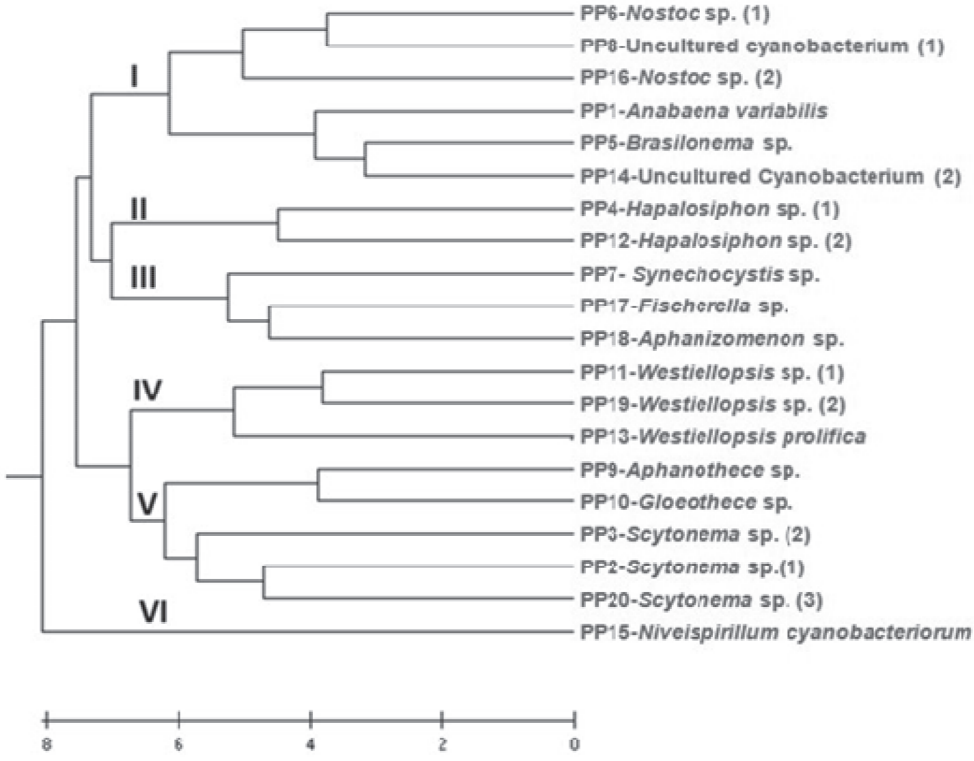


Fig 3. Cluster analysis of twenty cyanobacteria isolates using UPGMA methods.

3.4 Growth attributes

Cell dry weight, protein and carbohydrate contents varied significantly among the isolates (Table 4). A broad range of cell dry weight (3.47-17.20 mg/mL) was observed. The maximum cell dry weight was observed in *Scytonema* sp. (2), which was nearly same as in *Nostoc* sp. (1) and *Anabaena variabilis* and significant difference among the species was observed. Both protein and carbohydrate contents were maximum in *Anabaena variabilis,* which was significantly higher than of all other strains. The lowest value of protein content was found in *Hapalosiphon* sp. (1). Among the isolates, *Anabaena variabilis*, *Nostoc* sp. (1) and *Westiellopsis* sp. (1) had maximum carbohydrate accumulation whereas, the carbohydrate accumulation was minimum in *Brasilonema* sp. and *Hapalosiphon* sp. (1).

Table 4

Growth attributes (Cell Dry weight, Protein and Carbohydrate content) of different isolates

Species	Dry wt (mg/mL)	Protein (µg/mL)	Carbohydrate (µg/mL)	Chl a (µg/mL)	Car (µg/ mL)	PE (µg/mL)	PC (µg/mL)	APC (µg/mL)
Anabaena variabilis	12.37^{abc}	34.53^{a}	67.23^{a}	20.02^{ab}	7.98^{ab}	2.73^{b}	3.85^{b}	5.90^{a}
Scytonema sp. (1)	7.67^{cdefg}	19.88^{de}	36.76^{bcde}	6.01^{gh}	3.57^{hi}	1.56^{def}	1.94^{fg}	3.01^{cde}
Scytonema sp. (2)	17.20^{a}	29.05^{b}	45.09^{bcd}	17.37^{abcd}	5.58^{defg}	2.05^{c}	2.86^{c}	4.14^{b}
Hapalosiphon sp. (1)	8.60^{cdefg}	1.68^{j}	3.10^{j}	7.94^{fgh}	2.97^{i}	0.60^{ij}	0.84^{ij}	1.25^{fg}
Brasilonema sp.	3.47^{g}	1.72^{j}	2.92^{i}	4.88^{h}	1.25^{j}	0.55^{j}	0.84^{ij}	1.09^{ghi}
Nostoc sp. (1)	14.43^{ab}	22.34^{d}	50.24^{b}	22.23^{a}	7.27^{abcd}	1.75^{cde}	2.62^{cde}	4.08^{b}
Synechocystis sp.	7.30^{cdefg}	6.08^{ij}	11.53^{ghi}	18.17^{abcd}	6.20^{cdef}	2.68^{b}	3.89^{b}	6.06^{a}
Uncultured (1)	6.70^{cdefg}	20.93^{de}	47.10^{bc}	12.99^{def}	3.80^{hi}	1.58^{de}	2.35^{def}	3.62^{bc}
Aphanothece sp.	4.63^{fg}	14.16^{fg}	24.03^{efg}	8.59^{fgh}	3.24^{hi}	0.95^{hi}	1.39^{h}	2.12^{ef}
Gloeothece sp.	5.53^{efg}	5.05^{ij}	8.33^{hi}	5.31^{h}	1.26^{j}	1.05^{h}	1.47^{gh}	2.17^{ef}
Westiellopsis sp. (1)	8.40^{cdefg}	25.06^{c}	45.11^{bcd}	12.82^{def}	6.37^{bcde}	1.71^{cde}	2.16^{ef}	3.32^{bcd}
Hapalosiphon sp. (2)	7.33^{cdefg}	13.87^{fg}	30.52^{def}	11.42^{efg}	4.65^{fghi}	1.67^{de}	2.32^{def}	3.23^{bcd}
Westiellopsis prolifica	7.53^{cdefg}	15.96^{f}	35.09^{bcde}	13.17^{cdef}	7.11^{abcd}	1.90^{cd}	2.66^{cd}	3.79^{bc}
Uncultured (2)	7.97^{cdefg}	13.87^{fg}	27.76^{ef}	18.73^{abc}	8.08^{a}	1.55^{def}	2.08^{f}	2.62^{de}
N. cyanobacteriorum	5.80^{defg}	18.95^{e}	32.21^{cdef}	19.97^{ab}	7.92^{ab}	3.11^{a}	4.50^{a}	6.26^{a}
Nostoc sp. (2)	10.87^{bcde}	13.65^{fg}	23.51^{efg}	15.62^{bcde}	7.49^{abc}	1.43^{efg}	1.59^{gh}	1.16^{gh}
Fischerella sp.	11.40^{bcd}	13.16^{g}	18.31^{fgh}	14.71^{bcde}	8.21^{a}	1.07^{gh}	0.56^{ij}	0.31^{hi}
Aphanizomenon sp.	9.73^{bcdef}	9.64^{h}	18.76^{fgh}	8.28^{fgh}	4.09^{ghi}	0.31^{j}	0.37^{j}	0.26^{hi}
Westiellopsis sp. *(2)*	11.17^{bcde}	13.07^{g}	23.49^{efgh}	15.23^{bcde}	8.42^{a}	1.21^{fgh}	0.64^{ij}	0.63^{ghi}
Scytonema sp. *(3)*	7.37^{cdefg}	8.66^{h}	17.26^{fghi}	10.49^{efgh}	4.93^{efgh}	0.32^{j}	0.29^{j}	0.24^{i}
HSD	5.7972	2.5626	15.163	5.6772	1.7003	0.3719	0.4701	0.9178

* Means with at least one letter common superscripts are not statistically significant using Tukey's honestly significant difference (HSD) test at $p \geq 0.05$

PE-phycoerythrin; PC-phycocyanin; APC-allophycocyanin

3.5 Pigment Content

The chlorophyll *a* content of all isolates ranged from 4.88 to 22.23 µg/mL (Table 5). The highest chlorophyll *a* was found in *Nostoc* sp. (1) and *Anabaena variabilis*. The carotenoids content among isolates ranged from 1.25 to 8.42 µg/mL (Table 5). *Westiellopsis* sp. (2) exhibited the maximum carotenoids content which was at par with *Fischerella* sp., and uncultured cyanobacterium (2). Likewise, the isolates also showed significant variability with respect to PC, PE and APC contents (Table 5). The total phycobiliproteins ranged from 0.85 µg/mL in *Scytonema* sp. (3) to 13.87 µg/mL in *Niveispirillum cyanobacteriorum*. The PC (4.50 µg/mL) and PE (6.26 µg/mL) contents were observed the highest in *Niveispirillum cyanobacteriorum* while the lowest content was in *Scytonema* sp. (3) (0.24 µg/mL). Among the isolates analyzed, the maximum quantity of APC was found in *Niveispirillum cyanobacteriorum* which was statistically at par with *Synechocystis* sp. and *Anabaena variabilis*.

3.6 Activities of enzymes

The maximum nitrate reductase (NR) activity was recorded in *Nostoc* sp. (1) (32.08 µmoles/ml h), while *Gloeothece* sp. produced the lowest activity (17.25 µmoles/ml h) (Fig. 4). Glutamine synthetase (GS) activity was found highest in *Anabaena variabilis* (106.17 µmoles/ml h). *Hapalosiphon* sp. (2) had the lowest GS activity of 55.63 µmoles/ml h among the cyanobacterial isolates (Fig. 4).

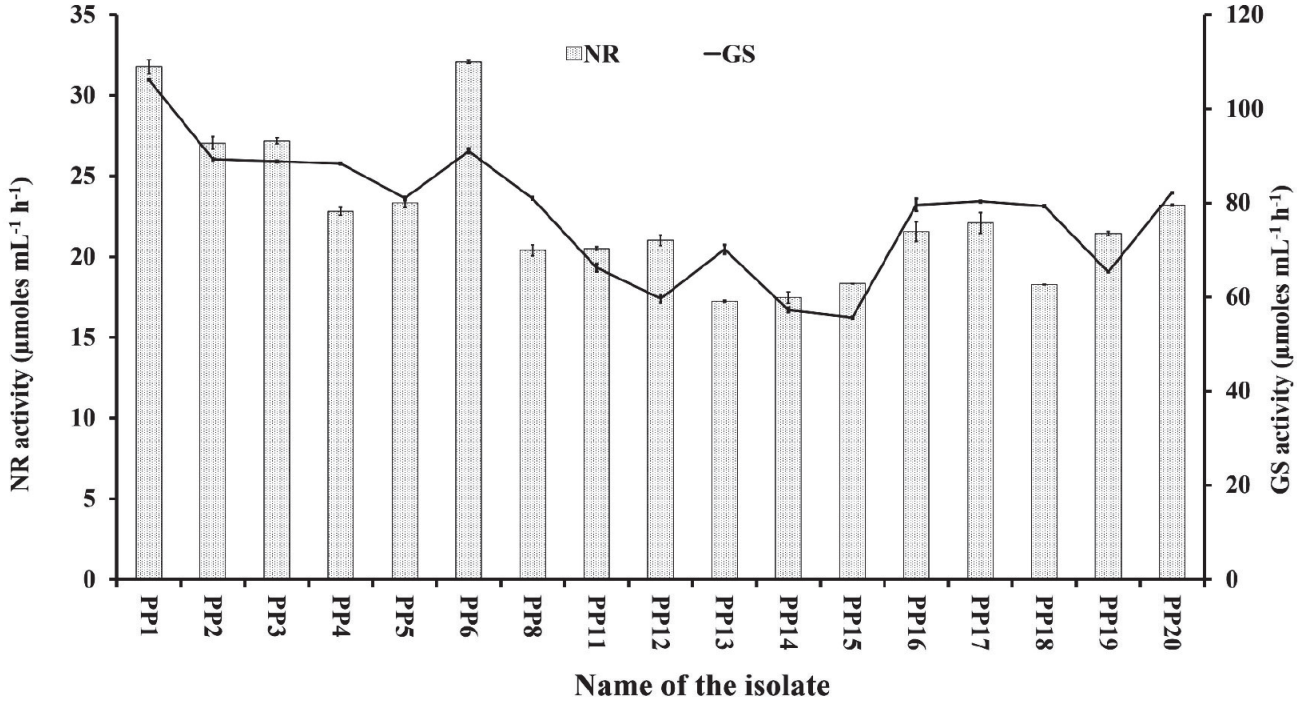


Fig 4. Nitrate Reductase (NR) and Glutamine Synthetase (GS) content of heterocystous cyanobacterial isolates

4. Discussion

Cyanobacteria are an oxygenic prokaryotic group and are present at all types of niches like terrestrial, sub-aerial, fresh water, saline water and hypersaline environments. Soil samples at experimental sites were slightly acidic to moderately alkaline. Cyanobacteria are ubiquitous in their distribution, but they prefer a neutral or alkaline pH for their best growth (Alghanmi and Jawad, 2019). Cyanobacteria such as *Anabaena, Scytonema, Hapalosiphon, Nostoc,* and *Westiellopsis* were profusely found in the soil within pH range of 6.5-7.5 and our finding was quite similar to these studies (Alghanmi and Jawad, 2019; Ghadage and Karanda, 2019). *Anabaena variabilis*, *Scytonema* sp. (1), *Scytonema* sp. (2), *Hapalosiphon* sp. (1), *Synechocystis* sp., Uncultured cyanobacterium (1), *Gloeothece* sp., *Westiellopsis* sp. (1), *Westiellopsis prolifica*, *Nostoc* sp. (2), *Fischerella* sp. and *Aphanizomenon* sp. were more abundantly found in slightly acidic soil pH. Whereas, *Brasilonema* sp., *Nostoc* sp. (1), *Aphanothece* sp., *Hapalosiphon* sp. (2), Uncultured

cyanobacterium (2), *Niveispirillum cyanobacteriorum*, *Westiellopsis* sp. (2), *Scytonema* sp. (3) were found in slightly alkaline paddy field soil.

EC is another important element that can affect the soil microbial community (Shariatmadari *et al.,* 2013). According to Singh *et al.*. (2014), *Anabaena constricta* were found abundantly in soil with high EC (801.8 µS cm^{-1}) whereas, in our study, *Fischerella* sp., *Scytonema* sp. (1) and *Westiellopsis* sp. (2) were found abundantly in soils with high EC and *Aphanizomenon* sp., *Westiellopsis prolifica* and *Anabaena variabilis* were more abundant in soils with low EC. The soils of this institute had generally unicellular *Aphanothece* sp. and filamentous *Gloeotrichia* sp. around 50 years ago (Singh, 1973; Pattnaik and Singh, 1978). This indicated a shift in the species distribution enforced by the agropractices and soil characteristics.

Cyanobacteria group occupy a high degree of morphological, physiological and developmental complexity. The size of *Aphanothece* sp., *Gloeothece* sp. and *Synechocystis* sp. were similar to the results obtained by Ghadage and Karande (2020). In our study, the cell width of cyanobacteria ranged from 4.23 to 8.9 µm. Similar results of *Scytonema* sp. (2) and *Westiellopsis* sp. were also found (Pattnaik and Samad, 2018). In certain cases, the morphological variability of cyanobacteria isolates was observed under the adverse environmental conditions.

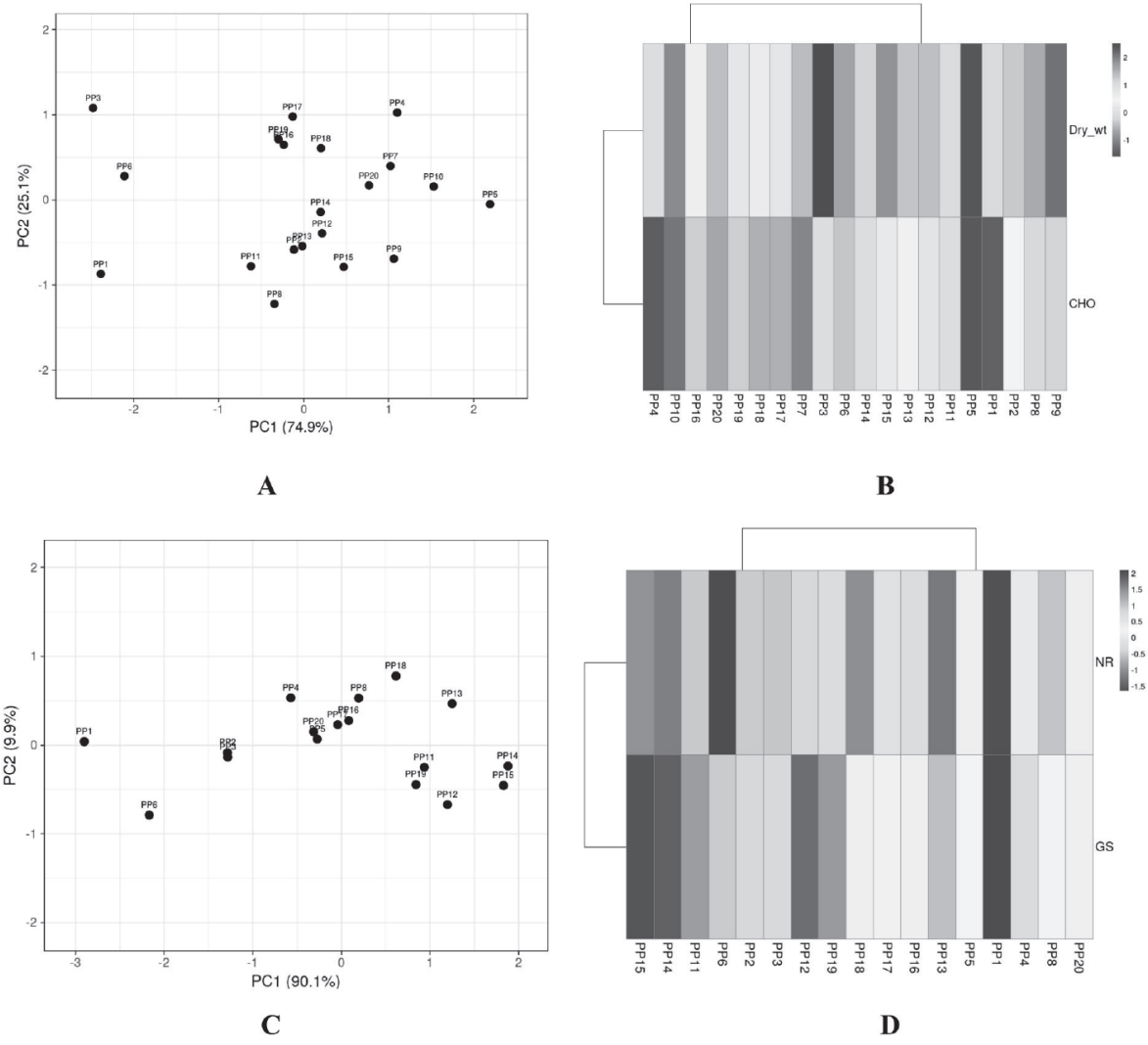
Most of the filamentous cyanobacterial species can generate heterocysts and akinetes with some exceptions like *Oscillatoria*. The abundant presence of heterocystous cyanobacteria may be indicating the lower nitrogen content at that particular location (Ghadage and Karande, 2019). The vegetative cell transformed into the heterocysts with certain morphological and physiological alterations in nitrogen deficient conditions. Heterocysts and vegetative cells are interdependent to each other for reduced carbon and nitrogen, respectively. These are the main attributes to compare the sizes between vegetative cell and heterocyst. The heterocysts size of *Aphanizomenon* sp. were larger as compared to vegetative cells as expected. Such features were recorded earlier in *Anabaena* species (Prasanna *et al.,* 2006). Heterocysts were mostly intercalary (Rippka *et al.,* 2015) but some exceptions were mentioned in Table 3, where both intercalary and terminal heterocysts were also observed. The large sized heterocysts may have greater potential to fix more nitrogen than smaller ones. Thus, the species with such attributes can be utilized for commercial purposes. The akinetes were found in away from the heterocyst with few exceptions. Similar results for the size of heterocysts and akinetes of *Anabaena* and *Nostoc* were also observed (Rajaniemi *et al.,* 2005).

The cell dry weight is a significant property to find out the growth rate of an organism. These broad range (14.52-9.43 mg/ml) of cell dry weight of different cyanobacterial species (*Lyngbya sp*., *Anabaena variabilis*) was also observed (Saxena *et al.,* 2007). As the cyanobacteria have more industrial value, the high growth rate gives a better opportunity to use them commercially. In cyanobacteria, carbohydrates are stored as small sized glycogens. This is the main reason behind the preference of cyanobacteria species in biofuel production. *Synechocystis* sp. and *Synechococcus elongatus* were able to produce biofuel along with other valuable chemicals (Machado and Atsumi, 2012). In our study, among the isolates, *Anabaena variabilis* (67.23 µg/ml) showed the highest content of carbohydrates followed by *Nostoc* sp. Principal component analysis and heat map study also depicted the similar picture (Fig. 5). Based on these *Anabaena variabilis*, *Nostoc* sp. (1) and *Scytonema* sp. (2). could be grouped together. The obtained results were similar to the results of several other workers (Prasanna *et al.,* 2006; Tiwari and Singh, 2005). The high protein content was observed in *Anabaena variabilis* and *Scytonema* sp. (2). The protein content of cyanobacteria was in the range from 6.1-497.8 µg/ml (Tiwari and Singh, 2005). The genera *Anabaena* had a wide range of protein content (31.17-447.69 µg/ml) because of a wide variation in their growth potential and biomass production (Prasanna *et al.,* 2006). Similarly, in our experiment the protein content of *Anabaena variabilis* was 34.53 µg/ml. The similar results were also reported by Narayan *et al.* (2006). There was a substantial variation in protein content among the 20 isolates. It may be because of the differences in the management practices of the crop fields resulting in different assimillable levels of nitrogen.

The maximum chlorophyll *a* content was found in *Nostoc* sp. (1) followed by *Anabaena variabilis*, *Niveispirillum cyanobacteriorum* and an uncultured cyanobacterium. Such results have also been observed by several workers in previous studies (Tiwari and Singh, 2005). The accessory pigments, carotenoids are abundantly found in cyanobacteria strains. Reports suggested that the cyanobacteria strains, that have high carotenoid content, possess more tolerance to high light intensity (Wilson *et al.,* 2006). Among the isolates the maximum carotenoids content was noted in *Westiellopsis* sp. (2) followed by *Fischerella* sp., an uncultured cyanobacterium and *Anabaena variabilis*. Thus, these isolates possessed high rate photosynthesis compared to other reported strains. The PC content varied from 0.29-4.49 µg/ml among the groups. The highest and lowest value has been exhibited by *Niveispirillum cyanobacteriorum* and *Scytonema* sp. (3), respectively. Similar reports by other workers suggested

that the APC content was higher than the PE and PC among the phycobiliproteins (Narayan *et al.,* 2006). Thus, our isolates *Niveispirillum cyanobacteriorum*, *Synechocystis* sp. and *Anabaena variabilis* be used as valuable resources for phycobiliprotein protein production as they have high content of this protein complexes. In some reports it was found that the carbohydrate accumulation and phycobiliprotein depletion had taken place at the same time (Mollers *et al.,* 2014).

The NR activity among these cyanobacteria varied from 17.48-32.08 µmoles/ml h. The minimum NR activities were found in *Westiellopsis prolifica*, uncultured cyanobacterium (2), *Niveispirillum cyanobacteriorum* and *Aphanizomenon* sp. whereas the maximum NR activity were shown by *Nostoc* sp. (1), *Anabaena variabilis* and *Scytonema* sp. (2). The activity of GS among these cyanobacteria ranged from 55.63-106.16 µmoles /ml h. Similar results have been obtained with *Anabaena* and *Nostoc* (Narayan *et al.,* 2006). Among the 20 isolates of the present experiment, the GS activity were found minimum in case of *Niveispirillum cyanobacteriorum*, Uncultured cyanobacterium (2) and *Hapalosiphon* sp. (2), whereas maximum GS activity was observed in *Nostoc* sp. (1), *Anabaena variabilis* and *Scytonema sp.* (2). Low GS with high NR enzyme activity found in *Westiellopsis* sp. (2) and *Hapalosiphon* sp. (2) are responsible for making of good quality biofertilizer (Shimkets, 2015). The attributes namely, heterocyst frequency and size, cell biomass and nitrogen fixation ability of cyanobacteria are the main determinants to qualify as biofertilizer. *Nostoc* sp. (1) and *Anabaena variabilis* can be used as biofertilizer as per the PCA and heat map analysis (Fig. 5). The isolate *Anabaena variabilis* showed a good result in all these growth attributes. This isolate can be utilized as biofertilizer and also in biofuel industry as a biofuel producer.



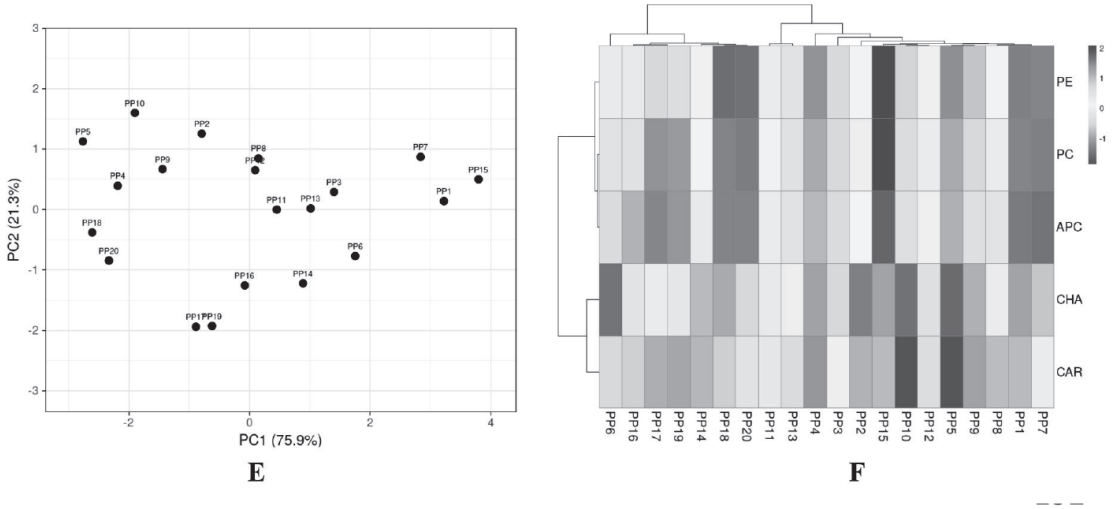


Fig. 5. Principal component analysis (PCA) and heat map (HM) study to differentiate cyanobacteria isolates as biofuel producer (A, PCA and B, HM), biofertilizer (C, PCA and D, HM) and pigment producer (E, PCA and F, HM) (Dry wt: dry weight; CHO: carbohydrate content; NR: Nitrate reductase; GS: Glutamine synthatase; CHA: chlorophyll *a*; CAR: carotenoids; PC: phycocyanin; PE: phycoerythrin and APC: allophycocyanin)

Reports suggested that the management practices like indiscriminate use of pesticides had detrimental effects on growth, photosynthesis, and nitrogen fixation of cyanobacteria (Shinde, 2018). The deleterious effect on photosynthesis may affect the nitrogen metabolism because photosynthesis supplies the energy to complete the process of N_2-fixation (Tiwari *et al.,* 2019). The nature, concentration and duration of expose to pesticides are found responsible for the toxicity to cyanobacteria. Thus, in our study, the different management practices were also playing the vital role as they have a duration based (long term and short term) pesticidal effect in the nitrogen fixation on the basis of the activity of enzymes i.e., NR and GS. This study also revealed that cyanobacteria can tolerate different groups of pesticides.

The rice ecosystem had both heterocystous and non-heterocystous cyanobacteria. The *Anabaena variabilis* and *Nostoc* sp. (1) may be utilized as biofertilizer and biofuel producer. Because of the high pigment content, *Westiellopsis* sp. (2), *Fischerella* sp., *Synechocystis* sp. and *Anabaena variabilis* may be used as valuable resources for cosmetic/ commercial industries. Based on the all parameters the *Anabaena variabilis* may be used as both agricultural and commercial sector. The long term and short term pesticide fields, organic fields and other experimented fields have various cyanobacterial strains which may have the ability to tolerate these stresses. These 20 abundant cyanobacterial isolates may be further studied for their role in bioremediation of pesticides. They can be analyzed for commercial suitability because of their huge genetic potential.

Acknowledgments

Authors are immensely thankful to Director, ICAR-NRRI, Cuttack-753006 and HOD, Department of Botany and Biotechnology, Ravenshaw University, Cuttack-753003 for providing necessary technical support.

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