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Fluoride (F) Accumulation and Genetic Diversity Studies in F Hyperaccumulator Plant *Prosopis juliflora* for Selection of the Best Germplasm for Phytoremediation Purpose

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Research on hyperaccumulation mechanism is increasing, but the genetic basis of fluoride (F) hyperaccumulation remains to be elucidated. The environmental pollution with F is a worldwide problem. Our earlier work demonstrated that *Prosopis juliflora* is a hyperaccumulator plant for F removal. We undertook the present study to screen the best germplasm of naturally growing plants of this species collected from five different locations of India viz. states of Rajasthan, Haryana, Tamil Nadu, Maharashtra and Madhya Pradesh based on F accumulation and ISSR genetic diversity. Significant variation was noted between F accumulation efficiency and genetic diversity of different population of *P. juliflora*. Genetic diversity parameters viz: percentage of polymorphic bands (PPB), Nei's genetic diversity (H) and Shannon's information index (I) within the populations of *P. juliflora* species was highest in Rajasthan population, i.e. 21.62%, 0.08, 0.11 and lowest in Maharashtra population, i.e. 2.70 %, 0.01, 0.01 respectively. The high diversity levels in Rajasthan populations acts as a buffer to combat F stress and be an important selection criterion in developing lines with greatest phytoremediation potential. This study suggests that Rajasthan genotype could be effectively used as hyperaccumulator for F phytoremediation. Further investigations on Rajasthan genotype to identify genomic regions linked to factors involved in F hyperaccumulation will be of great value.

Keywords: Fluoride, denetic diversity, hyperaccumulator, phytoremediation, *P. juliflora*

INTRODUCTION

Most research on hyperaccumulator has focused on the physiological mechanisms of metal uptake, transport and sequestration, but relatively little is known regarding the genetic basis of hyperaccumulation. There are few cases of major genetic polymorphisms in which some members of a species are capable of hyperaccumulation and others are not (Pollard *et al.* 2002).

Genetic variation between plants together with efficiency to accumulate metals and nonmetals is of great importance, because it is the raw material on which natural selection acts to influence the evolution of hyperaccumulation (Yang *et al.* 2005, Khan *et al.* 2012). Although some degree of hyperaccumulation occurs in all members of the species that can hyperaccumulate, there is an evidence of correlation between quantitative genetic variation and hyperaccumulation ability between and within populations (Assuncao 2001, Pilon-Smits 2002). The genotypic differences between populations described above are of great interest to researchers trying to understand and manipulate the genetics of hyperaccumulation. Relatively few studies have been designed to test the magnitude and genetics of within-population variability (Yang *et al.*

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2005). Hyperaccumulation efficiency is the key selection criteria for the phytoremediation, as these plants should combat metal and non metal stress and accumulates toxic substances effectively from the environment. Genetic variation indicates that diversity levels act as a buffer to combat F stress (Khan *et al.* 2012), and in turn it affects the hyperaccumulation efficiency. Genetic diversity studies together with the F accumulation efficiency provide a raw data to select the best germplasm for field use for phytoremediation purpose and important basis of hyperaccumulation improvement of plant traits through selective breeding.

Identifying the genes involved in heavy metal and nonmetal hyperaccumulation traits and evidencing the footprints of the natural selection acting on them are essential to understand the evolution of the hyperaccumulation trait (Pulford and Watson 2003). Persistent exposure of natural populations to inadequate or toxic micronutrient availability would be expected to provoke evolutionary adaptation, provided that the appropriate genetic variation is available in the populations in question (Yang *et al.* 2005). Populations vary in their metal hyperaccumulation and genetic makeup (Ent *et al.* 2013).

Fluoride (F) contamination is a global environmental problem, as there is no cure of fluorosis available yet. Fluorides are well recognized, widespread, non biodegradable and hazardous nonmetal pollutant (Agalakova 2012). Two kinds of defluoradation techniques, namely Nalgonda (based on addition of lime and alum) and Prasanthi (based on adsorption using activated alumina) are practiced (Bulusu and Nawlakhe 1990). However, these techniques are not effective, they remove only a small portion of F and are typically expensive and destructive. To overcome this problem phytoremediation is an eco-friendly environmental technology for remediation of contaminated soil and water using plants with great potential (Al-Qurainy and Abdel-Megeed 2009, Wani *et al.* 2012).

Genetic marker could be used to monitor whether environmental changes influence species at the level of DNA sequences to assess the nature of selection imposed by environmental changes and to assess the potential of populations to respond by evolution adaptation (Hoffmann and Willi 2008). The molecular marker best suited for detecting genetic diversity should be relatively easy and inexpensive and should evolve rapidly enough to be variable within populations. Ecotoxicological literature examples demonstrate that under strictly standardized conditions, the intersimple sequence repeat (ISSR) technique (Zietkiewicz *et al.* 1994) is simple, fast, sensitive, and relatively cheap method for those plants, whose genome has not been sequenced. It that has been successfully used to assess the effect of contaminants on genetic diversity of plants (Al-Qurainy 2010; Abdou *et al.* 2012). Therefore, ISSR technique is the marker of choice in present study.

Prosopis juliflora is widely distributed in the dry tropical and sub-tropical regions of Central and Northern South America, Africa and Asia, particularly in India. The roots penetrate to great depths in the soil (40 cm in eight weeks) and can grow in wide range of soils such as saline, alkaline, sandy and rocky soils (George *et al.* 2007). Pulford and Watson (2003) suggested that ability to grow on nutrient-poor soil, deep root system, fast rate of growth, high biomass and metal-resistance trait are important characteristics of plants for effective phytoremediation. It was earlier known to be tolerant to heavy metals and nonmetals, *e.g.* cadmium, chromium, copper and F also (Kumar *et al.* 2005, Saini *et al.* 2012). *P. juliflora* is widely distributed in India and exhibited great heterogeneity in the germplasm (Fig.1). Therefore, *P. juliflora* could be easily commercialized worldwide for phytoremediation purpose for F contamination. Thus, present study was undertaken to select the best germplasm of *P. juliflora* for phytoremediation purpose through genetic diversity and fluoride accumulation data, collected from five different states of India viz. (Rajasthan, Haryana, Tamilnadu, Maharashtra and Madhya Pradesh).

MATERIAL AND METHODS

2.1.Plant material

To investigate the accumulation of F in shoots and the genetic diversity, fully mature leaf samples of *P. juliflora* were collected from five different states of India *i.e.* Rajasthan, Haryana, Tamilnadu, Maharashtra and Madhya Pradesh listed in (Table 1, Fig 1). Adult trees were randomly selected for sampling. The minimum distance among the population is 100 meter. The leaves were shade dried at the collection sites, transferred in brown paper envelope and sealed. These were brought to the laboratory and stored at room temperature for further experiments (Thomson and Henry 1993).

2.2. Soil sample

Soil samples were collected from the surface (0–30 cm) within 100 meter of the site of collection of the plant material (Table1).

2.3. Determination of F content in soil and leaves of *P. juliflora*

The total F content in leaves of *P. juliflora* was determined by using the method of alkali fusion-ion selective electrode technique of McQuaker and Gurney (1977). The detection limit of method (LOD) was 0.05 mg/l.

2.4. Determination of bioaccumulation factor

Bioaccumulation factors (BF) for F were calculated as per Zhao *et al.* (2003).

BF = [F concentration in shoot] / [F concentration in soil]

2.5. DNA isolation

Table 1. Details of sample size (*N*), longitude and latitude of the sampling sites, for *Prosopis juliflora* from 15 locations.

Population	No of plants	States and sample code	Place	Latitude	Longitude
P1	8	Rajasthan (A)	Banasthali	26° 60' N	75° 54' E
P1	7	Rajasthan(B)	Jodhphur	26° 18' N	73° 4' E
P1	8	Rajasthan(C)	Kota	24° 14' N	75° 49' E
P2	6	Haryana (A)	Hisar	29° 10' N	75° 46' E
P2	4	Haryana (B)	Karnal	29° 40' N	76° 58' E
P2	4	Haryana (C)	Rohtak	28° 54' N	76° 34' E
P3	5	Tamilnadu (A)	Uppupallam	11° 11' N	76° 56' E
P3	6	Tamilnadu (B)	Coimbatore	11° 59' N	76° 58' E
P3	7	Tamilnadu (C)	Mettupalayam	11° 18' N	76° 56' E
P4	7	Madhya Pradesh (A)	Indore	22° 44' N	75° 50' E
P4	6	Madhya Pradesh (B)	Bhopal	23° 16' N	77° 36' E
P4	6	Madhya Pradesh (C)	Gwalior	26° 14' N	78° 10' E
P5	5	Maharashtra (A)	Kholapur	16° 42' N	74° 16' E
P5	7	Maharashtra (B)	Pune	18° 31' N	73° 55' E
P5	6	Maharashtra (C)	Akola	20° 42' N	77° 2' E

Table 2. Primer sequences of 20 ISSR that were used to characterize the 15 *Prosopis juliflora* populations in India and their specific annealing temperature (Tm) for PCR amplification.

S. No	Primer Code	Sequence	Length of oligomer	Calculated Tm ºC
1	PSBV101	ATGATGATGATGATGATG	18	48.0
2	PSBV102	AGCAGCAGCAGCAGCCG	17	58.0
3	PSBV103	ATGATGATGATGATGATG	18	48.0
4	PSBV104	TAGAGAGAGAGAGAGAGA	18	52.0
5	PSBV105	CTCTCTCTCTCTCTCTTG	18	54.0
6	PSBV106	CTCTCTCTCTCTCTCTAC	18	54.0
7	PSBV107	CTCTCTCTCTCTCTCTCG	18	56.0
8	PSBV108	CACACACACACACAGC	16	50.0
9	PSBV109	CACACACACACACAGT	16	48.0
10	PSBV1010	AGCAGCAGCAGCAGCCA	17	56.0
11	PSBV1011	AGAGAGAGAGAGAGAGC	17	52.0
12	PSBV1012	AGCAGCAGCAGCAGCAGCGT	20	58.0
13	PSBV1013	GAGAGAGAGAGAGACTT	17	60.0
14	PSBV1014	CACACACACACAGG	14	44.0
15	PSBV1015	GAGAGAGAGAGACC	14	44.0
16	PSBV1016	GATAGATAGATAGAT	15	38.0
17	PSBV1017	GACAGACAGACAGAC	15	46.0
18	PSBV1018	ACACACACACACACACG	17	52.0
19	PSBV1019	CCGCCGCCGCCGCCGCCG	18	72.0
20	PSBV1020	CTCCTCCTCCTCCTCCTC	18	48.0

Table 3. Genetic diversity within populations of *P. juliflora*, Na: observed number of alleles; Ne: effective number of alleles; H: Nei's (1973) gene diversity; I: Shannon's information index; PPB: percentage of polymorphic bands.

Populations	Na	Ne	H	I	PPB %
Rajasthan	1.21	1.13	0.08	0.11	21.62
Maharashtra	1.02	1.01	0.01	0.01	02.70
Haryana	1.18	1.15	0.08	0.11	18.92
Madhya Pradesh	1.05	1.02	0.01	0.02	05.41
Tamilnadu	1.16	1.12	0.06	0.09	16.22
Average	1.12	1.08	0.048	0.068	12.97
Species level		1.64	0.35	0.52	86.49

The total genomic DNA was isolated from various leaf samples of *P. juliflora* by a cetlytrimethyl ammoniumbromide (CTAB) method of Weising *et al.* (1995) except that viz. extracted aqueous layer was treated with 5M NaCl to remove polysaccharide contamination and incubation for precipitating DNA after adding isopropanol washing step was repeated twice. The extraction buffer contained 100 mM Tris-HCl (pH 8), 1.5 M NaCl, 25 mM EDTA, 2.5% CTAB (w/v), 2% β-mercaptoethanol and 1% polyvinylpyrrolidone. The purified pellet was dissolved in a minimal amount of Tris EDTA (TE) buffer (Tris-Cl 0.05M, EDTA 0.01 M). The isolated DNA was checked on 0.8% agarose gel by staining with ethidium bromide (0.5 mg/ml) and visualized under mini-transilluminator (Bio-Rad, USA). The gel was then photographed and analyzed by a Kodak gel documentation system (Model EDAS 290) using lamda DNA double digest (Bangalore Genei Pvt. Ltd., India) as standard.

2.6. ISSR- PCR amplification

A total of 20 ISSR primers (custom synthesized by Bangalore Genei Pvt. Ltd., India) were screened (Table 2). The primers with more than 60% GC content were used for ISSR analysis. ISSR-PCR reactions were carried out according to Zietkiewicz *et al.* (1994). Amplification products were separated on 1.5% (w/v) agarose gels (migration distance: 10 cm) with 1 x Tris Borate EDTA (TBE) buffer. Electrophoresis was performed at 100 V for 2 hrs and 10 µl of low range DNA ruler (Bangalore Genei, India) was run simultaneously as a molecular standard. The gel was analyzed as described in the previous section. The ISSR analysis on each primer was repeated twice to check the reproducibility. Only those gels which showed consistent amplification were considered in this study.

2.7. Data analysis

Only reproducible bands were scored as present (1) or absent (0) across all *P. juliflora* accessions. The binary character matrix of ISSR data were analyzed using the POPGENE ver 1.31 (Yeh *et al.* 1999) assuming Hardy – Weinberg equilibrium. The following genetic diversity parameters were estimated: Shannon's index of phenotypic diversity ($I = \Sigma\, Pi \log Pi$), Nei's gene diversity ($H = 1 - \Sigma\, Pi^2$; here Pi = the frequency of the ith allele in population, effective number of alleles per locus ($Ne = 1/n\ \Sigma\, \alpha i$ here αi = the number of alleles at locus i and n = the total number of alleles, polymorphic band percentage (PPB). Genetic similarity among collections was calculated using Jaccard's similarity coefficient using the SIMQUAL (Similarity for Qualitative Data) routine of NTSYS-pc (version 2.1). Similarity coefficient values were used to construct dendograms, using the unweighted pair group method with arithmetic averages (UPGMA) from the similarity data matrices. The numerical taxonomy and multivariate analysis system package for personal computer (NTSYS-pc 2.1) (Rohlf 1993) was used for this statistical analysis of data.

2.8. Statistical data analysis

Standard deviation (SD) and spearman's correlation were calculated by statistical software package (SPSS) to examine the differences between each treatment. The level of statistical significance was set at $P<0.05$ of the experimental data.

RESULTS

3.1. F concentration in soil and shoots of *P. juliflora* from selected sites

The F concentration in the shoot of *P. juliflora* ranged from 120.3 to 273.1 mg.Kg^{-1}dw in the five different states Rajasthan, Haryana, Tamilnadu, Maharashtra and Madhya Pradesh of India (Fig. 2). It was observed that maximum accumulation of F in shoot, i.e. 273.1 mg.kg^{-1}dw occurs in Rajasthan samples and minimum in Maharashtra (120.3 mg.Kg^{-1}dw). In contrast, although maximum soil F content (156.7 mg.Kg^{-1}dw) too was recorded in Rajasthan samples, the soils from other states did not show any significant variation (Fig. 2).

The highest bioaccumulation factor (BF) value of 1.74 was observed in Rajasthan. On the contrary, the Maharashtra samples showed the value of 1.17 (Fig. 3). A significant correlation was found between F concentration in shoot and F content in soil at ($P<0.05$) level of significance.

3.2. Genetic diversity analysis of selected *P. juliflora* samples by ISSR marker

Twenty ISSR primers were used for initial screening of 5 populations of *P. juliflora* which although 12 primers gave amplification, only 9 generated a clear reproducible pattern. The latter produced a total of 98 clearly identifiable bands of which 45 were polymorphic, i.e., the percentage of polymorphic bands (PPB) for this species was 86.49% (Table 3). At the

Fig 1. Distribution of *Prosopis juliflora* in India and collection sites for plant material and soil samples.

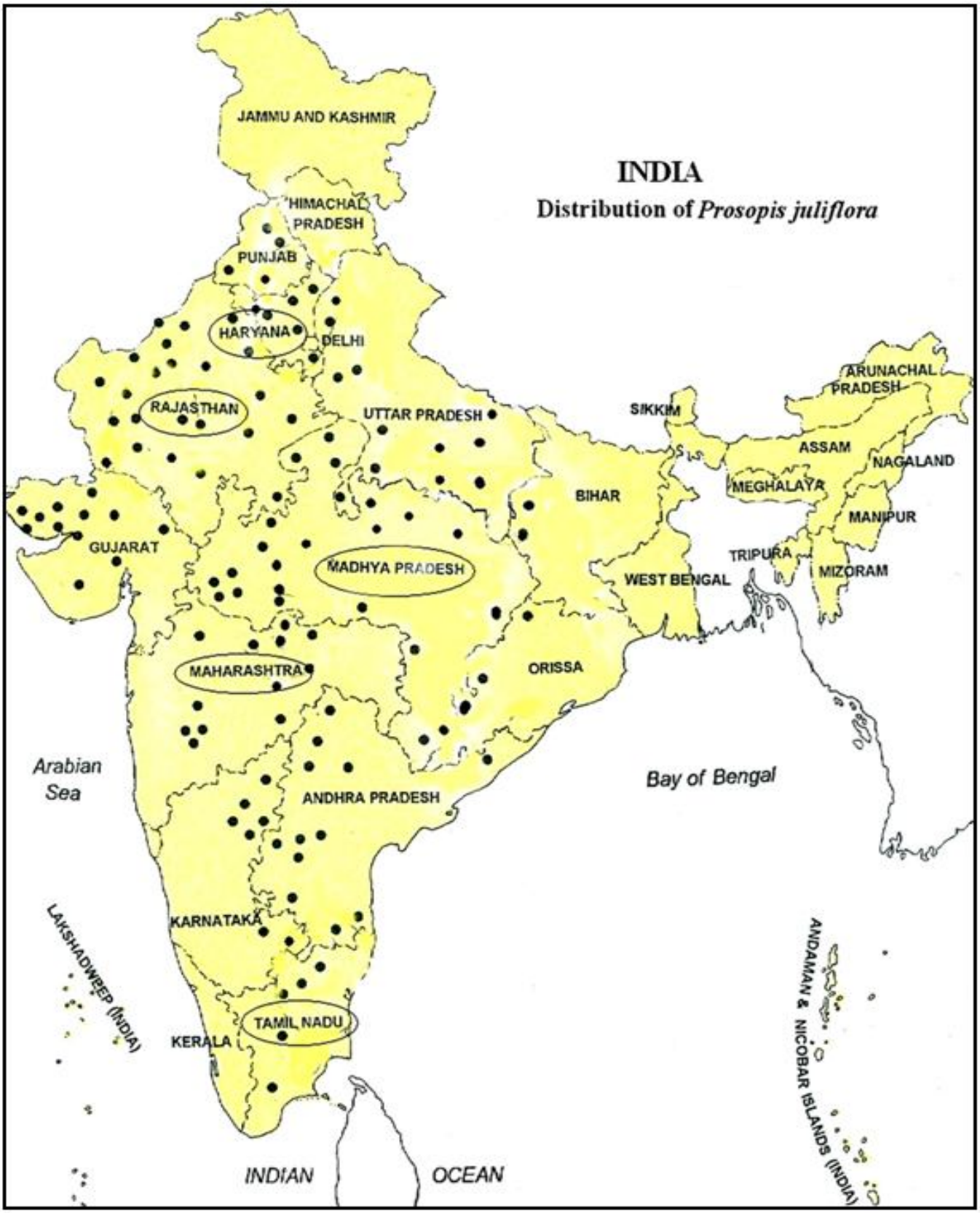
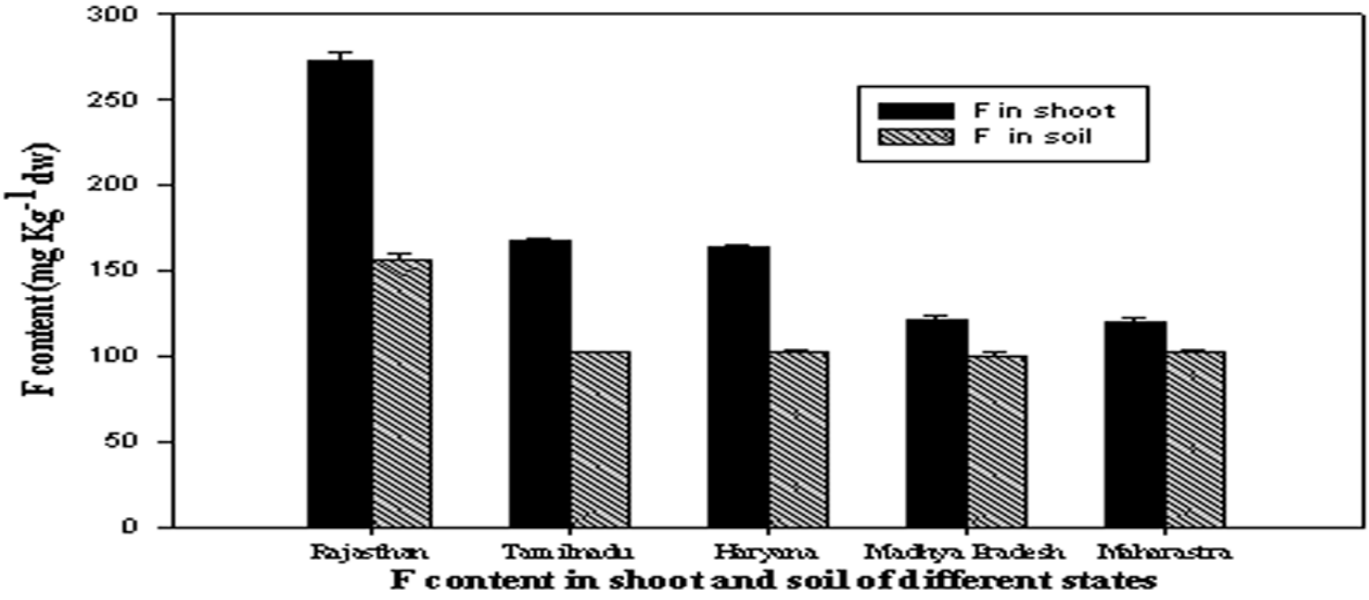


Fig 2. Fluoride content in shoot and soil of *Prosopis juliflora* collected from different states of India. Data presented as mean±SD (n=3).



population level, the PPB values ranged from 2.70% to 21.62%, with an average of 12.97%. The average effective number of alleles per locus was 1.08. Assuming Hardy-Weinberg equilibrium, Nei's gene diversity (H) varied between 0.01 to 0.08 with an average of 0.048 and Shannon's information index (I) ranged from 0.01 to 0.1, with an average of 0.068. The values of H and I showed a similar trend to PPB. However, the H and I values equaled 0.35 and 0.52 respectively at the species level, demonstrating a relatively high level of genetic diversity (Table 3).

The PPB value was found higher in Rajasthan population (21.62%) in comparison to other populations. Observed no of alleles (Na) and effective no of alleles (Ne) were also found be higher in Rajasthan population (Table 3). Number of alleles per locus (Na), mean number of allele over loci (Ne) *i.e.* the estimated allelic richness of populations, are significantly affected by sample size while no significant correlation between sample size and heterozygosities (He and Ho) was observed (Khan *et al.* 2012).

Binary data matrix of RAPD data were used to make pair wise comparison of the accessions based on shared and unique amplification products to generate a similarity matrix with NTSYS-pc 2.1. Similarity coefficient values were used to construct dendograms, using the unweighted pair group method with arithmetic averages (UPGMA) from the similarity data matrices. UPGMA analysis showed separate clusters of population (P1, P2, P3, P4 and P5). In the UPGMA tree, analyzed populations were mainly divided into three geographic groups (Fig. 4). Group I included the populations P1 (Rajasthan A, Rajasthan B, Rajasthan C) and P4 (Madhya Pradesh A, Madhya Pradesh B, Madhya Pradesh C), while the remaining populations were clustered into two other groups. This division reflected geographic distribution pattern of these populations. The populations P1 and P4 are geographically distant from the other populations. Group II included the populations from P2 and P3 which were sampled from Haryana (A, B, C) and (Tamilnadu A, B, C), while population P5 from Maharashtra (A, B, C) was unexpectedly not clustered with any other population (Fig. 4). P1 population showed close similarity with P4 population and distantly related with P5.

DISCUSSION

Investigations into natural population systems are of great importance for our understanding of the genetic basis of the hyperaccumulation trait and the underlying selective pressures that underlie it (Pollard *et al.* 2002). In the present study, five naturally growing populations of *P. juliflora* collected across different parts of India, for checking their F accumulation capacity and genetic diversity patterns, a considerable variation was found in the degree of F accumulation in different populations of *P. juliflora.* Individuals in a population have a specific genotype which is responsible to tolerance and extra ordinarily metal and nonmetal accumulation capacity. Earlier studies have shown that hyperaccumulation efficiency is a quantitative trait (QTL) trait (Yang *et al.* 2005). Consequently, significantly higher BF, values were observed in the Rajasthan

Fig 3. Bioaccumulation factor (BF) values in different populations of *Prosopis juliflora.* Data presented as mean±SD (n=3).

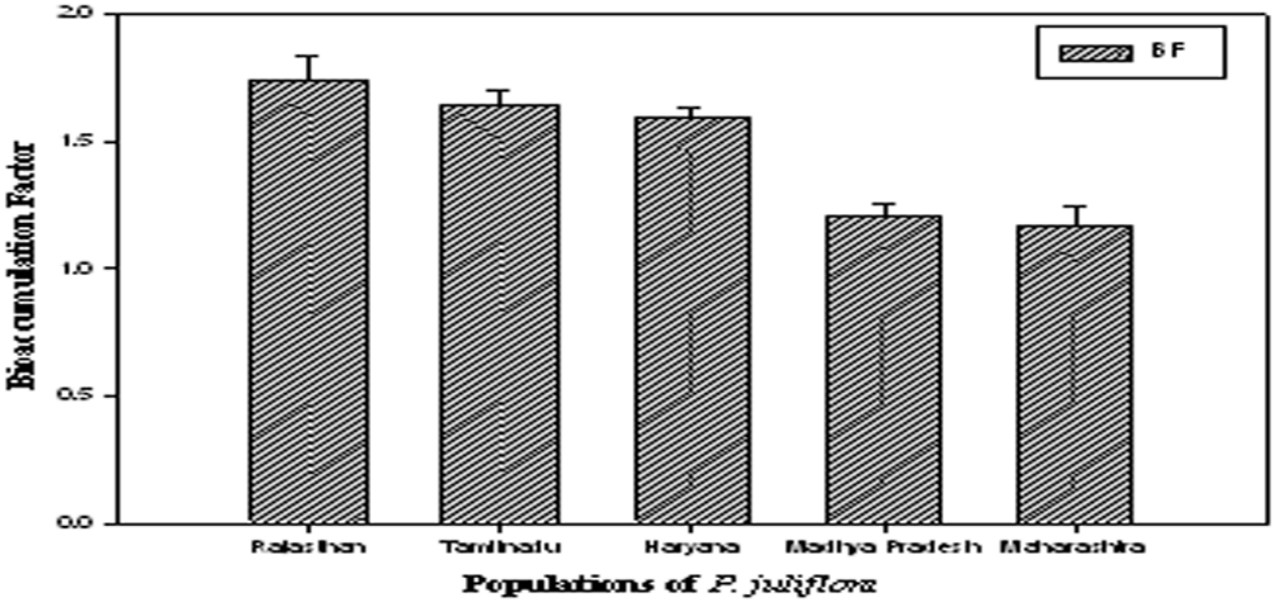
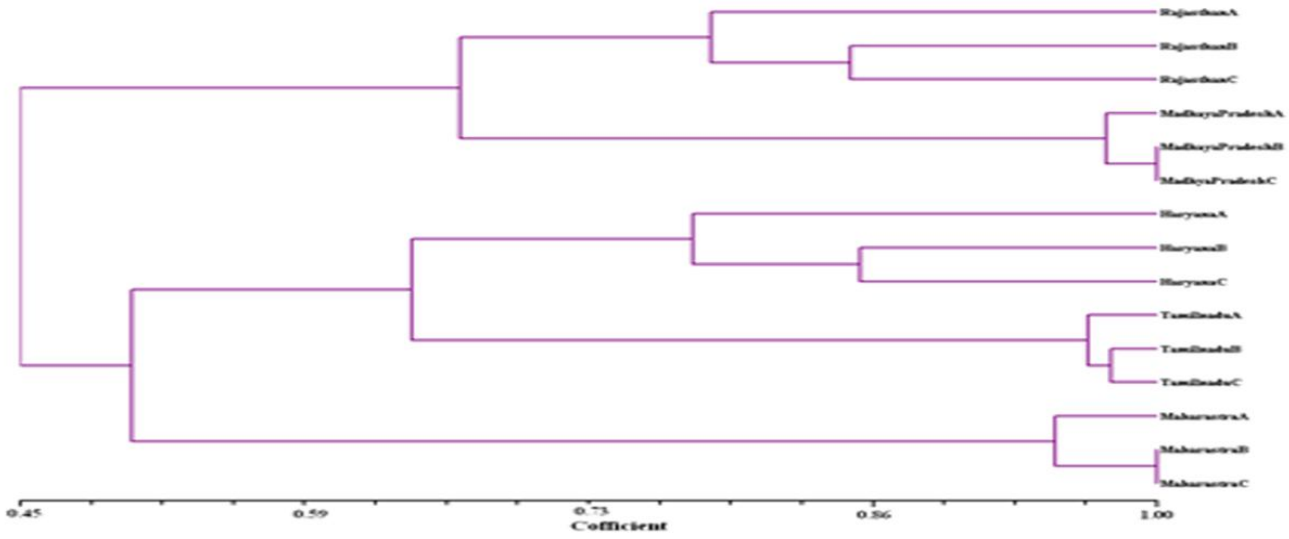


Fig 4. Dendogram obtained from 15 germplam of *Prosopis juliflora* based on unweighted pair group method with arithmetic averages (UPGMA) method.



populations collected from three different sites, when compared to the other populations. Obviously the Rajasthan population of *P. juliflora* carries out indicating more efficient translocation of F from soil to shoot. Our results showed that while F content in both shoot and soil was maximum in Rajasthan, it was minimum in Maharashtra and Madhya Pradesh respectively among the five populations of *P. juliflora.*

ISSR proved to be successful method for detection of fluoride stress on genetic variability. ISSR is an effective marker system for ecotoxicological studies (Abdou *et al.* 2012). Advances in molecular biology have led to the development of selective and sensitive assays for DNA analysis, which could also be applied to the field of genetic toxicology. By using ISSR primers, we demonstrated that there was considerable genetic diversity within populations of *P. juliflora*, 86.49% of bands were polymorphic in 5 populations. There is a positive correlation between high genetic diversity and F accumulation efficiency. It was known that there is considerable variation in a members of a species to accumulate metal and non metal, this was due to the presence of positive correlation between quantitative genetic variation and hyperaccumulation ability between and within populations (Assuncao 2001, Pilon-

Smits 2002).

Genetic diversity parameters viz: percentage of polymorphic bands (PPB), Nei's genetic diversity (H) and Shannon's information index (I) within the populations of *P. juliflora* species was highest in Rajasthan population, and lowest in Maharashtra population. In comparison with the other populations of *P. juliflora* Rajasthan population is expected to be more tolerant as it grows in more severe habitats, viz. high temperatures upto 48ºC dry climate, sandy to rocky soils, high pH, suggesting that germination, vegetation and propagation will be difficult even when the seeds disperse. F hyperaccumulating plants accumulate huge amounts of F and can therefore provide a specific environment for other plants that could be adapted to survive in high non metal concentrations. Contaminants may increase the average fitness of the population in a short term but it may be accompanied by a loss of genetic variation if the selective differential associated with tolerance to contaminants has been large (Baker *et al.* 2001). Thus, as pointed out by McMillan *et al.* (2006), rapid adaptation to novel environmental stressors is no guarantee of long-term population sustainability. An increase in genetic variation has been reported when the toxicant is mutagenic (Baker *et al.* 2001; Slomka *et al.* 2011). Moreover, differential selection associated with contaminants could theoretically contribute to increased genetic diversity under the hypothesis that tolerance to contaminants may increase as a function of individual heterozygosity (Van Straalen and Timmermans 2002).

The present study showed high genetic diversity of Rajasthan population *P. juliflora* populations which is confirmed with high PPB and high gene diversity values. The high diversity levels in Rajasthan populations acts as a buffer to combat F stress and be an important selection criterion in developing lines with greatest phytoremediation potential. Favorable alleles selected during natural selection may have become part of the *P. juliflora* genome and allow its populations to grow in fluoride stress area. Differences in diversity parameters may be attributed to the genetic response to environmental stress. These results were expected, since similar tolerance to metals has been reported in literature for a wide range of organisms; bacteria, protists, plants and animals (Lopes *et al.* 2005). The increase in genetic variation has been reported when the toxicant is mutagenic (Chen *et al.* 2003). Moreover, differential selection associated with contaminants could theoretically contribute to increased genetic diversity under the hypothesis that tolerance to contaminants may increase as a function of individual heterozygosity (Van Straalen and Timmermans 2002). On the other hand, although it may increase the average fitness of the population in the short-term, selection may be accompanied by a loss of genetic variation if the selective differential associated with tolerance to contaminants has been large (Baker *et al.* 2001). Field studies in the natural habitats of hyperaccumulator plants will also be of great importance in testing the ecological significance of metal hyperaccumulation as a defense (Yang *et al.* 2005). When we compare our results of genetic diversity analysis and hyperaccumulation we find that Rajasthan germplasm shows higher F accumulation and higher genetic diversity. Therefore, it seems that Rajasthan genotype is the best germplasm for F removal and phytoremediation purpose.

CONCLUSION

The genotypic differences between populations described above are of great interest to researchers trying to understand and manipulate the genetics of hyperaccumulation. Significant variation was noted between F accumulation efficiency and genetic diversity of different population of *P. juliflora.* Our present study concludes, that highly diversed population has greater tendency to accumulate F. This study suggests that Rajasthan genotypes effectively used in F contaminated fields for phytoremediation. Further investigations on Rajasthan genotype to identify genomic regions linked to factors involved in F hyperaccumulation will be of great value.

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