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Full Length Research Paper

RAPD analysis reveals genetic variation in different populations of *Trichodesma indicum* - A perennial medicinal herb

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Random amplified polymorphic DNA (RAPD) markers were used to assess genetic variation in different populations of *Trichodesma indicum* (family - Boraginaceae), a useful medicinal herb. 50 plants were collected from 4 different locations of Chhattisgarh, one location of Madhya Pradesh and *in vitro* regenerated plants. Genetic variation was detected among the populations and within each population including micropropagated plants regenerated from zygotic embryos. 20 primers generated a total of 121 polymorphic bands out of 125 total bands (96.8% polymorphism), with an average of 6.05 amplified bands per primer. The results reveal very high level of genetic diversity in the species.

Key words: Boraginaceae, polymorphism, *in vitro*, RAPD, amplification.

INTRODUCTION

Trichodesma indicum (L.) R. Br. is a valuable medicinal herb. The plant is used to expel dead fetus (CSIR, 1986). It inhibits diarrhea (Perianayagam et al., 2005) and sulfur dioxide-induced cough reflex in mice (Srikanth et al., 2002). In Chhattisgarh state, it is used for treatment of breast cancer (Tirky, 2002). It is a cross-pollinated species, in which buzz-pollination is carried out by two species of *Anthophora* (Ahmed et al., 1995).

T. indicum (family-Boraginaceae) is a perennial herb commonly known as a*dhpuspi* or *oundhapuspi*, that is, flowers are bent downward. It is distributed in India, Bhutan, Burma (Banerjee and Pramanik, 1975) and Pakistan (Martin et al, 2001). Banerjee and Pramanik, (1975) reported 3 varieties of *T. indicum* namely *T. indicum* var. *indicum*, *T. indicum* var. *amplexicaule* and *T. indicum* var. *subsessilis. T. indicum* var. *amplexicaule* is common in grasslands and bunds of fields, noted at different parts of India; Hague, Alirajpur, Khujawa and Dhar (Samvastsar, 1996). *Trichodesma mudgalii* is described and illustrated as a new species from Madhya Pradesh, India. The *T. mudgalii* is closely allied to *T. indicum* and *T. stocksii* (Kumar et al., 2002). This suggests that the species is variable in morphological and reproductive characters. Random amplified polymorphic DNA (RAPD) markers were used to assess genetic variation in different populations of *T. indicum*.

Random amplified polymorphic DNA (Williams et al., 1990) technology is a reliable method for characterizing variation among species, within a species and among populations (Gustine and Huff, 1999). RAPD profile construction has several advantages, such as rapidity of process, low cost and the use of small amounts of plant material (Jain et al., 1994; He et al., 1995; Lopez et al., 1996). In recent years, RAPD analysis has become a popular method for estimating genetic diversity in plant populations. RAPD has been used for the assessment of genetic relationships and variation in *Paspalum vaginatum* (Lin et al., 1994), variation in populations of *Ranunculus reptans* (Fischer et al., 2000) and *Changeful smyrnioides* (Fu et al., 2003). The present paper reports for the first time genetic variation in the populations of *T. indicum*.

MATERIALS AND METHODS

Plant material

T. indicum plants are found on rice field bunds, grazing grounds and forests during rainy season. However, it is possible to grow

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Table 1. Location of *T. indicum* collection.

S/ N	Location	No. of plants	Sample number
1.	Raipur, Chhattisgarh	20	Ti 1 - Ti 20
	a University campus	10	Ti 1 - Ti 10
	b College premises	5	Ti 11 - Ti 15
	c Kharun river bank	5	Ti 16 - Ti 20
2.	Champa, Chhattisgarh	5	Ti 21 - Ti 25
3.	Kawardha, Chhattisgarh	5	Ti 26 - Ti 30
4.	Korba, Chhattisgarh	5	Ti 31 - Ti 35
5.	Sagar, Madhya Pradesh	5	Ti 36 - Ti 40
6.	*In vitro* propagated plants	10	Ti 41 - Ti 50

plants and keep them in flowering state throughout the year under moist condition. A total of 50 plants were analyzed; 40 plants from states of Chhattisgarh and Madhya Pradesh and 10 plants regenerated from zygotic embryo explants *in vitro*. Five samples were collected from each of the 4 sites namely Champa, Kawardha and Korba (Chhattisgarh state) and Sagar (Madhya Pradesh) and 20 samples were obtained from three sub-areas of Raipur (Chhattisgarh state); university campus, college campus and the bank of river Kharun. The 5 sites are spread over a distance of approximately 500 km and the minimum distance between any 2 sites is more than 40 km.

DNA isolation

Genomic DNA was extracted from fresh tender leaf samples using CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1990) with modifications as described by Khanuja et al. (1999). 4 g of fresh leaf of each 50 samples were ground to a fine powder in a mortar in liquid nitrogen and then transferred to Tarson tubes filled with 15 ml of freshly prepared and preheated 2.5 x CTAB extraction buffer. Extraction buffer consisted of 100 mM Tris - Cl (pH 8.0), 25 mm EDTA (pH 8.0), 1.5 M NaCl, 1% PVP and 0.2% β - merceptoethanol. The suspensions of samples were incubated at 65^0C for 1 h. After cooling at room temperature an equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 10 K rpm for 10 min at room temperature. DNA from aqueous layer was precipitated by adding 5 M NaCl (30% of supernatant) and 0.6 volumes of chilled isopropanol. The mixture was centrifuged at 10 K rpm for 10 min. Pellets were washed with 80% alcohol and let the pellet dry. Pellets were dissolved in high salt TE buffer then RNase A were added and incubated at 37^0 C for 30 min. Again, it was extracted with equal volume of chloroform - isoamylalcohal (24:1). 2 volumes of cold ethanol were added in aqueous layer and centrifuged at 10 K rpm for 10 min. Pellets were washed with 80% alcohol and dissolved in TE (Tris- Cl- EDTA) buffer after drying.

RAPD

PCR for amplifying the DNA preparations was carried out in a 25 µl volume of reaction mixture. A PCR tube contained 20 ng DNA, Taq DNA polymerase (0.6 unit), 100 mM dNTPs, 2.5 µl 10x polymerase buffer and 5 pmol decanucleotide primers. Amplification was carried out in the DNA engine thermal cycler (MJ Research USA.) using following parameters: 94, 35 and 72^0C for 44 cycles and final extension at 72^0C. The PCR products were subjected to separate on 1.2% agarose gel containing 0.5 μl^{-1} ethidium bromide along with 1 kb ladder (Bangalore Genie) and photographed with image master VDS (Pharmacia).

Screening of specific RAPD amplicon

80 random primers (OPA 1-20, OPB 1-20, OPO 1-20 and MAP 1-20) were screened by RAPD for identification of specific marker. The screening of primers resulted in 20 MAP decamer primers (Khanuja et al., 2000) which showed polymorphisms with all 50 samples. Other 60 primers could not amplify all the 50 samples.

RAPD data analysis

RAPD bands were transformed into a binary character matrix, “1” for the presence and “0” for the absence of the bands. Similarity matrices were computed based on Jaccard’s similarity coefficient, using the SPSS. The average of similarity matrices was used to generate dendrogram by UPGMA (unweighted paired group method of cluster analysis).

RESULTS AND DISCUSSION

Genomic DNA was isolated from fresh tender leaf tissues of all the 50 plants of *T. indicum* collected from different locations (Table 1). The procedure yielded 500-800 ng of DNA per 4.0 gm of tissue in 0.8% agarose gel electrophoresis. Of the 80 primers screened, MAP (= M) 1-20 primers (Table 2) produced distinct, highly reproducible amplification profile for all the screened samples (Figure 1). These 20 primers were selected for further analysis of the plant materials.

The polymorphic bands and level of polymorphism of distinguishable RAPD bands in different plants of *T. indicum* populations are shown in Table 3. The 20 primers produced total 125 bands; the number of bands ranged from 3 to 11 bands (Table 3), with an average of 5.70 bands per primer; 114 bands (91.0%) of these were polymorphic. The level of polymorphism for each primer ranged from 0.67 to 1.0. Level of polymorphism within population for each primer in Champa ranged from 0.14 (M7) to 1.0 (M8), in Kawardha from 0.20 (M19) to 1.0 (M2), in Korba from 0.14 (M7) to 1.0 (M11 and M13), in Raipur from 0.50 (M9, M18) to 1.0 (M3, M4, M5, M7, M8, M11, M13, M15, M16), in Sagar from 0.17 (M2 and M17) to 0.86 (M8) and in tissue culture plants derived from individual zygotic embryos from 0.25 (M18) to 1.0 (M2,

Table 2. Sequences of primers.

S/N	Primer	Sequence of primer	S.N.	Primer	Sequence of primer
1.	MAP 1	AAATCGGAGC	11.	MAP 11	CCCTGCAGGC
2.	MAP 2	GTCCTACTCG	12.	MAP 12	CCAAGCTTGC
3.	MAP 3	GTCCTTAGCG	13.	MAP 13	GTGCAATGAG
4.	MAP 4	TGCGCGATCG	14.	MAP 14	AGGATACGTG
5.	MAP 5	AACGTACGCG	15.	MAP 15	AAGATAGCGG
6.	MAP 6	GCACGCCGGA	16.	MAP 16	GGATCTGAAC
7.	MAP 7	CACCCTGCGC	17.	MAP 17	TTGTCTCAGG
8.	MAP 8	CTATCGCCGC	18.	MAP 18	CATCCCGAAC
9.	MAP 9	CGGGATCCGC	19.	MAP 19	GGACTCCACG
10.	MAP 10	GCGAATTCCG	20.	MAP 20	AGCCTGACGC

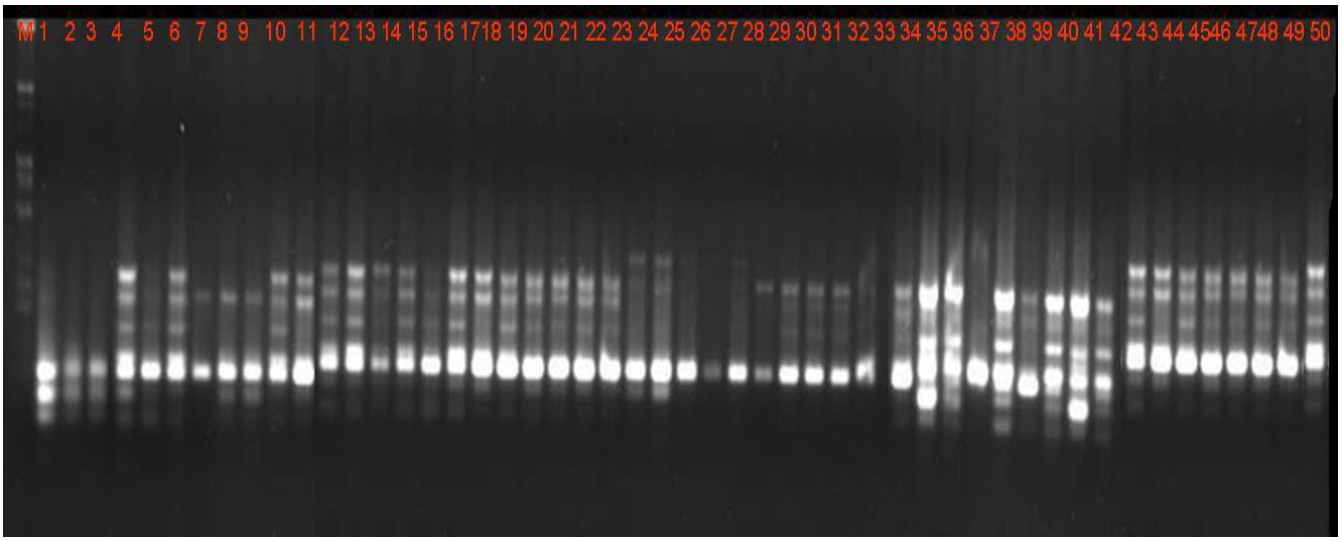

Figure 1. RAPD profile of *T. indicum* DNA generated with the primer MAP7. Lane 1: Marker λHind III + *Eco*RI. Lanes 2-21: Profile of Ti1-Ti20. Lanes 22-26: Profile of Ti21- Ti25. Lanes 27-31: Profile of Ti26- Ti30. Lanes 32-36: Profile of Ti31-Ti35. Lanes 37-41: Profile of Ti36- Ti40. Lanes 41-51: Profile of Ti41- Ti50.

M5, M6, M13, M15, M20) (Table 3). Raipur population exhibited highest variability (0.83) followed by Champa (0.57), Kawardha (0.49) and Korba (0.48) population. Lowest variability (0.44) was observed in the population of Sagar. The diversity within and among the populations is related to different primers and different population. Primers M10 and M14 produced the maximum 11 bands, while M1 primer amplified only 3 fragments. The similarity indices (not shown) indicate that *T. indicum* plants collected from different provenances have high level of variation.

The dendrogram reveals 3 major sub-clusters (Figure 2). The sub-clusters in the dendrogram could be to some extent correlated to the geographical distribution. The samples Ti 1-Ti 20 form the major sub-cluster, which represents all the plants from Raipur. Surprisingly, the samples Ti 21-Ti 40, which belong to widely distributed areas Champa, Kawardha, Korba of Chhattisgarh state and Sagar of Madhya Pradesh are grouped in a common sub-cluster. It suggests that the plants of Raipur make independent population and do not frequently share gene pool of other 4 populations. Sub-cluster Ti 41-Ti 50 represents *in vitro* plantlets. All the tissue culture plants were regenerated from individual zygotic embryos produced by a small group of plants of Raipur. However, they formed a separate sub-cluster. The population of Raipur is highly polymorphic. Similarly, the *in vitro* regenerated plants derived from zygotic embryos are also highly variable. These plants exhibited 71% polymorphism. The high level of RAPD polymorphism reveals genetic variability in cross pollinated species *T. indicum*.

In this study, we have developed RAPD marker for *T. indicum*. It has been used for diversity analysis of many plants like *Vatica guangxiensis* (Li et al., 2002), *Changium smyrnioides* (Fu et al., 2003) and *Rauvolfia serpentine* (Padmalatha and Prasad, 2007) and authen-

Table 3. Total number of bands with number of polymorphic bands for *T. indicum.*

S/N	Primer	Amplified bands	Polymorphic bands (No)						Total No of Polymorphic bands
			Raipur	Champa	Kawardha	Korba	Sagar	*In vitro*	
1	MAP 1	3	2 (0.67)	1 (0.33)	2 (0.67)	2 (0.67)	1 (0.33)	1 (0.33)	2 (0.67)
2.	MAP 2	6	5 (0.83)	5 (0.83)	6 (1.0)	3 (0.50)	1 (0.17)	6 (1.0)	6 (1.0)
3	MAP 3	8	8 (1.0)	2 (0.25)	3 (0.38)	2 (0.25)	2 (0.25)	5 (0.62)	8 (1.0)
4.	MAP 4	5	5 (1.0)	4 (0.80)	4 (0.80)	2 (0.40)	2 (0.40)	4 (0.80)	5 (1.0)
5.	MAP 5	5	5 (1.0)	3 (0.60)	4 (0.80)	2 (0.40)	1 (0.40)	5 (1.0)	5 (1.0)
6.	MAP 6	7	6 (0.86)	4 (0.58)	3 (0.43)	3 (0.43)	3 (0.43)	7 (1.0)	7 (1.0)
7.	MAP 7	7	7 (1.0)	1 (0.14)	2 (0.29)	1 (0.14)	4 (0.58)	6 (0.86)	7 (1.0)
8.	MAP 8	7	7 (1.0)	7 (1.0)	3 (0.43)	3 (0.43)	6 (0.86)	5 (0.71)	7 (1.0)
9.	MAP 9	6	3 (0.50)	4 (0.67)	2 (0.33)	3 (0.50)	4 (0.67)	2 (0.33)	6 (1.0)
10.	MAP 10	11	10(0.91)	3 (0.27)	5 (0.45)	3 (0.27)	5 (0.45)	8 (0.73)	11 (1.0)
11.	MAP 11	5	5 (1.0)	1 (0.20)	2 (0.40)	5 (1.0)	3 (0.60)	3 (0.60)	5 (1.0)
12.	MAP 12	4	3 (0.75)	3 (0.75)	2 (0.50)	2 (0.50)	2 (0.50)	2 (0.50)	4 (1.0)
13.	MAP 13	4	4 (1.0)	3 (0.75)	3 (0.75)	4 (1.0)	2 (0.50)	4 (1.0)	4 (1.0)
14.	MAP 14	11	6 (0.55)	5 (0.45)	4 (0.36)	2 (0.18)	5 (0.45)	10(0.91)	11 (1.0)
15.	MAP 15	5	5 (1.0)	3 (0.60)	3 (0.60)	2 (0.40)	2 (0.40)	5 (1.0)	5 (1.0)
16.	MAP 16	8	8 (1.0)	2 (0.25)	2 (0.25)	3 (0.38)	2 (0.25)	3 (0.38)	7 (0.88)
17.	MAP 17	6	4 (0.67)	2 (0.33)	2 (0.33)	2 (0.33)	1 (0.17)	3 (0.50)	5 (0.83)
18.	MAP 18	8	4 (0.50)	7 (0.88)	2 (0.25)	2 (0.25)	2 (0.25)	2 (0.25)	8 (1.0)
19.	MAP 19	5	3 (0.60)	2 (0.40)	1 (0.20)	2 (0.40)	2 (0.40)	3 (0.60)	4 (0.80)
20.	MAP 20	4	3 (0.75)	3 (0.75)	2 (0.50)	2 (0.50)	3 (0.75)	4 (1.0)	4 (1.0)
Average		6.25	5.15 (0.83)	3.25 (0.57)	2.85 (0.49)	2.65 (0.48)	2.75 (0.44)	4.40 (0.71)	6.05 (0.97)

tication of traditional Chinese medicinal plant species ginseng (Um et al., 2001). Naugzemys et al. (2007) analyzed *Lonicera caerulea* germplasm accessions using RAPD markers and found that RAPD analysis is efficient for genotyping of accessions. DNA polymorphism significantly exceeds the morphological diversity of the samples. A total of 105 bands were scored with 11 random primers; 83.9% of scored bands were polymorphic. Krecher et al. (2000) used RAPD to survey the genetic diversity of *Vaccinium stamineum* within and among patches and found 67 unique RAPD profiles among the 99 sampled individuals from 22 patches. Auge et al. (2001), reported high level of genetic diversity within populations of clonal herb *Viola riviniana* and investigated the pattern of seedling recruitment, the amount of geno-typic variation and the partitioning of genetic variation among and within population. RAPD yielded 45 scorable bands that were used to identify multilocus genotype of *V. rivinian.* Jain et al. (2003) studied molecular diversity in *Phyllanthus amarus* by RAPD profiling of 33 collections that revealed 65% variation among the accessions. Intra-population variation was found to be much larger in the some accessions. Jayram and Prasad (2008), used RAPD markers to assess genetic diversity in *Oroxylum indicum,* a vulnerable medicinal plant collected from 8 locations in Andhra Pradesh, India. High level of genetic similarity was observed in the collected accessions. Overall genetic similarity based on 40 random primers was 87%. Wolff and Morgan-Richards, (1999) observed large diversity among populations of *Psammochloa villosa*, a self-pollinating species. In our RAPD analysis, significant genetic polymorphism was observed within each populations of *T. indicum*, which was expected due to cross pollination in the species. In addition, polymorphism was also detected among the plantlets micro propagated from zygotic embryo explants. The analysis reveals that the species is endowed with high level of genetic variation that enabled its wide distribution and formation of new varieties and species.

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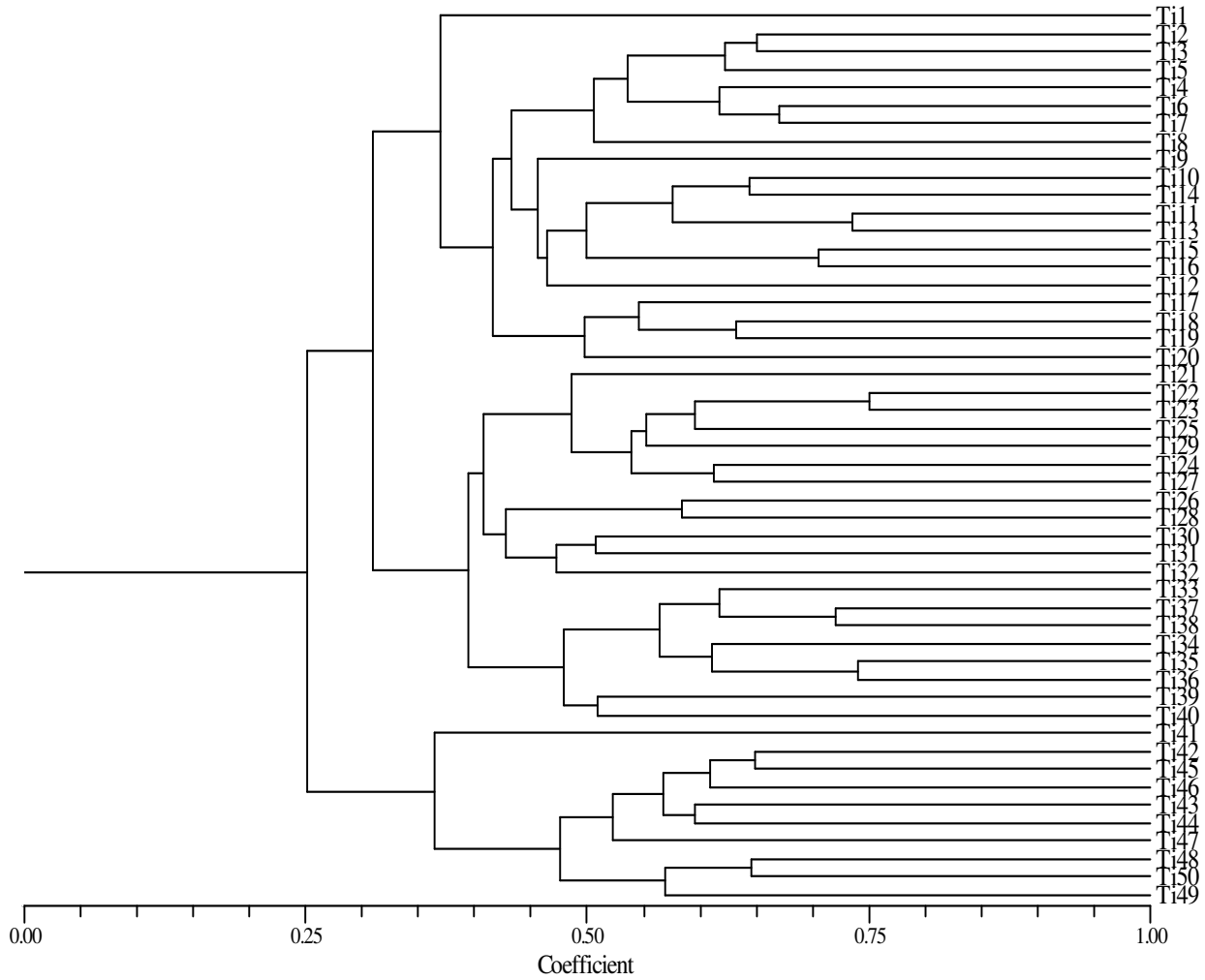

Figure 2. RAPD dendrogram shows sub cluster of Raipur (Ti 1 - 20) population, a single sub cluster of Champa, Kawardha, Korba and Sagar (Ti 21 - 40) populations and a sub cluster of tissue culture pants of *T. indicum*.

REFERENCES

Ahmed T, Sarwar GR, Ali T, Qaiser M (1995). Buzz Pollination in *Trichodesma indicum* (L.) R. Br. (Boraginaceae). Pak. J. Bot. 27(1): 93-99.

Auge H, Neuffer B, Erlinghagen F, Grupe R, Brandl R (2001). Demographic and RAPD analyses reveal high levels of genetic diversity in a clonal violet. Mol. Ecol. 10: 1811-1819.

Banerjee SP, Pramanik BB (1975). A Taxonomic revision of Indo Burmese *Trichodesma* Boraginaceae. Bulletin of the Botanical Survey of India, 17(1-4): 108-123.

CSIR (1986). The useful plants of India (Ed. Ambusta S.P.) CSIR. Publication, New Delhi, p. 648.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue, Focus, 12: 13-15.

Fischer M, Husi R, Daniel P, Peintinger M, Mark VK, Bernhard S (2000). RAPD variation among and within small and large populations of the rare clonal plant *Rananculus reptans* (Rananculaceae). Am. J. bot. 87(8): 1128- 1137.

Fu C, Qiu Y, Kong H, (2003). RAPD analysis for genetic diversity in *Chagium smyrnioides* (Apiaceae), an endangered plant. Bot. Bull. Acad. Sin. 44: 13-18.

Gustine DL, Huff DR (1999). Genetic variation within and among white clover populations from managed permanent pastures of the northeastern US. Crop Sci. 39: 524- 530.

He G, Prakash CS, Jarret RL (1995). Analysis of genetic diversity in a sweet potato (*Ipomea batatas*) amplification fingerprinting germ plasm collection using DNA. Genome, 38: 689-696.

Jain A, Bhatia S, Banga SS, Prakash CS, Laxmikumaran (1994). Potential use of RAPD technique to study the genetics diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis. Theor. Appl. Genet. 88: 116- 122.

Jain N, Shasany AK, Sundaresan, Rajkumar S, Darokar MP, Bachi GD, Gupta AK, Kumar S, Khanuja SPS, (2003). Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis. Curr. Sci. (85)10: 1454-1458.

Jayram K, Prasad MNV (2008). Genetic diversity in *Oroxylum indicum* (L.) Vent. (Bignoniaceae), a vulnerable medicinal plant by random amplified polymorphic DNA marker. Afr. J. Biotechnol. 7(3): 254-262.

Khanuja SPS, Shasany AK, Darokar MP, Kumar S (1999). Rapid isolation of PCR amplifiable DNA from the dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils by modified CTAB procedure. Plant Mol. Bio. Rep. 17: 1-7.

Khanuja SPS, Shasany AK, Srivastava A, Kumar S (2000). Assessment of genetic relationships in *Mentha* species. Euphytica, 111: 121-125.

Krecher SA, Fore SA, Collins BS (2000). Genetic variation within and among patches of the clonal species, *Vaccinium stamineum*. Mol. Ecol. 9: 1247-1252.

Kumar A, Khanna KK (2002). *Trichodesma mudgalii* (Boraginaceae)-a

new species from Madhya Pradesh, India. Botanical survey of India. 22(2): 223-225.
Li Q, Xu Z, He T (2002). *Ex situ* genetic conservation of endangered *Vatica guangxiensis* (Dipterocarpaceae) in China. Biol. Conser. 106: 151-156.
Lin ZW, Jarret RL, Dunccan RR, Kresovich S (1994). Genome. 37: 1011-1017.
Lopez- Brana I, Romero MD, Delibes A (1996). Analysis of *Heteroderma avenae* populations by the random amplified polymorphic DNA technique. Genome, 39: 118-112.
Martin A, Khan MJ, Ashraf M, Qureshi RA (2001). Traditional use of herbs, shrubs and trees of Shorgran Valley, Mansehra, Pakistan. Pak. J. Biol. Sci. 4(9): 1101-1107.
Naugzemys D, Zilinskaite S, Denkovskij J, Patamsyte J, Literskis J, Zvingila D (2007). RAPD based study of genetic variation and relationships among Lonicera germplasm accessions. Biologija, 53 (3): 34-39.
Padmalatha K, Prasad MNV (2007). Inter and intra population genetic variation in *Rauvolfia serpentine* Benth. ex. Kurz- an endangered medicinal plant. Med. Arom. Plant Sci. Biotechnol. 1: 118-123.
Perianayagam JB, Sharma SK, Pillai KK (2005). Evaluation of antidirrheal potential of *Trichodesma indicum* root extract in rats. Methods Find Exp. Clin. Pharmacol. 27(8): 533-537.
Samvastsar S (1996). The flora of Western tribals Madhya Pradesh (India). Scientific Publishers, Jodhapur.
Srikanth K, Murugesan T, Kumar A, Suba V, Das AK, Sinha S, Arunachalam G, Manikandan L (2002). Effect of *Trichodesma indicum* extract on cough reflex induced by sulphur dioxide in mice. Phytomedicine-(Jena). 9(1): 75-77.
Tirky A (2002). Ethanomedicinal investigation on plants of district Raigarh (Madhaya Pradesh). Ph.D. thesis, submitted to Barkatullah University, Bhopal, (M.P.), India.
Um JY (2001). Molecular authentication of Panax ginseng species by RAPD analysis and PCR- RFLP. Biol. Pharm. Bull. 24: 872 875.
Williams JGK, Kubelik AR, Livak KT, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: p. 6531.
Wolff K, Morgan-Richards M (1999). The use of RAPD data in the analysis of population genetic structure: case studies of *Alkanna* (Boraginaceae) and *Plantago* (Plantaginaceae). Molecular systematics and plant evolution, London: Taylor & Francis. pp. 51-73.