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Chemical and molecular characterisation of *Artemisia annua* L. genotypes grown under *Tarai* conditions of Uttrakhand

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ABSTRACT

***Artemisia annua* L. is an important medicinal plant belonging to the family Asteraceae having potential value as a source of artemisinin and essential oils. Artemisinin, a secondary plant product has been found to have strong anti-malarial properties with little or no side effects. Considering the importance of plant against malaria, present investigation was conducted to study various morphotypes of *A. annua* growing in *Tarai* areas of Uttarakhand by characterizing them using chemical and molecular determinants. Chemical characterization was done by preparative TLC. The percent artemisinin ranged from 0.39% (dry wt) to 0.78% (dry wt) in P.A-IV (S.P.C.) and P.A-I (I.D.F. Nagla) respectively. Further, twelve populations of three high yielding morphotypes of *Artemisia annua* were selected for studying genetic variation using 16 random decamer primers. The RAPD profiles for all 12 populations showed an average of 10.375 fragments per primer in the size-range of 326-1354 bp. A total of 166 bands with 122 polymorphic and 44 monomorphic were obtained by these primers. A maximum of 29 bands were observed with primer PR-6. Some primers also produced unique alleles (15) in specific genotypes which could be used to distinguish them. Based on RAPD profiles, 12 populations were categorized into two major clusters with 35 to 79% similarities. Cluster I comprised of 9 populations while cluster II comprised only 3. Population 11 and 12 showed highest degree of similarity (78%) with each other.**

Key words: *Artemisia annua*, artemisinin, morphotypes, TLC, RAPD.

INTRODUCTION

Malaria, one of the world's most serious parasitic diseases, caused by *Plasmodium* results in at least 500 million cases globally every year, tolling in more than one million deaths (Ro *et al*., 11). The biggest challenge facing the fight against malaria is the multi-drug resistance of *Plasmodium* strains to the widely used anti-malarials such as chloroquine, mefloquine and sulfadoxine-pyrimethamine (Liu *et al*., 8). The rise of multi-drug resistant *Plasmodium* species and the pesticide resistance of the infection vector, the anopheles mosquito, have made the eradication of this disease very difficult. Therefore, artemisinin an endoperoxide containing sesquiterpene isolated from artemisia, is becoming more and more popular for the production of anti-malarial drug. *A. annua* L., a member of family Asteraceae also known as qinghao, annual or sweet wormwood, is an annual herb native of China. The importance of the plant is due to presence of artemisinin, a sesquiterpene lactone endoperoxide that has been found to have strong anti-malarial properties (Klayman, 3; Sangwan *et al*., 14). Artemisinin content in *A. annua* is very low (0.01-1.0% dry weight) and the demand for artemisinin is increasing every year in tropical and sub-tropical regions of the world (Namdeo *et al*., 10). Although total synthesis of artemisinin has been achieved, the synthesis yields low yields and is uneconomical. Hence, the isolation of artemisinin from the plant still holds the best alternative.

The Indian population of *Artemisia annua* is a racial mixture of multiple introductions from diverse secondary populations and these populations have freely outcrossed during years of vicinal cultivation (Sangwan *et al*., 13). Therefore, for optimization of breeding for desirable characters from such introduced populations, it is essential to screen and evaluate the available variability to find out plants with maximum content of artemisinin. In the present study, morphotypic variants representative of the introduced *Tarai* population growing at various sites in Pantnagar, were selected for chemical and molecular analysis on the basis of artemisinin content and RAPD profiling respectively.

MATERIALS AND METHODS

Characteristics of different morphotypes such as plant spread, plant architecture, plant height and leaf-stem ratio are given in Table 1. Chemotyping of these morphotypes was done using TLC. During flowering stage, plants were again screened for more variability and a total of four variabilities were identified from the three high yielding morphotypes based on pigmentation in the leaf nodes and early flowering

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Table 1. Vegetative characters of the selected *Artemisia* morphotypes.

Morphotype	Plant height (cm)	Plant spread (cm)	Leaf-stem ratio
PA- I	210.6	127.4	1:4
PA- II	218.1	117.0	1:4
PA- III	248.0	126	1:3
PA-IV	284.0	85.4	1:5
PA-V	284.0	87.0	1:3
PA-VI	242.0	88.0	1:4

character (Table 2). Seeds were collected from these plants separately and were sown in separate raised beds in the poly house. Three populations each, belonging to variabilities V-I, V-II, V-III and V-IV were selected and were transferred to individual pots after 45 days of sowing. These 12 populations *viz*., V-I (V-1), V-I (V-2), V-I (V-3), V-II (V-4), V-II (V-5), V-II (V-6), V-III (V-7), V-III (V-8), V-III (V-9), V-IV (V-10), V-IV (V-11), V-IV (V-12) were used for further studies with respect to molecular characterization.

The procedure of Gupta *et al*. (2) was employed for determination of artemisinin content by plotting calibration curve of standard artemisinin in the range of 2 to 20 µg. Artemisinin was isolated from the plant and its identity was confirmed by TLC. Extraction was done by soxhlet apparatus in n-hexane for 24 h. Samples were prepared by pipetting out 10 µl of crude solution in different test tubes. Freshly prepared dye (comprising of glacial acetic acid, concentrated H_2SO_4 and anisaldehyde (50:1:0.5) was used followed by incubation at 110°C for 30 min. and concentration of artemisinin in µg/µl (Table 3) was calculated using standard curve.

For diversity analysis, genomic DNA from the young leaves was isolated using the modified CTAB

Table 2. Differentiating characters of varaiabilities in *Artemisia* genotypes.

Variability	Morphotype	Characters	
V-I	PA-I	Pigmented	Normal flowering
V-II	PA-I	Non-Pigmented	Early flowering
V-III	PA-II	Pigmented	Normal flowering
V-IV	PA-III	Non-Pigmented	Early flowering
V-V	PA-IV	Non-pigmented	Early flowering
V-VI	PA-V	Non-pigmented	Early flowering
V-VII	PA-VI	Pigmented	Normal flowering
V-VIII	PA-VI	Pigmented	Early flowering

Table 3. Artemisinin content in different *Artemisia* morphotypes**.**

Genotype	Optical Density	Artemisinin content (µg /10 µl)	Dry wt. conc. (mg/g)	Dry wt. conc. (%)
PA- I	0.3	130.0	7.80	0.78
PA- II	0.25	108.00	6.48	0.64
PA- III	0.27	118.0	7.08	0.70
PA-IV	0.15	66.00	3.96	0.39
PA-V	0.18	80.00	4.80	0.48
PA-VI	0.16	70.0	4.20	0.42

method (Sangwan *et al.*, 12). RNA was removed by RNAse treatment. For further purification, DNA solution was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1; pH 8.0). Quantity was checked spectrophotometrically from the absorbance data of the sample DNA at 260 and 280 nm. The purity of DNA was calculated from OD_{260}/ OD_{280} ratio. Sixteen random primers, procured from Operon Technologies (USA) were used (Table 4) for PCR amplification. The reaction mixture consisted of 1 X PCR assay buffer (60 mM Tris-HCl, 25 mM KCl, 1.5 mM $MgCl_2$ 10 mM 2-mercaptoethanol, 0.1 % Triton X-100), 200 µM each of dNTPs, 50 ng of primer, 0.5 unit of *Taq*DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore) and 50 ng of DNA template . The volume was made to 25 µl with sterile double-distilled water. The solution was mixed gently and amplification was carried out for 40 cycles. After initial heat denaturation of the DNA at 94°C for 5 min., the thermal cycling was performed with the following temperature regimes; primer annealing (35°C) for 1.5 min. and primer extension (72°C) for 1.5 min. In the next 39 cycles, the period of denaturation was reduced to 1 min and temperature 95°C, while the primer annealing and the primer extension time remained as in the first cycle. The last cycle consisted of only primer extension (72°C) for 5 min.

PCR products were separated on a 1.5% agarose gel in 1X TAE buffer. The gel was visualized using ethidium bromide stain (0.5 µg/ml) and photographed in a gel documentation system (Alpha Imager™ 2200). To test the reproducibility of the profiles, the reactions were repeated twice. The RAPD products were scored and Jaccard's coefficient of similarity was measured. A dendrogram based on similarity coefficients was genetrated by using Unweighed Pair Group Method with Arithmetic Mean (UPGMA). The computer package Statistica version 5 was used for cluster analysis. Average expected gene diversity (H_i) was calculated by method given by Vicente Fulton (16) in learning module on genetic diversity analysis with molecular marker data as obtained from the website http://www.ipgri.cgiar.org/publications (Using Molecular Marker Technology in Studies on Plant Genetic Diversity). On the basis of the scoring pattern, allelic frequencies were calculated which are then used to calculate average expected gene diversity. Polymorphic information content (PIC) that provides an estimate of the discriminatory power of a locus or loci, by taking into account, not only the number of alleles that are expressed, but also relative frequencies of those alleles (Kumar *et al.*, 5) was estimated.

RESULTS AND DISCUSSION

Both the representative as well as samples pooled from different morphotypes of the plant contained artemisinin. The artemisinin content was maximum in P.A-I (0.78% dry wt) and minimum in P.A-IV (0.39% dry wt). % artemisinin among different morphotypes ranged in the order, P.A-I (0.78%) > P.A-III (0.70%) > P.A-II (0.64%) > P.A-V (0.48%) > P.A-VI (0.42%) > P.A-IV (0.39%) (Table 3). Thus, variation in the artemisinin content was seen among the different morphotypes. Artemisinin is accumulated in the glandular trichomes of *A. annua* (Duke *et al.*, 1994). A possible explanation for the variable artemisinin content could be related to variability in the number of trichomes. Plants having higher average plant spread have higher % artemisinin (Tables 1&3). TLC-densitometry studies of 90 samples of *A. annua* plantlets showed high variability of the artemisinin content, ranging from 0.02 to 0.67% w/w dry weight (Koobkokkruad *et al.*, 4).

The RAPD primers (Table 4) used for assessing genetic diversity of different populations of *A. annua* and also to determine phylogenetic relationships among them (Fig. 1). A total of 166 bands, comprising of 122 polymorphic and 44 monomorphic bands were observed against the sixteen primers used for amplification. RAPD profiles showed an average of 10.375 fragments per primer in the size range of 0.32625 to 1.35437 kb (Table 5).The percentage polymorphism for all the samples varied from 40 to 100% (average 67.20%). A maximum of 29 bands were observed with PR-6 (Fig.1). The polymorphism was highest in PR-3 and 6 primers, while it was the lowest in LC-5 and 6 primers. Primer PR-4, PR-5, LC-3 and LC-5 produced one unique band each in V-11, V-8, V-7 and V-9 respectively. Four unique bands were obtained with primer PR-3 in the V-1, V-2, V-3, and V-10 of size 0.3, 0.38, 0.4 and 0.45 kb respectively. Different variabilities of *A. annua* can be easily distinguished using unique RAPD loci.

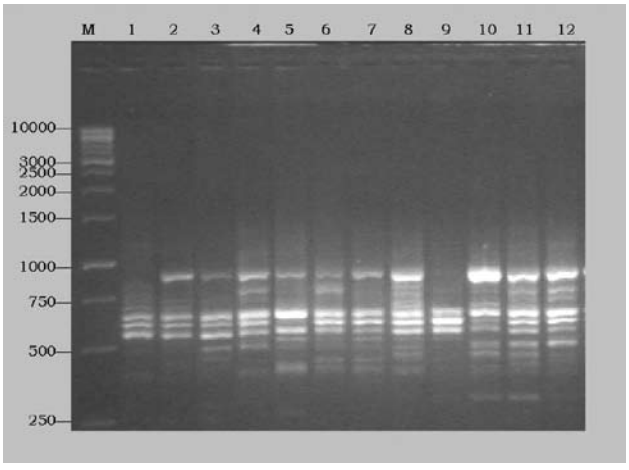

Fig. 1. The RAPD profile of different *Artemisia annua* genotypes with RAPD primer PR-6.

Table 4. Details of RAPD primers used in the study.

S. No.	Code	Primer sequences (5'→3')	GC content (%)
1.	OPD-20	ACCCGGTCAC	70
2.	OPD-07	TTGGCACGGG	70
3.	OPE-06	AAGACCCCTC	60
4.	LC-124	ATCGGGTCCG	70
5.	LC-137	CCGGCCCCAA	80
6.	LC-91	TGGACCGGTG	70
7.	LC-90	GTGAGGCGTC	70
8.	LC-123	GCGATCCCCA	60
9.	OPA-02	TGCCGAGCTG	70
10.	OPA-10	GTGATCGCAG	60
11.	OPA-20	GTTGCGATCC	60
12.	OPB-06	TGCTCTGCCC	70
13.	OPB-10	CTGCTGGGAC	70
14.	OPC-02	GTGAGGCGTC	70
15.	OPC-04	CCGCATCTAC	80
16.	OPC-08	TGGACCGGTG	70

The dendrogram constructed based on banding pattern using UPGMA method revealed two distinct clusters comprising of 9 and 3 populations (Fig. 2). These clusters were further divided into different sub-clusters. The cluster analysis comprising the 12 populations showed 35 to 79.1% similarity in RAPD analysis. The result of pair wise combinations indicated that the two populations, V-11 and V-12 were highly related as indicated by the high value of similarity coefficient (0.78). One cluster indicated that the populations 10, 11 and 12 are genetically more similar. The other cluster comprising of 9 populations (V-1, V-2, V-3, V-4, V-4, V-5, V-6, V-7, V-8 and V-9) showed 37.3 to 66.7% similarity within cluster. Cluster I was further divided into two sub-clusters. Sub-cluster I comprised of 7 populations, while cluster II consisted of 2 populations, V-8 and V-9 with 0.47 similarity coefficient. Sub-Cluster I further divided into two groups. Group II of Sub cluster I has two populations of *A. annua*, *i.e.* V-6 and V-7 with 0.618 similarity coefficient. Group I of sub-cluster I is divided into two sub-groups. The sub-group I comprises two populations, V-1 and V-2 with 0.667 similarity coefficient. Sub-group II again comprised two sub-clusters *i.e.* sub-cluster I comprising of two populations, *i.e.* V-2 and V-5 having similarity coefficient of 0.684 while the sub-cluster II comprised of single population *i.e.*, V-4 having 0.559 and 0.637 similarity coefficients with V-2 and V-5 respectively.

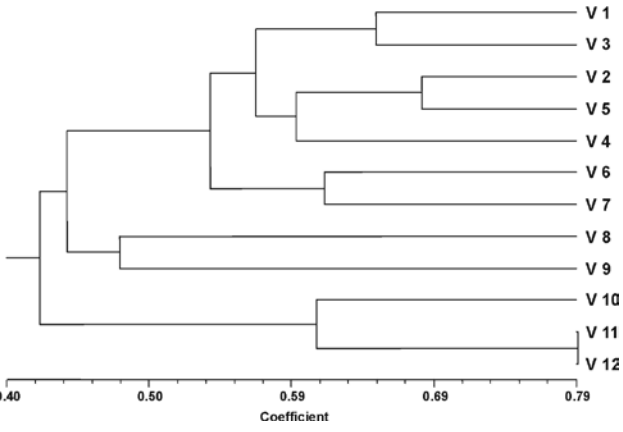

Fig. 2. Dendrogram of 12 variabilities of *A. annua* L. genotypes constructed by using UPGMA based on Jaccard's coefficient.

The Polymorphic Information Content (PIC) was determined based on the frequency of occurrence of each in all the genotypes and it ranged from 0.3167 to 1.00 in primer LC-5 and PR-1; average was found to be 0.5926. The observed PIC for the RAPD markers amongst the twelve populations of *A. annua* represented a substantially moderate degree of genetic diversity of about 59.2%. The high PIC is related to reportedly high levels of outcrossing amongst the different populations. All the primers showed moderate value (average 0.5926) of PIC which is a measure to know the usefulness of the primer for diversity analysis. The gene diversity (H_i) ranged from 0.076 to 0.344 in primer LC-5 and PR-1 respectively. The average gene diversity was 0.196 which showed moderate diversity

Table 5. Number of RAPD loci, polymorphic information content and gene diversity generated by the DNA primers.

Primer code	Total No. of loci	Amplified product range (bp)	Polymorphic bands		Monomorphic bands		Unique bands	PIC	H_i
			No.	%	No.	%	No.		
LC-1	6	400-910	3	50	3	50	-	0.607	0.146
LC-2	8	300-900	6	75	2	25	2	0.552	0.175
LC-3	8	300-1500	4	50	4	50	1	0.395	0.096
LC-4	5	400-1510	3	60	2	40	-	0.466	0.124
LC-5	5	300-1520	2	40	3	60	1	0.316	0.076
LC-6	7	300-1440	3	42.8	4	57.1	-	0.447	0.145
LC-7	5	340-1390	3	60	2	40	-	0.400	0.271
LC-8	8	280-1490	6	75	2	25	-	0.552	0.175
PR-1	2	300-520	1	50	1	50	-	1.000	0.344
PR-2	21	300-2000	14	66.6	7	33.3	-	0.472	0.324
PR-3	7	280-1500	7	100.0	0	0	4	0.826	0.167
PR-4	12	380-1200	9	75.0	3	25.0	1	0.589	0.157
PR-5	11	350-1150	10	90.9	1	9.0	1	0.724	0.232
PR-6	29	400-1500	29	100.0	0	0.0	2	0.715	0.248
PR-7	12	300-1600	9	75.0	3	25.0	3	0.760	0.200
PR-8	20	290-1540	13	65.0	7	35.0	-	0.655	0.260
Total/ Mean	166	326.25-1354.37	122	67.2	44	32.7	15	0.592	0.196

among the different populations. Vaishali *et al*. (15) reported gene diversity values in the range of 0.57-0.79 in *Butea monosperma* collected from different agro-ecological regions of India.

For discrimination of medicinal plants, subjective identification methods, based on the plants morphological features, are generally used; however it is very difficult to discriminate between medicinal plants on morphological basis only. Therefore, various molecular biology techniques, utilizing the different genetic information of organisms, are employed for species discrimination of plants. In particular, random amplified polymorphic DNA (RAPD) analyses were widely used in assessing genetic diversity within a species (Sangwan *et al*., 14). Sangwan *et al*. (14) suggested that wide phytochemical diversity of *Artemisia annua* is included within genetic diversity. The present study also reports RAPD based segregation of different morphotypes of *Artemisia annua*, which also varied in their chemical content. In essence, the present investigation displayed intra-population variation not only among different morphotypes, but also in populations belonging to a morphotype and this can be attributed to the crop's reproductive behavior *i.e*., the cross-pollinated nature and local adaptation changes. The dendrogram also established genetic relatedness among different morphotypes. The fingerprint data obtained in this study can be further utilized in identification and development of improved artemisia varieties. Use of SCAR and ISSR markers also differentiated various populations of artemisia (Lee *et al*., 6; Mohsen and Ali, 9).

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