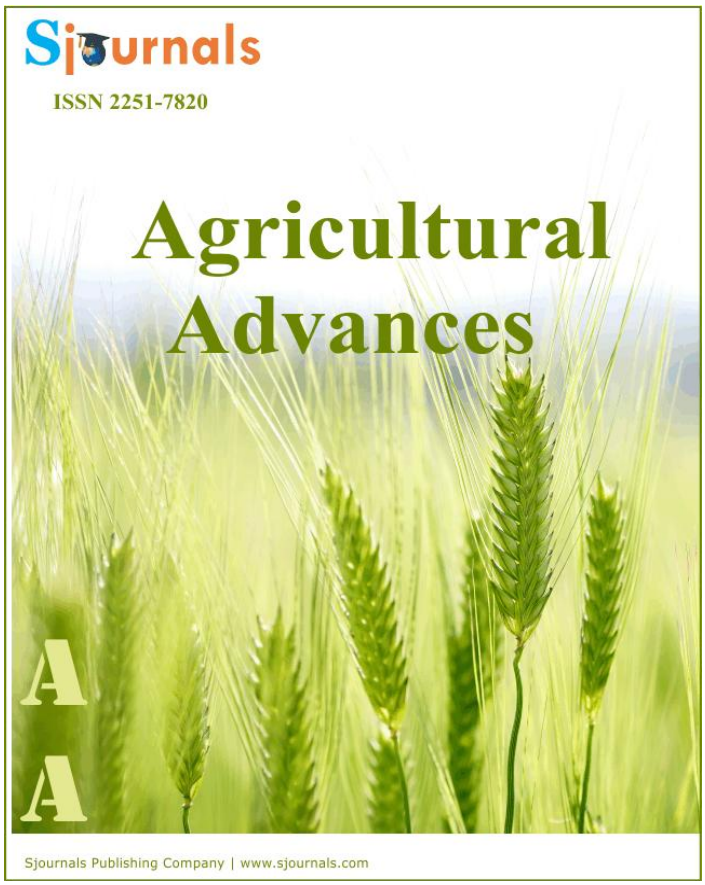

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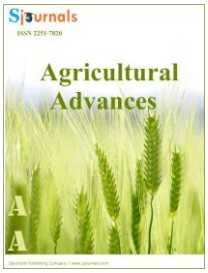


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Original article

# Correlation between genetic diversity, phenotype and Esculin content in *Taraxacum officinale* Weber

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ABSTRACT

Attempts were made to correlate the secondary metabolite content with genetic diversity in *Taraxacum*. In the present study, esculin, one of the important cytotoxic coumarins was analysed in populations of *Taraxacum officinale* collected from Districts Baramullah (Jammu and Kashmir) and Chamoli Garhwal (Uttarakhand) respectively. The two populations differed in a number of morphological and phenotypic characters. Genetic profiling of esculin quantified individuals was done using RAPD markers for the identification of high esculin yielding genotypes.



## 1. Introduction

*Taraxacum officinale* Weber (Asteraceae), commonly known as Dandelion is a very important medicinal plant. The plant shows extensive geographical distribution and grows vigorously in temperate regions. *Taraxacum* multiply by three different modes of reproduction: allogamy, autogamy and apomixis. Usually sexually reproducing individuals are restricted to warmer regions than that of polyploids (van Dijk, 2003). Molecular studies have revealed high genetic diversity in *T. officinale* (van der Hulst et al., 2003).

Dandelions have long been used in herbal medicines for their choleretic, diuretic, anti-inflammatory and anti-carcinogenic activities (Yang et al., 1996; Ahmad et al., 2000; Choi et al., 2002; Yun et al., 2002; Ilu and Kitts, 2003).

Dried *Taraxacum* leaves and roots are available as herbal teas, and the powdered root is sold in capsule form. Roasted root is a substitute for coffee (Lee et al., 2004). A large number of medicinally active compounds have been isolated from *T. officinale*, including taraxacin, taraxasterol, inulin, esculin, guaianolide, desacetylmatricarin, ß- glucopyranosyl ester and sonchuside A (Williams et al., 1996; IIo et al., 1998; Zielinska and Kisiel, 2000), etc. Triterpenoids (taraxasterol and taraxerol) obtained from dandelion roots exhibit anti-tumor activity (Takasaki et al., 1999). But esculin is among the most important compounds used by pharmaceutical industry (Buszewski et al., 1993). It helps in providing elasticity and sealing of blood vessels (Pietrogrand et al., 1992). Esculin is a well known natural UV- B protective agent and one of the phytomedicines in practice for the treatment of various peripheral vascular disorders (Stanic et al., 1999). Esculin belongs to coumarins and has been extensively examined for its cytotoxic effects on tumors under in vitro and in vivo conditions (Kostova, 2005). Hence, it represents an exploitable source of anti-cancer agents. Seasonal variability in esculin content in *Aesculus hippocastanum* has been established in bark samples (Stanic et al., 1999). Since *Taraxacum* shows high genetic diversity it would be worthwhile to locate natural variants in a population and compare the yield of secondary metabolite, esculin.

*Taraxacum officinale* is a good source of esculin and produces more or comparable levels of esculin as others. The plants show ploidy variation; therefore, higher yielding genotypes need be selected. There are reports on diversity and taxonomic controversy of *Taraxacum* (Menken et al., 1995; Vavrek, 1998; Vasut, 2003), but not much work has been done on the esculin content of the plant. Williams et al. (1996) have analysed esculin in the leaves of *T. officinale*. A number of PCR-based molecular markers have been used for detecting polymorphism at DNA level. Among them, random amplification of polymorphic DNAs (RAPD) gained much popularity because of its simplicity, nonrequirement of prior information of nucleotide sequence and can be performed with a very small amount of genomic DNA. Random amplification of polymorphic DNA technique has been successfully employed for the estimation of genetic diversity; some of which include *Plantago ovate* (Singh et al., 2009), *Ricinus communis* (Gajera et al., 2010), *Jatropha curcas* (Zhang et al., 2011), *Curcuma longa* (Singh et al., 2012), *Aloe vera* (Nejatzadeh-Barandozi et al., 2012) and *Clitoria ternatea* (Yeotkar et al., 2012). Hence, molecular and chemical characterization can go hand in hand rather than in isolation because such studies would be quite helpful for conservation strategies and the selection of population containing maximum content of active compound. In the present study, we have tried to correlate morphological and genetic variability, and mode of reproduction in *T. officinale* with the yield of esculin.

## 2. Materials and methods

### 2.1. Sample collection

Two populations of *T. officinale* were collected at six-month old stage from temperate regions, one from Kashmir 'K' and the other from Garhwal 'G'. The two states constitute the northern extremity of India, both having temperate climatic conditions and abound rich in fauna. Kashmir is situated between 32.17 degree and 36.58 degree north latitude and 37.26 and 80.30 degrees east longitude with an average annual rainfall of 650.5 mm. Garhwal is located between 13 degree and 35 degree north latitude and 33 degree and 46 degree east longitude. The annual rainfall varies between 1400-2800 mm and a minimum and maximum temperature of 4 ºC and 30 ºC respectively. While in Kashmir temperature varies between – 8 ºC to 30 ºC.

### 2.2. Genetic diversity analysis

#### 2.2.1. DNA isolation

DNA isolation was done by the modified method of Doyle and Doyle (1990). 0.2 g of lyophilized leaves was pulverised in liquid nitrogen. 20 ml of CTAB (2 %) (Sigma Aldrich-Germany); (1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl purchased from Himedia-India) buffer was added and incubated at 65 ºC for 40 minutes. Equal volume of Chloroform : Isoamyl alcohol (24: 1) was added and centrifuged at 6000 rpm for 20 minutes at room temperature. Aqueous phase was collected in fresh tubes and treated with 10 µl of RNase (50 µg / ml) at 37 ºC for 30 minutes. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added again and centrifuged at 10000 rpm for 20 minutes at 25 ºC. 0.6-1.0 volume of chilled iso-propanol was mixed to the aqueous phase by inversion and kept at -20 ºC for one hour. Precipitated DNA was centrifuged at 13000 rpm at room temperature for 20 (minutes. The supernatant was discarded and the DNA pellet washed with 70% alcohol. Pellet was then dried, dissolved in sterile water and further purified with Gen Elute Plant Genomic Extraction Kit (G2N - 70) (Sigma Aldrich-Germany).

**2.2.2. RAPD analysis**

Ten randomly selected individuals of 'K', labeled as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 and ten from 'G' 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 were used for the study. The isolated DNA was quantified on 0.8 % agarose gel by comparing with 25 ng/µl uncut Lambda DNA (Banglore GeNei-India). DNA was diluted to a working concentration of 25 ng/µl. A master mix comprising of DNA (25 ng/µl), 10 X PCR Buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, Taq Polymerase (1 unit/µl) (Sigma Aldrich-Germany), 10 µM primer (Microsynth-Germany) and sterile water was assembled for multiple reactions. The assay was carried out in 15 µl reaction volume.

DNA was amplified with random decamer primers; G-01 (TGC TCT GCC C), G-02 (GGT GAC GCA G), G-03 (TGG GGG ACT C), G-04 (GGT CAC CTC A), G-06 (GAT GAC CGC C), G-07 (AAA GCT GCG G), G-08 (GTC GCC GTC A), G-09 (CCG CGT CTT G), G-10 (GGA CCT GCT G) and G-14 (CCC CGA TGG T). RAPD assay was set up by adding genomic DNA along with the master mix in PCR tubes. The cycling parameters were:

One cycle at 94 ºC for 4 min, 32 ºC for 2 min and 72 ºC for 2 min; 35 cycles at 94 ºC for 1 minute, 32 ºC for 1 min and 72 ºC for 2 min. Final extension at 72 ºC for 10 min and hold at 4 ºC. The products were size fractioned on 1.2 % agarose gel and viewed under UV light.

**2.3. Esculin quantification**

**2.3.1. Sample preparation**

Leaves of selected individuals (1 to 10 of 'K' and 11 to 20 of 'G') were harvested during August, dried at 40 ºC and powdered. 1 g of each sample was extracted in Soxhlet with 150 ml methanol. Extracted samples were concentrated to 2 ml and air dried. To check the presence of esculin in the samples TLC was run on standard silica gel 60G (mean particle size 15 µM, E. Merck, Germany) coated glass plates (20 cm X 20cm, 0.2 mm wet thickness) with 15 % acetic acid in water and chloroform in 1 : 1 ratio (Williams et al., 1996). Rf value of 0.62 was measured for standard esculin (Sigma Aldrich) and samples after one day when yellow spots developed. Samples were then completely dried in vials and weighed.

**2.3.2. HPLC analysis**

Reverse phase HPLC was performed by following modified protocol of Stanic et al. (1999) in a Waters HPLC unit. DW was purified using Milli-Q system (Millipore, 0.2 µm filter), all other chemicals used were of HPLC grade. Mobile phase consisting of 1.5 % acetic acid, methanol and acetonitrile (E. Merck, India) in 90 : 3 : 7 ratio was filtered using a vaccum pump and membrane filter (0.45 µm pore dia) and degassed in a sonicator for 15 min before use. Esculin was dissolved in methanol to give a stock solution of 5 mg/ml. Dilutions per ml of 2 mg, 1 mg, 100 µg, 75 µg, 50 µg and 25 µg were made from the stock. All standard solutions were stored at 4 °C. The mobile phase was run at a flow rate of 1.0 ml/min maintained by a Binary Pump (LC 600) Separation was performed on a Merck LiChroCART, RP-18 (125 mm x 4 mm inner dia) HPLC column packed with LiChrospher 100 beads of 5 µm dia and a functional surface of octadecylsilane (reverse phase, $C_{18}$). A diode array detector (PDA 996) was employed for UV spectrophotometric detection of the effluent from the column, set at a wavelength of 340 nm. The extracted samples were dried, weighed and dissolved in 2 ml methanol. Samples and standard dilutions were filtered via Whatman filter paper and syringe filters. 20 µl of filterate from standard dilutions and samples was injected in the HPLC column. Standard curve was plotted between concentration and peak area of the six dilutions of the standard. The retention time of the samples was compared with that of standards. Areas of the relevant peaks of each sample were interpolated with the standard curve to determine the concentration of esculin (µg/g dw). Quantification was done by calculating the peak area.

**3. Results**

**3.1. Phenotypic variations**

The two populations of *T. officinale* investigated in the present study could be distinguished from each other by a number of morphological features (Fig. 1). Individuals from 'K' have smaller leaves with deeply serrate margins in comparison to genotypes from 'G'. 'K' individuals remain compressed to the ground while 'G' has an upright rosette appearance. The flowering phenology of the plants also differ. 'K' population flowers throughout the year except July to October. The 'G' population flowers only during the months of March to May which is also

the peak flowering season for 'K'. Analysis of flower heads of 'K' and 'G' showed that 'K' florets possess weakly formed anthers in comparison to well developed anthers in 'G'.


**Fig. 1.** *Taraxacum officinale*, morphological variations. (a) Individual from 'K' population, (b) Individual from 'G' population, (c) Difference in leaf size and (d) Difference in seed ball size. 728x471mm (72 x 72 DPI).

### 3.2. Esculin content

The 20 experimental individuals could be separated into three groups on the basis of variation in their esculin content (Table 1; Fig. 2). Group A (individuals 1, 3 and 4 of 'K') with 40-45 µg/g dw, group B (11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 of 'G') containing 50-60 µg/g dw and group C (2, 5, 6, 7, 8, 9 and 10 of 'K') having highest esculin content of 445-700 µg/g dw. The highest esculin content correlates with their apomictic nature. Group A having the least amount of esculin is from 'K' population. These may be the individuals produced by cross fertilization between apomicts and diploid sexually reproducing plants.

**Table 1**
Three groups of *T. officinale* on the basis of esculin content (µg/g dw).

| Groups | Genotypes | Esculin content (µg/g dw) Mean ± S.E | Esculin content (µg/g dw) Group Mean |
|---|---|---|---|
| A | 1 | 40.278 ± 0.209 | 41.58 ± 1.36 |
| | 3 | 44.294 ± 0.798 | |
| | 4 | 40.170 ± 1.151 | |
| B | 2 | 659.055 ± 5.667 | 539.499± 39.19 |
| | 5 | 573.768 ± 3.185 | |
| | 6 | 694.537 ± 3.149 | |
| | 7 | 445.785 ± 2.129 | |
| | 8 | 453.193 ± 3.931 | |
| | 9 | 464.153 ± 1.779 | |
| | 10 | 485.963 ± 1.494 | |
| C | 11 | 55.533 ± 2.591 | 54.48 ± 1.63 |
| | 12 | 53.706 ± 3.082 | |
| | 13 | 54.46 ± 2.359 | |
| | 14 | 46.24 ± 0.831 | |
| | 15 | 60.367 ± 1.057 | |
| | 16 | 50.05 ± 0.429 | |
| | 17 | 64.615 ± 1.864 | |
| | 18 | 51.345 ± 1.702 | |
| | 19 | 51.635 ± 1.491 | |
| | 20 | 56.093 ± 1.508 | |

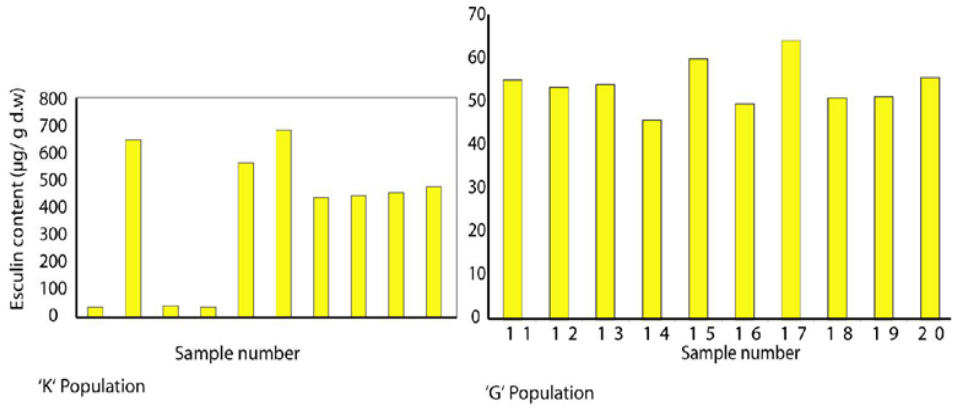


**Fig. 2.** Esculin content (*g/g dw) in Taraxacum officinale, 1-10 = 'K' population and 11-20 = 'G' population. 675x287mm (72 x 72 DPI).

### 3.3. Genetic variation

To further establish correlation between esculin content and genotype, of the seventeen random decamer primers, ten were used for their clear, unambiguous and reproducible banding pattern (Fig. 3). A total of 108 bands were produced, of which 103 were polymorphic (95.37 % polymorphism). From the genetic similarities among the genotypes and using UPGMA aggregation method a dendrogram was obtained using NTSYS-pc (version 2.11 w) (Fig. 4). Three clusters were formed. All the genotypes of 'G' were grouped together and 'K' population was divided into two. These were represented by three clusters: cluster I (genotypes 1, 3 and 4, group A); cluster II (genotypes 2, 5, 6, 7, 8, 9 and 10, group B) and cluster III (genotypes 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20, group C). Cluster I has a similarity coefficient varying from 0.63 to 1.00, cluster II from 0.98 to 1.00 and cluster III from 0.08 to 1.00. It was possible to correlate the esculin content with the three clusters of dendrogram. The presence or absence of a band can be used as a marker for the identification of a particular genotype (Table 2). The molecular markers G-$01_{737.5}$, G-$08_{1060}$, G -$08_{900,}$ G-$09_{1320}$, G-$10_{1040}$, G-$14_{1215.5}$ and G-$14_{867}$ were present in genotypes 1, 3 and 4 having lowest levels of esculin (40 - 44 µg/g dw). Similarly, markers G-$03_{550}$, G-$04_{1170}$, G-$06_{1150}$ amplified in genotypes 11 - 20 (50-65 µg/g dw of esculin), and G-$01_{1415}$, G-$08_{738}$ and G-$10_{1353}$ in genotypes 2, 5 - 10 (445-695 µg/g dw) signifying higher yield, respectively. Mostly, the molecular markers that amplified in a group were absent in the other except G-$07_{564}$, G-$09_{600}$, G-$10_{564}$, G-$14_{1078}$ and G-$14_{737}$ showing monomorphic band pattern.

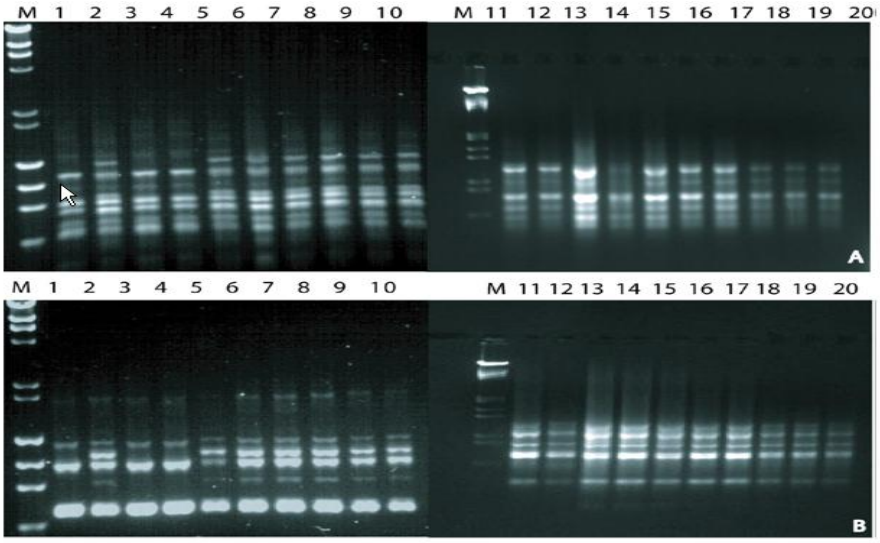


**Fig. 3.** RAPD profile of *Taraxacum officinale* 'K' and 'G', M = Marker (Mix of 7 DNA digested with Hind III and _ X 174 DNA digested with Hae III), 1-10 = genotypes of 'K' population, M* = Marker (Mix of 7 DNA digested with Hind III and Eco R1) and 11-20 = genotypes of 'G' population. (a) Primer = G-04, (b) Primer = G-07. 622x565mm (72 x 72 DPI).

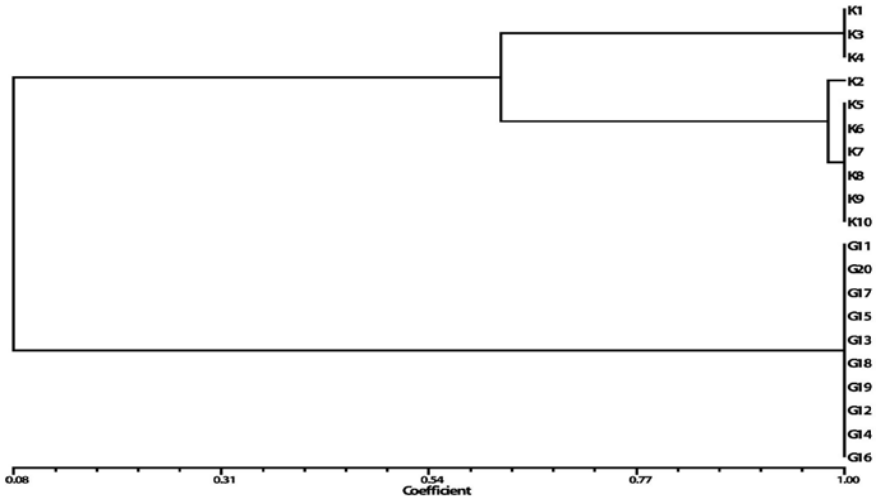


**Fig. 4.** Dendrogram constructed from binary matrix of RAPD analysed data. 215x160mm. (300x300 DPI).

**Table 2**
Markers amplified in different genotypes.

| S.No. | Genotypes | Markers |
|---|---|---|
| 1 | 1, 3, 4 | G-01$_{737.5}$, G-08$_{1060/900}$, G-09$_{1320}$, G-10$_{1040}$, G-14$_{1215.5/867}$ |
| 2 | 2, 5, 6, 7, 8, 9, 10 | G-01$_{1415/795}$, G-02$_{1200/867}$, G-04$_{1353/890}$, G-06$_{1215}$, G-07$_{1215.5/872}$, G-08$_{738}$, G-09$_{1380/1290}$, G-10$_{1353}$, G-14$_{660/603}$ |
| 3 | 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 | G-01$_{1167/1100/831/572}$, G-02$_{1584/1375/1161/947/831}$, G-03$_{1225/947/697/600/550}$, G-04$_{1170/800/682/564/500}$, G-06$_{1150/947/697.5/564/450/225}$, G-07$_{1150/947/831/760}$, G-08$_{1340/1161/947/831/504/500/450/415}$, G-09$_{947/564/500/355}$, G-10$_{825/697.5/450}$, G-14$_{564}$ |

**4. Discussion**

*Taraxacum officinale* shows high variation because of its ability to reproduce apomictically besides sexual reproduction. Sexually reproducing plants are diploids (n=16) and apomictics are polyploids, commonly triploids (n=24) (van Dijk and Bakx-Schotman, 2004). Apomictics are mostly obligate but occasionally they do reproduce sexually (Richards, 1973). Although phenomenon of geographical parthenogenesis i.e., different geographical distribution of sexuals and parthenogens is common, mixed population also occurs (van Dijk, 2003).

Collier and Rogstad (2004) have reported variation in flowering phenology involving capitulum and seed ball size of *T. officinale*. Sexual reproduction is facilitated by bee-pollination in 'G' which may ensure cross pollination between 'K' and 'G', as individuals of 'K' are not visited by bees otherwise (when only 'K' is in flowering stage). However, occasional visitors to 'K' can be seen when both populations are in the same territory, which may be responsible for cross fertilization between the two populations. Otherwise, triploids (3N) are common in *T. officinale*. Apomixis in *Taraxacum* takes place by meiotic diplospory (van Baarlen et al., 2002). Difference in flowering phenology and presence of well developed anthers in only 'G' population lead us to believe that 'G' reproduces by sexual means while population 'K' predominantly retains apomixis producing triploids and occasionally diploids (2n) and triploids (3n) and tetraploids (4n) through sexual reproduction. Menken et al. (1995) and Tas and van Dijk (1999) have reported cross fertilization between sexual diploids and apomicts.

Though studies have been carried out to characterize the genetic diversity of medicinal plants using molecular markers (Sarwat et al 2008), only few reports are available correlating the secondary metabolite content with the RAPD profile of the genotypes. Sandhu et al. (2006) generated markers specific to genotypes and tried to correlate Pilocarpine content with the genetic profile of the plant, *Pilocarpus jaborandi*. Secondary metabolite content of a plant is affected by biotic and abiotic factors (Dixon and Paiva, 1995). The variation in the concentration of esculin may be correlated to the variation in the breeding and ploidy pattern of *T. officinale.* The

biosynthetic pathways of secondary metabolites are known to have altered during the evolution process modulating the final content of the compounds (Haslam, 1986) as in caffeine, the content varies even within the cultivars of the same species (Mazzafera et al., 1994). In addition to DNA markers, phytochemical markers high performance liquid chromatography (HPLC) play a role to portray genetic variability and authentication of medicinally important plants (Li et al., 2008).

The differences and the similarities of the 20 *T. officinale* genotypes suggest a genetic structure with intraspecific and intrapopulation diversity. The differences between the two populations of *T. officinale* (interpopulation) can be either because of the different geographical location or because of the difference in their breeding patterns. Intrapopulation differences i.e., genotypes 1, 3 and 4 forming one group and 2, 5 – 10 another may be attributed to the facultative apomictic nature of *T. officinale* due to which the individuals are capable of reproducing by sexual reproduction also (Richards, 1973).

With the data generated in this work, it is implicit that individuals can be selected on the basis of their genetic profile for higher yield of the secondary metabolite and propagated on a large scale for commercial exploitation. Our studies would also help conservation strategies aimed at this plant and others.

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