Difference in in vitro response and esculin content in two populations of *Taraxacum officinale* Weber

Sumiya Jamshieed · Sandip Das · M. P. Sharma ·
P. S. Srivastava

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Abstract In vitro micropropagation has been achieved in medicinally important plant, *Taraxacum officinale* collected from two different regions, Kashmir (J & K) and Garhwal (Uttarakhand). Leaf segments inoculated on MS supplemented with different combinations of Indole-3-acetic acid (IAA) and Benzyladenine (BA) produced indirect regeneration. For root induction MS fortified with Indole-3-butyric acid (IBA) was used. *Taraxacum officinale* collected from Garhwal responded two weeks earlier and showed shoot regeneration whereas in Kashmir population only callus proliferation occurred. Esculin content was also higher in the samples from Garhwal. The content was affected by both, the hormone concentration as well as age of the cultures. RAPD of the in vitro raised regenerants confirmed genetic stability.

Keywords *Taraxacum officinale* · Different populations · Micropropagation · RAPD · Esculin

Abbreviations

IAA Indole-3-acetic acid
BA Benzyladenine
IBA Indole-3-butyric acid

RAPD Randomly amplified polymorphic DNA analysis
'K' Kashmir
'G' Garhwal

Introduction

Taraxacum officinale of Asteraceae, a traditional herbal medicine commonly known as Dandelion is reported to cure a number of ailments. Whole plant extracts possess choleric, diuretic, anti-inflammatory and -carcinogenic activities (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 tea. The powdered root is also sold in capsule form. Roasted root is substitute for coffee (Lee et al. 2004). Though the plant shows extensive geographical distribution, it grows vigorously in temperate regions. Taraxacin, taraxasterol, inulin, esculin, guaianolide, desacetylmaticarin, β -glucopyranosyl ester and sonchuside A are among the medicinally active compounds (Ilo et al. 1998). Esculin is one of the most important components used in pharmaceutical industry (Buszewski et al. 1993). It is an elasticity giving and sealing material for blood vascular system (Pietrogrande et al. 1992). Esculin is also 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). Triterpenoids (taraxasterol and taraxerol) obtained from roots exhibit anti-tumor activity (Takasaki et al. 1999).

Micropropagation of *T. officinale* may help alleviate pressure on natural resources as well as in the enhanced yield of secondary metabolites. An efficient micropropaga-

S. Jamshieed · M. P. Sharma
Department of Botany, Faculty of Science, Jamia Hamdard,
New Delhi, 110062, India

S. Jamshieed · S. Das · P. S. Srivastava (✉)
Department of Biotechnology, Faculty of Science,
Jamia Hamdard,
New Delhi, 110062, India
e-mail: pss410@rediffmail.com

tion protocol will facilitate exploitation of higher yielding individuals for commercialization and also genetic transformation for enhanced yield in individuals with low content.

Material and methods

Plant material, sterilization and culture medium

Populations of *T. officinale* were collected from Kashmir (J & K, India) and Garhwal (Uttarakhand, India) and maintained in the Herbal Garden at Hamdard University. Two individuals from each population were selected as mother plants and designated 'K' (Kashmir) and 'G' (Garhwal) for in vitro propagation. For establishing micropropagation protocol, leaf explants were inoculated on MS medium (Murashige and Skoog 1962) supplemented with different concentrations of IAA and BA. The explants were washed under running tap water for ten min and treated with 0.25% cetrimide for seven min. The leaf segments were surface sterilized with 0.2% streptomycin sulfate and 0.5% bavistin in 1 : 1 ratio for 25 min. The explants were then washed with 0.1% mercuric chloride and rinsed with 70% alcohol for three and one min, respectively. The sterilized leaf segments were thoroughly rinsed with sterile double distilled water (DDW) before inoculation on MS medium solidified with 0.63% agar and pH adjusted to 5.69. After pouring in culture vials the medium was autoclaved at 121°C (1.06 kg cm²) for 20 min. Regenerated shoots were excised and transferred to MS medium containing IBA for root induction. All the cultures were maintained in a culture room at 23±2°C, 55% relative humidity and 8 h photoperiod (15 µmolm⁻²s⁻¹). Morphogenic response was monitored periodically.

For hardening, the plantlets were transferred to MS medium lacking any hormones for 2 weeks. Hardening was achieved sequentially by transferring the plantlets to 1/4th strength of MS and then to the medium lacking organic nutrients. The plantlets were transplanted to plastic pots containing autoclaved soil and soilrite (1: 1) and maintained in the culture room. The plants were covered with self-sealing polyethylene bags in which holes were made after

1 week. The bags were removed subsequently after 15 days as the plantlets grew further. Finally, they were transplanted to 6" earthen pots filled with garden soil.

Analysis of clonal fidelity of regenerants

RAPD was used to establish genetic stability of the regenerants. DNA was isolated from the mother plants and ten randomly selected regenerants of each population by the modified method of Doyle and Doyle (1990). 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 MgCl₂, 10 mM dNTP mix, 10 µM primer, Taq Polymerase (1 unit/µl) 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°C for 4 min, 32°C for 2 min and 72°C for 2 min; 35 cycles at 94°C for 1 minute, 32°C for 1 min and 72°C for 2 min. Final extension at 72°C for 10 min and hold at 4°C. The products were size fractionated on 1.2% agarose gel and viewed under UV light.

Esculin analysis

Leaf samples were harvested in the months of December, March and July. This coincided with the pre-flowering, flowering and post-flowering stage of plants of Garhwal ('G') region; 'K' flowers throughout the year except July to October. 2- and 6-month-old in vitro cultures of both the populations were harvested from the following media: MS (mg/l) + IAA (0.5) + BA (1.0), MS + IAA (0.5) + BA (2.0), MS + IAA (1.0) + BA (1.0), MS + IBA (1.0) + BA (1.0) and MS + IBA (1.0) + BA (2.0). Samples were dried at 40–50°C and powdered. The extraction procedure was optimized by performing TLC of the extracts. Optimal

Fig. 1 *Taraxacum officinale*. Medium: MS (in mg/l) + IAA (1.0) + BA (1.0); Age: 10 weeks; (a) Callusing and shoot multiplication in 'K', (b) Shoot multiplication in 'G'

Table 1 Percent callusing and regeneration of leaf explant in *Taraxacum officinale* ‘K’ and ‘G’ after 4 and 8 weeks

Medium	Percent callusing		Percent regeneration		Percent callusing		Percent regeneration	
	After 4 weeks				After 8 weeks			
	‘K’ Mean $\pm$ S.E	‘G’ Mean $\pm$ S.E	‘K’ Mean $\pm$ S.E	‘G’ Mean $\pm$ S.E	‘K’ Mean $\pm$ S.E	‘G’ Mean $\pm$ S.E	‘K’ Mean $\pm$ S.E	‘G’ Mean $\pm$ S.E
MS (in mg/l) IAA + BA								
0.5+0.5	47.27 $\pm$ 4.29	55.15 $\pm$ 0.21	39.33 $\pm$ 1.76	47.27 $\pm$ 4.29	75.10 $\pm$ 0.59	47.61 $\pm$ 0.36	45.93 $\pm$ 3.20	78.71 $\pm$ 0.15
0.5+1.0	60.36 $\pm$ 0.48	63.59 $\pm$ 0.23	58.49 $\pm$ 2.31	62.98 $\pm$ 1.57	80.27 $\pm$ 0.41	61.51 $\pm$ 0.29	74.93 $\pm$ 4.75	83.03 $\pm$ 0.32
0.5+2.0	72.09 $\pm$ 1.67	71.23 $\pm$ 0.72	65.19 $\pm$ 2.17	69.58 $\pm$ 1.68	91.35 $\pm$ 0.94	72.14 $\pm$ 0.20	83.84 $\pm$ 0.71	97.51 $\pm$ 0.30
1.0+0.5	54.82 $\pm$ 2.40	61.30 $\pm$ 0.22	22.62 $\pm$ 1.53	29.71 $\pm$ 1.13	76.67 $\pm$ 1.97	54.51 $\pm$ 0.29	41.27 $\pm$ 0.41	96.99 $\pm$ 0.29
1.0+1.0	95.63 $\pm$ 2.82	99.65 $\pm$ 0.43	82.51 $\pm$ 2.64	89.61 $\pm$ 0.39	99.86 $\pm$ 0.14	95.80 $\pm$ 0.14	98.30 $\pm$ 0.88	99.98 $\pm$ 0.01
1.0+2.0	98.12 $\pm$ 1.15	99.97 $\pm$ 0.02	42.17 $\pm$ 1.92	46.52 $\pm$ 0.59	98.98 $\pm$ 0.99	98.90 $\pm$ 0.56	12.67 $\pm$ 0.10	100 $\pm$ 0.00
2.0+0.5	29.88 $\pm$ 0.76	37.97 $\pm$ 0.34	4.72 $\pm$ 0.83	15.93 $\pm$ 0.26	54.96 $\pm$ 2.14	30.65 $\pm$ 0.26	64.04 $\pm$ 0.85	68.18 $\pm$ 0.34
2.0+1.0	95.55 $\pm$ 1.27	98.99 $\pm$ 0.01	31.35 $\pm$ 0.35	41.23 $\pm$ 2.06	99.73 $\pm$ 0.27	97.46 $\pm$ 0.33	9.36 $\pm$ 0.34	99.66 $\pm$ 0.31
2.0+2.0	28.88 $\pm$ 1.39	41.51 $\pm$ 0.29	44.07 $\pm$ 0.25	10.75 $\pm$ 0.14	51.44 $\pm$ 0.62	29.56 $\pm$ 0.33	79.75 $\pm$ 0.65	61.07 $\pm$ 0.58

extraction was performed by heating 1 g of each sample (stapled in Whatman filter paper) dipped in methanol (NG) in a Soxhlet. Tissue samples, concentrated to 1 ml and standard esculin purchased from Sigma Aldrich Chemical Co. were then dissolved in methanol and applied on standard silica gel 60G (mean particle size, 15 μ m, E.M, Germany) coated glass plates (20 cm $\times$ 20 cm, 0.2 mm- wet thickness) dipped in 15% acetic acid : chloroform (1:1). Appearance of yellow spot and matching of R_f values of standard and samples confirmed the presence of esculin. Reverse phase HPLC was performed following the modified protocol of Stanic et al. (1999) in a Waters HPLC unit. Distilled water was purified using Milli-Q system (Millipore, 0.2 μ m filter), all other chemicals used were of HPLC grade. Mobile phase consisting of 1.5% acetic acid, methanol and acetonitrile (E. Merck, India Limited) in 90:3:7 ratio was filtered using a vacuum pump and membrane filter (0.45 μ m 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 μ g, 75 μ g, 50 μ g and 25 μ g were made from this stock. The mobile phase was run at a flow rate of 1.0 ml/min maintained by a Binary Pump (LC 250). Separation was performed on a Merck LiChroCART, RP-18

(125 mm $\times$ 4 mm inner dia) HPLC column. A diode array detector was employed for UV spectrophotometric detection of the effluent from the column, set at a wavelength of 340 nm. Standard curve was plotted between concentration and peak area of the six dilutions of the standard. Areas of the relevant peaks of each sample were interpolated with the standard curve to determine the concentration of esculin (μ g/g dw).

Results

Micropropagation protocol

A reproducible micropropagation protocol could be established from leaf explants of two populations, ‘K’ and ‘G’. Regeneration was indirect. Callus initiated after two and four weeks in *T. officinale* ‘K’ and ‘G’, respectively. MS supplemented with (in mg/l) IAA (1.0) and BA (2.0) proved to be the best medium for callus proliferation, while MS (mg/l) + IAA (1.0) + BA (1.0) induced best regeneration. The response initiated 2 weeks earlier in population ‘G’ than in ‘K’. ‘K’ showed higher amount of callusing while ‘G’ showed regeneration (Fig. 1). The

Fig. 2 *Taraxacum officinale*. Medium: MS (in mg/l) + IAA (0.5) + BA (2.0) + 2, 4-D (0.5), (a) 10-wk-old callus of ‘K’ population, (b) Regeneration in 6-wk-old culture of ‘G’ population

Fig. 3 RAPD fingerprint of in vitro regenerants of populations 'K' and 'G' of *T. officinale* (primer G-01, Lanes: M = Marker (λ DNA digested with *Hind* III and *Eco* RI); 'K' = Mother plant, a–j = Regenerants, G = Mother plant, k–t = Regenerants

percent shoot regeneration was 82.51 and 89.61 in 'K' and 'G', respectively (Table 1). Highest average callusing was 99.97 in 'K' and 98.12% in 'G'. For root induction, the shoots were transferred to MS supplemented with (in mg/l) IBA (0.25 to 2.0). Percent rooting and length of roots was optimal on IBA (1.0 mg/l).

For induction of embryogenesis 3-wk-old callus grown on MS (in mg/l) + IAA (1.0) + BA (0.5) was transferred to liquid MS medium containing (in mg/l) BA (0.5) and 2,4–D (5.0). The cultures were placed on a horizontal shaker at 150 rpm for 1 week at $25 \pm 2^\circ\text{C}$. The 'G' callus upon transfer to MS + 2, 4-D (0.5 mg/l) regenerated shoots after 4 weeks that multiplied rapidly after 6 weeks. Only profuse white callus, however, generated from 'K' after 4 weeks, but shoots emerged after 10 weeks (Fig. 2).

RAPD analysis confirmed the fidelity of the regenerants to their respective mother plants as 100% monomorphism was observed in both the populations. Figure 3 clearly indicates the monomorphic band pattern of the regenerants with their respective mother plants. Individuals on the left represent the mother plant, 'K' and the regenerants (a–j). On the right are mother plant, 'G' and the regenerants (k–t). The clonal fidelity ensured that there are no variations in

the genetic make up of the in vitro raised plants of *T. officinale*.

Esculin content

For in vivo analysis, samples were harvested from in vivo plants of the two populations, 'K' and 'G'. In both populations the content (in mg/g dw) was highest in December samples (1.52 in 'K', 7.21 in 'G') followed by March (0.09 in 'K', 0.22 in 'G') and the least in July (0.05 in 'K', 0.09 in 'G'). At all the three stages, 'G' population had higher content of esculin.

Under in vitro conditions also esculin content was higher in 'G' and it increased with age of the cultures (Table 2). Esculin, accumulation (21.44 mg/g dw in 'G' and 16.12 mg/g dw in 'K'), was the highest in 6-month-old cultures grown on MS (in mg/l) + IAA (0.5) + BA (1.0). Increased concentration of BA (2 mg/l) decreased the content. On MS (in mg/l) + IAA (1.0) + BA (1.0) decrease was more severe. When IAA (1.0 mg/l) was replaced with IBA (1.0 mg/l) and BA (1.0 or 2.0 mg/l), similar trend was observed up to 2-months. In 6-month-old cultures esculin content was higher at BA (2.0 mg/l). Thus, under in vitro

Table 2 Esculin content (mg/g dw) of 2 and 6-month-old in vitro raised regenerants of *T. officinale* 'K' and 'G'

Medium	2 month old		6 month old	
	'K' Mean $\pm$ S.E	'G' Mean $\pm$ S.E	'K' Mean $\pm$ S.E	'G' Mean $\pm$ S.E
MS (in mg/l) + IAA + BA				
0.5+1.0	0.22 $\pm$ 0.01	0.27 $\pm$ 0.003	16.12 $\pm$ 0.10	21.44 $\pm$ 0.29
0.5+2.0	0.015 $\pm$ 0.0	0.048 $\pm$ 0.0	8.56 $\pm$ 0.04	10.91 $\pm$ 0.03
1.0+1.0	0.006 $\pm$ 0.0	0.014 $\pm$ 0.0	0.974 $\pm$ 0.0	1.13 $\pm$ 0.0
MS (in mg/l) + IBA + BA				
1.0+1.0	3.81 $\pm$ 0.06	5.32 $\pm$ 0.05	5.49 $\pm$ 0.03	8.21 $\pm$ 0.04
1.0+2.0	0.95 $\pm$ 0.0	1.40 $\pm$ 0.06	7.43 $\pm$ 0.08	8.56 $\pm$ 0.04

conditions both, the hormone concentration, as well as age of the cultures affected the secondary metabolite production; in our studies, esculin accumulation increased with increasing age of the cultures. It is the first report quantifying esculin content at different stages of plant growth under in vitro and in vivo conditions in *T. officinale*.

Discussion

Regeneration in *Taraxacum mongolicum* has been reported by Song et al. (1999) on MS medium supplemented with (in mg/l) IAA (0.2) and BA (1.0). Lee et al. (2004) obtained 100% shoot formation when transformed and non-transformed root explants of *T. platycarpum* were cultured on MS (in mg/l) + BA (1.0). Bae et al. (2005) observed shoot regeneration from leaf explants of *T. platycarpum* on MS (in mg/l) + NAA (0.1) + BA (1.0). In our study, shoot induction occurred from callus on MS medium enriched with BA. Multiple shoot induction has been recorded by Chen et al. (2005) in *T. officinale* on MS (in mg/l) + IAA (0.2) + TDZ (1.0). They obtained regeneration from fragile callus cultured on MS + BA (1.0 mg/l). Callusing and shoot regeneration in *T. officinale*, however, varied with the concentration of IAA and BA. The response is also dependent upon geographical distribution of plants. In the present study although MS (mg/l) + IAA (1.0) + BA (1.0) induced best regeneration, the response initiated 2 weeks earlier in population 'G' than in 'K'. 'K' showed higher amount of callusing while 'G' showed regeneration. In general, auxin in combination with cytokinin is required to achieve shoot initiation and elongation (Chen et al. 2003). In our experiments, lower levels of IAA and higher BA concentration always promoted more shoot development.

Shoots of *T. officinale* when transferred to MS (in mg/l) + IBA (0.25 to 2.0) + BA produced roots in 4% cultures. At IBA (1.0 mg/l) and BA (1.0 mg/l) percent rooting and length of roots was optimal. However, when BA was withdrawn from the medium, roots appeared after only 2 weeks on MS containing IBA. Kaur and Jain (2005) reported similar results in *Abrus precatorius* indicating BA concentration-dependent inhibition of root differentiation in cultures. Likewise, in *Phaseolus vulgaris* L. IBA + BA combination reduced the number and length of roots in cultures, while IBA alone resulted in maximum rooting (Drewes and Staden 1989). Khawar and Ozcan (2002) used 0.25, 0.5, 1.0 and 2.0 mg/l of IBA in lentil. IBA (0.25 mg/l) induced best results after 4 weeks. Only the callus of *T. officinale* 'K' could be maintained in non-regenerated state for a longer duration on 2,4-D supplemented medium. The result also indicates the difference in response of the two populations to 2,4-D.

RAPD analysis confirmed the genetic fidelity of the in vitro raised regenerants with the mother plants of 'K' and 'G'. The genetic stability of in vitro raised multiple shoots of *T. officinale* was also confirmed by Chen et al. (2005) through RAPD. RAPD has been used to determine the genetic stability of regenerated plants in a number of taxa (Rani and Raina 2000; Sharma et al. 2004; Narula et al. 2007; Mandal et al. 2008; Mandal et al. 2009).

Secondary metabolite content under in vitro and in vivo conditions

Rehman et al. (2003) have reported variation in esculin content during juvenile, rosette, bolting and flowering stages in *Cichorium intybus*. Likewise, esculin content in bark samples of *Aesculus hippocastanum* is age and season dependent (Stanic et al. 1999). According to Dixon and Paiva (1995) the concentration of secondary metabolites in plants is strongly affected by biotic and abiotic factors. In *Taraxacum officinale* also (our studies) there was difference in esculin yield of the two populations. Under in vitro conditions esculin content was higher than in the samples of in vivo mother plants. The content was affected by both, age as well as medium composition. Indeed in *Cichorium intybus*, according to Rehman et al. (2003) esculin content increased under in vitro conditions with increasing age and differentiation. An enhancement in shikonin content was observed in cultured *Onosma paniculatum* cells when brassinolide, IAA and BA were added (Yang et al. 1999). Pectin administered to in vitro cultures of *Uncaria tomentosa* enhanced the production of triterpene acids (Ursolic and oleanic acid) without affecting growth and sterol accumulation (Flores-Sanchez et al. 2002). These results and that of Kumari et al. (2007) corroborate our findings in *Taraxacum*.

The micropropagation protocol will not only help produce large number of plants of *T. officinale* but also ensure expected quantity of esculin. Since the plant shows high diversity owing to its mode of reproduction, higher yielding individuals can be selected and conserved in vitro.

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