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ORIGINAL ARTICLES

In vitro Clonal Propagation, Caffeic Acid Production and RAPD Analysis of Some Varieties of *Echinacea Purpurea* Plant.

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ABSTRACT

An efficient multiple shoot has been developed for three varieties of *Echinacea purpurea* using shoot tip explant. Multiple shoots originated when shoot tip cultured on MS-medium supplemented with 0.5 mg/L BA and different concentrations of NAA. Optimum shoot multiplication was observed on MS-medium containing 0.5 mg/L BA for the three varieties. The best results for rooting experiment were obtained with MS-basal medium for Rubinstern variety, however in Double decker and Baby swan white varieties, the addition of IBA to the medium improve rooting. Analysis of caffeic acid derivatives in the dried flowering tops of *in vivo Echinacea* varieties and produced *in vitro* plants *Echinacea* varieties indicated that the total caffeic acid derivatives were higher in the dried flowering tops of *in vivo Echinacea* varieties in comparison with produced *in vitro* plants *Echinacea* varieties. Whereas HPLC analysis of caftaric acid and chicoric acids which the most important compounds in the caffeic acid derivatives showed that the higher productivity of caftaric and chicoric acids were recorded in the *in vitro* plants *Echinacea* varieties compared to the dried flowering tops of *in vivo Echinacea* varieties. The Data of RAPD–based DNA fingerprint analysis revealed that the highest percentage of polymorphism (22.2%) was recorded with B4 primer.

Key words: *Echinacea*, Multiplication, Caffeic acid derivatives, RAPD, Medicinal plants.

Introduction

Echinacea purpurea L. (EP) is one of the most important medical herbs and a kind of *Asteraceae* natively perennial grown in North America. It is used pharmacologically and for aesthetic enjoyment. In 2005, *Echinacea* products ranked among the top botanical supplements sold in the United States. Its root and subterranean stem were used by North America in early period to treat trauma and alleviate symptoms of infection and inflammation. The *E. purpurea* have been proven to show good immunoregulation, antiinflammation and antioxidant capacity (Lee *et al*, 2009 and Zhai *et al*, 2007) with no hypersensitivity or other side effects during clinical trial stages (Saunders *et al*, 2007). Varieties of *E. purpurea* all contain similar main ingredients including caffeic acid derivatives, alkamides, flavonoids, essential oils, and polyacetylenes, and medical activities of which are yet to be exactly identified with corresponding diseases (Thygesen *et al*, 2007). However, caffeic acid derivatives and alkamides have been proven to be active ingredients with immunoregulation effects (Matthias *et al*, 2008). Moreover, synergistic antioxidative effect of caffeic acid derivatives, alkamides and polysaccharide fractions was demonstrated by measuring their inhibition of *in vitro* Cu(II)-catalyzed oxidation of human low-density lipoprotein (LDL) (Brown *et al*, 2005).

In vitro tissue culture protocol, was tested for propagation of *Echinacea purpurea*, *E. pallida* and *E. angustifolia* in order to obtain biomass for the production of cichoric acid (Butiue-Keul *et al*, 2012).

Micropropagation offers an approach that is capable of producing large numbers of genetically similar, disease free plants in a short period of time and limited space. Lata *et al*, (2004) reported that *Echinacea* species have been regenerated from a rang of tissue types varying from *in vitro* seedlings to nature field-grown plants, with a number of studies having described the biomass production by *in vitro* culture. Coker and Camper (2000) used MS medium with NAA and kinetin to induce shoots from sterile seedlings of *E. purpurea*. Furthermore, plantlets of *Echinacea purpurea* were rooted on MS medium alone or in combination with different concentration of IBA. Also, Koroch *et al,* (2002) reported that high rooting and survival percentage were achieved using MS medium without plant growth regulators. The use of Random Amplified Polymorphic DNA (RAPD) for identification of cultivars through DNA profiling is the current method of choice in measuring genetic variation within germplasm collections (Williams *et al,* 1990; Trujillo *et al,* 1995; Paull *et al*, 1998 and

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Hernendez *et al*, 2001). Due to technical simplicity and speed, RAPD methodology was used for diversity analyses in several crops (Demek *et al*, 1996). PCR-based RAPD markers are dominant markers that are extensively used in genetic mapping (Chalmers *et al*, 2001) and identification of genetic polymorphisms (Bai *et al*, 2003 and Sun *et al*, 2003). Therefore, the current study was achieved on three varieties of *Echinacea purpurea* (Rubinstern, Double decker and Baby swan white) to investigate the effect of various media ingredients on shoots proliferation and roots formation *in vitro*. Also, the active components in the produced *in vitro* plants were determined compared with the *in vivo* flowering tops. In addition, the *in vitro* multiplication randomly amplified polymorphic DNA (RAPD) technique was applied to detect the genetic variation between *in vitro* and *in vivo* plants.

Materials and Methods

Plant Material:

Seeds of the three varieties of *Echinacea purpurea* (Rubinstern, double decker and baby sawn white) were kindly supplied through SEKEM Company, Egypt. Seeds were sterilized with 70% of commercial solution Clorox 20 min, rinsed thoroughly with sterile distilled water seven times and germinated aseptically on basal medium of Murashige and Skoog, (1962), (MS medium). The medium was containing 3% sucrose and solidified using 0.7% agar. The pH of the medium was adjusted to 5.8 and after one-month the seedlings reached about 1-3 cm in height and were used as starting plant material.

In Vitro Propagation of Echinacea Varieties:

a. Initiation and Multiple Shoot Production:

When the seedling reached 30 days old , the shoot tips (0.5-1.0 cm) were cultured in MS-basal-medium or MS-medium supplemented with BA (0.5 mg/L) alone or in combination with different concentrations of NAA (0.0,0.005, 0.01, 0.02 mg/L). The cultures were maintained at 26±1ºC for 16 h photoperiod.

b. Rooting Experiment:

The shoots which formed in the previous experiment were transferred into Ms-medium supplemented with different concentrations of IBA (0.0, 1.0, 3.0 mg/L) to form roots. The cultures were maintained at 26±1ºC for 16 h photoperiod.

Total Caffeic Acid Derivatives Determination:

The plants which produced from the previous experiment (*in vitro* plants three months old) were taken to determine the total caffeic acid derivatives and compared with the dried flowering tops of *in vivo Echinacea* varieties according to Bauer and Wagner (1988). The absorbance was read at 330nm against chicoric acid which was used for standard curve preparation. Pure chicoric acid was kindly supplied through SEKEM Co. The standard curve of chicoric acid was prepared using different concentrations prepared in 80% methanol.

HPLC Analysis of Caftaric and Cichoric Acids:

HPLC analysis of caftaric acid and chicoric acid was performed for *in vivo Echinacea* flowering tops and *in vitro* plants of *Echinacea purpurea* varieties according to Bauer and Wagner (1988). Chlorogenic acid is used as external standard with response correction factor. Stock solution of chlorogenic acid prepared in 20% methanol. Five grams of the dried sample were extracted with 70% aqueous ethanol (5ml) for 24 h. Samples were filtered and remacerated with 2.5 ml aqueous ethanol for 24 h, then filtered and collected together. The extracts were concentrated to contain 50% solid content. Volume of each sample was adjusted to 1ml with 20% aqueous methanol then filtered using 0.45µm membrane filter and to be ready for injection. Caffeic acid fractions were analyzed using HPLC with Hypersil Rp C18 (250×4 mm) column. The mobile phase was (A) 0.1% Orthophosphoric acid in water and (B) Acetonitril. Flow rate was 1.5 ml/min and injection volume was 20 µl. Caffeic acid derivatives were detected at 330nm.

Calculation:

The percentage of chicoric acid was calculated using the following equation:

$$\frac{A_3 \times C_2 \times 100 \times 0.695}{A_2 \times C_1}$$

The percentage of caftaric acid was calculated using the following equation:

$$\frac{A_1 \times C_2 \times 100 \times 0.881}{A_2 \times C_1}$$

Where,

A_1= peak area due to caftaric acid in the chromatogram obtained with test solution
A_2= peak area due to chlorogenic acid in the chromatogram obtained with the reference solution
A_3= peak area due to chicoric acid in the chromatogram obtained with test solution.
C_1= concentration of the tested solution in mg/ ml
C_2= concentration of chlorogenic acid in reference solution in mg/ml 0.695= peak correlation factor based upon the liquid chromatography response observed 0.881=peak correlation factor between caftaric acid and chlorogenic acid.

RAPD Analysis:

The *in vitro* and *in vivo* plants were also characterized by using RAPD technique as the following steps:

a- Isolation of DNA:

DNA was isolated by the CTAB (cetyl trimethyl ammonium bromide) method according to Doyle and Doyle (1990).

b- Polymerase Chain Reaction (PCR):

Sex different synthetic random primers (Promega Madisonwi USA) which shown in Table (1) were selected and used in this experiment. MasterMix was purchased from Applied Biotechnology Co. Amplification reactions were performed as described by Williams *et al.* (1990) with slight modifications. The reaction volume was 25 µl and the PCR reaction was run on Hybaid thermal Cycle. Amplification conditions include a preliminary 4 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 32°C for 30 sec and extension at 72°C for 1 min. The amplification was terminated with incubation at 72°C for 7 min and then held at 4°C until the reaction tubes were removed. The PCR products were separated on 2 % agarose gel in 1 x TBE buffer. The gel was stained with 1% ethidium bromide then visualized and photographed under a UV transilluminator.

Table 1: The random primers sequences used for RAPD analysis.

No.	Name	RAPD Primers Sequences 5'--------- 3'
1	B1	TCT GCG GTA GTT CCA GT
2	B2	CTT CGG CAG CAT CTC TTC AT
3	B3	CAG TGT GGA AGC CGA TTA TG
4	B4	ATG TGT TGT CTG GCT TGG TA
5	B5	TGG TCA GTG A
6	B6	TCA CGA TGC A

Results and Discussion

1- In vitro Propagation of Echinacea Varieties:

1-A- Initiation and Multiple Shoot Production:

Table (2) and Fig. (1) show the initiation and multiplication shoots of three *Echinacea purpurea* varieties from shoot tip after four weeks. Results indicate that BA singly or combination with NAA induced shoots in three varieties of *Echinacea purpurea*. In the Rubinstern *Echinacea* variety the medium which contain 0.5 mg/L BA improved shoot length (3.3±0.44 cm) and gave the highest number of shoots/ explant (4.3±0.88) compared with the others media. Whereas the number of leaves was improved with different concentrations of NAA addition, and the highest number of this parameter (20.0±0.0) was achieved with the medium which contain 0.5 mg/L BA+0.01 mg/L NAA. The same results were obtained with Double decker *Echinacea* variety that the medium which contains 0.5 mg/l BA improved shoot length (3.5±0.29 cm) and number of shoot/ explant

(4.0±0.88). However the medium which contains 0.5 mg/l BA+0.01 mg/L NAA gave the best results for the number of leaves parameter. While in Baby swan white *Echinacea* variety the medium which contain 0.5 mg/L BA improved only the number of shoot/explant parameter, whereas shoot length and number of leaves parameters of this variety enhanced with the medium which contain 0.5 mg/l BA+ 0.005 mg/l NAA.

The effect of BA on the establishment of shoot multiplication system of *E. pallida* and *E. purpurea* was studied by Lakshmanan *et al*, (2002) who found that BA proved to be highly effective in inducing axillary bud proliferation in *Echinacea*, shoot production increased almost three times with 2µM BA. Kim *et al*, (2010) studied the effect of different concentrations of cytokinins (BAP and kinetin) on the efficiency of shoot organogenesis in *E. angustifolia* from excised stem explants. They found that shoot development did not occur in the absence of exogenous BAP and kinetin. The treatment with BAP significantly promoted shoot regeneration from stem explants of *E. angustifolia* but kinetin was much lower than BAP. Also the authors studied the effects of different auxins on shoot regeneration and growth and found that all tested auxin treatments in basal medium marginally increased the shoot regeneration and growth rates of *E. angustifolia* stem culture.

Table 2: Multiplication of the *Echinacea Purpurea* Varieties Shoots from Shoot Tip after Four Weeks of Cultivation on MS-medium Supplemented with Different Concentrations of BA and NAA.

Varieties	BA (mg/L)	NAA (mg/L)	Shoot Length (cm)	No. of Leaves/explant	No. of Shoots/explant
Rubinstern	0.5	0.000	3.3±0.44	12.7±2.3	4.3±0.88
	0.5	0.005	2.3±0.33	14.3±5.4	2.3±0.33
	0.5	0.010	2.3±0.25	20.0±0.0	2.3±0.33
	0.5	0.020	1.7±0.17	17.3±2.7	1.3±0.33
Double decker	0.5	0.000	3.5±0.29	13.0±3.5	4.0±0.88
	0.5	0.005	2.8±0.17	11.3±2.4	3.0±1.02
	0.5	0.010	3.0±0.29	13.3±1.3	4.0±0.0
	0.5	0.020	1.5±0.00	11.3±2.4	3.3±0.3
Baby swan white	0.5	0.000	2.3±0.17	12.0±1.51	4.3±0.33
	0.5	0.005	3.5±0.50	15.0±0.58	2.3±0.33
	0.5	0.010	2.5±0.86	14.0±5.03	2.0±0.57
	0.5	0.020	1.8±0.75	6.5±1.5	1.0±0.0

1-B- Rooting Experiment:

Multiple shoots which produced in the pervious experiment on MS-medium with 0.5 mg/l BA were taken individually in the media contain different concentrations of IBA to induce roots. The effect of IBA on root formation after four weeks are presented in Table (3). Data reveal that in Rubinstern *Echinacea* variety the MS-basal medium without IBA addition induced 100% rooting, the number of roots/explant (4.0±0.91) and root length (4.2±0.52) which the highest compared with the others media. The addition of IBA at 1mg/l decreased the number of roots/explant and root length. When IBA was increased to 3 mg/l, the root formation was inhibited. From these observations we can conclude that for Rubinstern *Echinacea* variety the basal medium proved the best medium of rooting. Whereas for Double decker *Echinacea* variety, the addition of 1mg/l IBA enhanced % rooting (100 %), also increased the number of roots/ explant (5.3±0.25), but decreased root length (1.6±0.24). In Baby swan white *Echinacea* variety, the addition of 3 mg/l IBA improved the root length, and induced 100% rooting. From these results it can be concluded that the addition of IBA in the culture media for rooting is not preferred for Rubinstern variety, whereas this addition was effective with Double decker and Baby swan white varieties. Our data are in agreement with Harbage (2001) who studied the effect of auxin addition on the rooting of *Echinacea pallida*, *E. angustifolia* and *E. purpurea* shoots and reported that auxin was not necessary for root induction and did not significantly affect the number of roots produced. On the other hand, Mechanda *et al*. (2003) found that the addition of 1mg IBA inducing rooting (100%) of *E. purpurea* which agreed with Koroch *et al*. (2002) who found that the highest number of roots per shoot of *E. purpurea* was induced with the addition of IBA at concentrations of 2.46 and 4.9µM to the culture medium.

Table 3: Rooting of *Echinacea purpurea* varieties after four weeks on MS-medium supplemented with different concentrations of IBA.

Varieties	IBA (mg/L)	% of Rooting	No. of Root/explant	Root Length (cm)
Rubinstern	0	100	4.0±0.91	4.2±0.52
	1	100	3.8±1.03	0.63±0.13
	3	0	0.0±0.00	0.0±0.00
Double decker	0	88	2.0±0.00	4.0±0.29
	1	100	5.3±0.25	1.6±0.24
	3	92	2.8±0.75	1.5±0.25
Baby swan white	0	100	3.5±0.50	2.3±0.20
	1	88	4.3±0.85	3.0±0.69
	3	100	4.3±0.25	3.2±0.76

Fig. 1: Shoots multiplication of Rubinstern (R), Double decker (D) and Baby sawn white (S) *Echinacea* varieties on the MS-medium supplemented with 0.5 mg/l BA.

Fig. 2: Rooting of Rubinstern (R), Double decker (D) and Baby sawn white (S) Plants after Four Weeks.

2- Total Caffeic Acid Derivatives Contents:

Analysis of caffeic acid derivatives was applied on the dried flowering tops of *in vivo* and *in vitro* plants *Echinacea* varieties according to Bauer and Wagner (1988). Data summarized in Table (4) indicate that the concentration of total caffeic acid derivatives was higher in the dried *in vivo* flowering tops of *Echinacea* varieties in comparison with *in vitro Echinacea* varieties plants. Also the caffeic acid derivatives concentration was the highest in the Rubinstern Variety in both *in vivo* and *in vitro Echinacea* varieties as compared to the Double decker and Baby sawn white varieties. Furthermore, the lowest value (0.86%) was recorded with Double decker Variety of the dried flowering tops of *in vivo Echinacea* and for *in vitro* plant of Baby sawn white variety (0.29%). Wu *et al*, (2008) detected the optimum yields of phenolics (52.3 mg/g DW) in the adventitious roots of *Echinacea purpurea*, while Lee *et al*, (2010) found that the total phenolic contents of the *in vitro E. Purpuea* extract was 22.3 mg gallic acid equivalent/g DW.

Table 4: Comparative Analysis of Caffeic Acid Deirvatives of *In vivo* and *Iin vitro* Rubinstern, Double decker and Baby sawn white Plants.

Varieties	Total Caffeic Acid Derivatives			
	In vivo Plants		*In vitro* Plants	
	mg/g D W	% (w/w)	mg/g D W	% (w/w)
Rubinstern	14.90	1.49	6.23	0.62
Double decker	8.60	0.86	5.19	0.52
Baby swan white	1.02	0.10	2.86	0.29

3- HPLC Analysis of Caftaric and Cichoric Acids:

Caftaric acid and cichoric acid which represent the most important caffeic acid derivatives, were evaluated in the dried flowering tops of *in vivo Echinacea* varieties and compared with that produced *in vitro* plants of *Echinacea* varieties. Data present in Table (5) show that the higher productivity of caftaric and cichoric acids were recorded with *in vitro* plants of *Echinacea* varieties compared to the dried flowering tops of *in vivo Echinacea* varieties. The highest value of caftaric acid was recorded in *in vitro* Double decker variety (0.977%) which was 2.4 fold of caftaric acid in *in vivo* Double decker variety, while the highest value of cichoric acid was detected in *in vitro* Baby swan white variety (8.44%) which was 12.1 fold of cichoric acid in *in vivo* Baby swan white variety. It could be noticed that the lowest values of caftaric and cichoric acids in the *in vivo* plants were found in the Baby swan white variety (0.3% and 0.7% respectively). However, the lowest values of caftaric and cichoric acids for the *in vitro* plants were recorded in Rubinsterin variety (0.791% and 5.296% respectively). Caftaric and cichoric acids were detected by Wu *et al*, (2007) who determined caftaric and cichoric acids in the adventitious roots of *Echinacea purpurea* which cultured in airlift bioreactors and found that cichoric acid content was higher than caftaric acid. Also the contents of caftaric acid (1.6-fold) and cichoric acid (3.6-fold) were higher in the adventitious roots than in the roots of field grown plants. These results are in agreement with

our data. Wu *et al*, (2008) examined different extraction solvents and different extraction temperatures for the extraction of caffeic acid derivatives from the adventitious roots of *E. purpurea*. Their results showed that optimum yields of caftaric acid and cichoric acid were 4.9 mg/g DW and 24.6 mg/g DW respectively which achieved with the use of 60% ethanol. Abbasi *et al*, (2007) studied the effect of light on the caffeic acid derivative biosynthesis in hairy root cultures of *E. purpurea* and reported that cichoric acid, the most important component of caffeic acid derivative reached the maximum accumulation (27.0 mg/g DW) in the light-grown purple root cultures at day 45.

4- RAPD Analysis:

Sex random primers (B1,B2,B3,B4,B5 and B6) were used in RAPD analysis for their ability for sufficient amplification production. The results of DNA fingerprints generated by PCR amplification using the sex primers are presented in Table (6) and Fig (3). The six primers yielded 85 bands, including 6 polymorphic bands. The number of fragments generated per each primer varied between 3-21 bands. The primer B3 gave the highest number of bands (21), while the B6 primer gave the lowest number of bands (3). The highest percentage of polymorphism (22.2%) was recorded with B4 primer, whereas B2,B5 and B6 primers gave the lowest percentage of polymorphism (0%).

Table 5: HPLC Analysis of Caftaric and Cichoric Acids in *In vivo* and *In vitro* Different *Echinacea* Varieties.

Varieties	*In vivo* Plants		*In vitro* Plants	
	Caftaric Acid (%)	Cichoric acid (%)	Caftaric acid (%)	Cichoric acid (%)
Echinacea purpurea var. Rubinstern	0.4	1.0	0.791	5.3
Echinacea purpurea var. Baby swan white	0.3	0.7	0.871	8.4
Echinacea purpurea var. Double decker	0.4	0.8	0.977	8.3

Distribution and size of polymorphic bands from *in vivo* and *in vitro* three *Echinacea* varieties using six tested primers presented in Table (7). It was observed that with the primer B1, two polymorphic bands (lane 1) ranging size 300-400 bp are presented in *in vivo* Baby sawn white variety. Using the primer B3 there are two polymorphic bands one of them (lane 2) has molecular size about 900 bp. This band was absent in *in vivo* Double decker variety. Band in lane 1, which ranging size of 200-300 bp was absent in *in vivo* Baby sawn white variety. From the profile obtained with B4 primer, there are two polymorphic bands (lane 3), with size about 600 bp which was found in *in vivo* Rubinstern variety, while the other polymorphic band has size about 500 bp which was absent in *in vivo* Rubinstern variety.

In this respect, RAPD technique was used by Kapteyn *et al*, (2002) used RAPD technique to determine the genetic relationships between the three *Echinacea* species (*E. purpurea*, *E. angustifolia* and *E. pallida*). Kapteyn and Simon (2002) used RAPD markers to evaluate the level of diversity present within germplasm of *E. purpurea* , *E. angustifolia* and *E. pallida*. They compare of the accessions of each species available from different sources including the USDA National Plant Germplasm System and commercial sources. Chen *et al*, (2009) also used RAPD markers generated from 45 pre-selected primers to predict the contents of total phenol and alkamide in aerial parts of 70 *E. purpurea* accessions through stepwise regression analysis. The same technique was used by Taha *et al*, (2010) to evaluate the similarity between *in vitro* callus of *E. angustifolia* derived from different explants and the mother plant.

Table 6: RAPD-PCR Amplification Product of DNA Extracted from *In vivo* and *In vitro Echinacea* Varieties Using Six Random Primers.

Primer	Sequence 5'-3'	Total no. of Bands	No. of Polymorphic Bands	% of Polymorphism
B1	TCT GCG GTA GTT CCA GT	18	2	11.1
B2	CTT CGG CAG CAT CTC TTC AT	18	0	0
B3	CAG TGT GGA AGC CGA TTA TG	21	2	9.5
B4	ATG TGT TGT CTG GCT TGG TA	9	2	22.2
B5	TGG TCA GTG A	16	0	0
B6	TCA CGA TGC A	3	0	0
Overall Totals		85	6	7.0

% of polymorphism= no. 0f polymorphic bands/ no. of total bands x 100

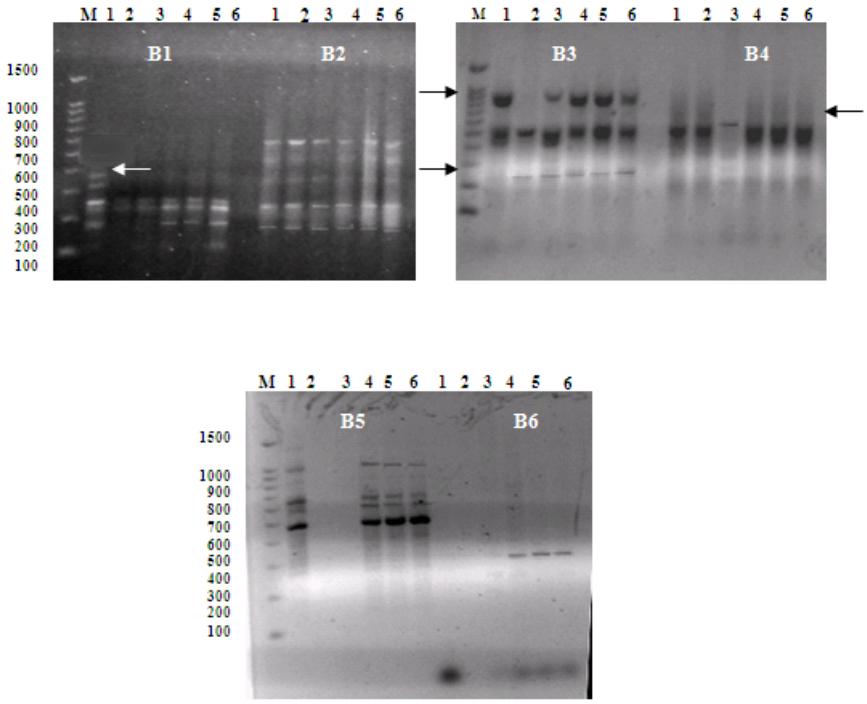

Fig. 3: Agarose Gel Electrophoresis of RAPD Fragments Generated by Primers B1,B2,B3,B4,B5 and B6 of *In vivo* and *In vitro* Three *Echinacea* Varieties.

Where: M. DNA marker (100-1500 pb). (1) *in vivo* Baby sawn white plant. (2) *in vivo* Double decker plant. (3) *in vivo* Rubinstern plant. (4) *in vitro* Baby sawn white plant. (5) *in vitro* Double decker plant. (6) *in vitro* Rubinstern plant.

Table 7: Distribution and Size of Polymorphic Bands of *In vivo* and *In vitro* Three *Echinacea* Varieties.

Primer	Size of polymorphic bands (bp)	Distribution of Polymorphic Bands					
		1	2	3	4	5	6
B1	300-400	+	-	-	-	-	-
	300-400	+	-	-	-	-	-
B3	900	+	-	+	+	+	+
	200	-	+	+	+	+	+
B4	600	-	-	+	-	-	-
	500	+	+	-	+	+	+

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