In vitro studies and RAPD analysis of *Echinacea angustifolia*.

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ABSTRACT: The goal of this research was to establish protocol for micropropagation and RAPD analysis of *Echinacea angustifolia*. The obtained data revealed that seeds sterilization with 5% clorox for 20 min and 0.1% mercuric chloride (MC) for 1 min, gave the best results for seeds germination and survival percentage. Three type of explants i.e., leaf, petiole and root were used for callus formation. The best results were obtained with MS medium supplemented with 1.0 mg/L-1 BA + 1.0 mg/L-1 NAA from leaf and petiole explant, respectively. However, supplementation of MS medium with 0.2 mg/L of BA gave the highest number of shootlets regeneration from root explants compared with other explants. Moreover, culturing the regenerated shoots on MS medium supplemented with 0.2 mg/L-1 IBA and 1 g/l activated charcoal enhanced of roots formation within two weeks. Furthermore, there is no evidence of somaclonal variations were recorded between the *in vitro* derived plantlets and those mother plant that revaluated highly similarity using RAPD–based DNA fingerprint analysis. [Journal of American Science 2010;6(10):781-790]. (ISSN: 1545-1003).

Key words: Calli, regeneration, *Echinacea angustifolia*, RAPD

INTRODUCTION

Echinacea species are members of the family Asteraceae **(perry *et al.*, 2001).** The word *Echinacea* is derived from the Greek Echinos for sea urchin or hedge hog, a reference to the spiny appearance of the plant **(Speroni *et al.,* 2002)**. *Echinacea* species, commonly known as purple coneflower, comprise a genus native to north America, occurring primarily in the USA and in South- Central of Canada **(Mc Gregor, 1968).**

Echinacea species have long been recognized as important medicinal plants used by Native Americans for the treatment of many diseases, including colds, toothaches, snake bites, rabies and wound infections **(Bauer and Wagner, 1991)**. For sterilization of explants clorox and murcuric chloride can be used as reported by **Sujatha (1997)** on *Guizotia abyssinica*, and **Abou Dahab *et al.*, (2005)** on *Russcus hypoglossum*. Murch *et al.*, (2006) reported that seeds of *E. purpurea* were surface sterilizated by immersion in 70% ethanol for 30 s and then in 5.4% sodium hypochloride for 18 min which proved highly efficient in ensuring contamination free seeds germination. Furthermore, **Korach *et al.*, (2003)** in their study on *Echinacea pallida* they found that callus were derived from leaf tissue explants when placed on MS medium supplemented with BA and NAA combinations. In addition when **Subbaiah *et al.*, (2003)** used another strains of *Echinacea* they reported that callus were induced from leaf segments of mature plants of *E. purpurea* placed on MS medium supplemented with 0.5 mgl-1 NAA and 2.5 mgl-1BA. Plant regeneration from petiole explants of *E. purpurea* was achieved using only a small amount of BA **(Choffe *et al.,* 2000)**. However, **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. Moreover, **Koroch *et al.* (2002)** reported that high rooting and survival percentage were achieved using MS medium without plant growth regulators. The use of (RAPD) Random Amplified Polymorphic DNA 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 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 expermints were achieved on *E. angustifolia* to investigate the effect of various media ingredients on callus production, shootlets proliferation and roots formation *in vitro*.

MATERAILS AND METHODS

This investigation was carried out on *Echinacea angustifolia* at the Department of Plant Biotechnology, National Research Centre. The objective of this study was to establish protocol for callus production, micropropagation and RAPD

analysis of the *in vitro* derived plantlets compared with the mother plant.

Explants source and disinfection

Seeds of *E. angustifolia* were obtained from Horticulture Division, Faculty of Agriculture, Cairo University, Egypt. These seeds were soaked for 1 min in 70% ethanol under aseptic condition and treated with 5% of commercial Clorox with a few drops of tween-20 for 20 min followed by 0.1% of mercuric chloride (MC) for 1 min. Finally, they were rinsed three times with steril-distiled water. Sterilized seeds were cultured onto growth regulator-free MS-medium (five replicate per treatment). After three weeks of culturing germinated seeds were used as a source of leaf, petiole and root explants..

Culture media and conditions

For callus induction, shootlets proliferation and rooting experiments, all used media were enriched with 30 g/l sucrose and 7 g/l agar. All type of media were adjusted to pH 5.8±0.1 and autoclaved at 121°C and 1.2 kg/cm^2 for 20 min before using. The different explants i.e. leaf; petiole and root were placed vertically in 200 ml capacity galss containing 25 ml media. The culture media were incubated at 26 ±1°C under fluorescent white lamps with light intensity of 3000 lux at 16-hours photoperiod and the other cultures were maintained in dark conditions at 25°C to determined the callus formation.

Callus induction

Leaf, petiole and root segments (3-4mm) were excised from sterilized *in vitro* germinated plantlets cultured on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of BA at the rate of 0, 1, 3, 5 (mg/l) and 1mg/l NAA. In all treatments twenty explants in five replicates were cultured for one month and subcultured onto same treatment for three times. The callus value were recorded by presented as coefficient (+ or -).

Shootlets regeneration

An equal pieces of calli cultures (~ 250 mg/jar) produced from leaf, petiole and root explant were recultured on MS medium supplemented with different concentrations of BA at the rate of 0, 0.2, 0.4, 0.6, 0.8, or 1.0 (mg/l). In all treatments twenty derived calli in five replicates were cultured for one month and subcultured onto same treatment for three times. The number of shootlets, shootlet length (cm) and number of leaves per shootlet were recorded.

Roots formation

In this experiment, shootlets (1-2 cm length) were recultured onto MS-free- growth regulators medium supplemented with 1 g/l activated charcoal (AC) or on MS medium supplemented with different concentration of IBA. Twenty shootlets in five replicated were used in each treatment for one month and then the percentage of root formation, number of roots per shoot and root length (cm) were recorded.

The obtained results were statistically analyzed using SPSS multiple range test at 0.001 & 0.05 level of significance according to **Snedecor and Cochran (1982)**.

Genomic DNA extraction and RAPD analysis.

Genomic DNA was isolated using CTAB method according to **Doyle and Doyle (1990).** RAPD-PCR analysis, PCR amplification was performed in 20 ul reaction mix containing 20 ng genomic DNA, 1 unit taq polymerase (Gibco), 200 uM each of dATP, dCTP, dGTP, dTTP, 20 pmole of random primers (Operon) as shown in Table (1) and appropriate amplification buffer. The mixture was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed according to **Williams *et al.*, (1990)** for 45 cycles, using Biometera Uno Thermal Cycler, one cycle at 92°C for 3 min and then 44 cycles at 92°C for 30 sec, 35°C for 60 sec and 72 °C for 2 min (for denaturation, annealing and extension stages, respectively). Reaction was finally incubated at 72 °C for 10 min and further for 10 min at 62°C.

Table (1): The random primer names and sequences used for RAPD analysis.

No.	RAPD primers	
	Name	Sequences 5'--------- 3'
1	AM1	CTTCGGCAGCATCTCTTCAT
2	AM2	CAGTGTGGAAGCCGATTATG
3	AM3	ATGTGTTGTCTGGCTTGGTA

Electrophoresis, the amplification products were analyzed by electrophoresis according to **Sambrook *et al.*, (1989)** in 2% agarose in TAE buffer (for each litre of 50X TAE Stock solution: 242 g Tris Base, 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA). Then the products were stained with 0.2 ug/ml ethidium bromide. Nucleic acids bands were photographed and detected under short wave of UV light.

Experimintal design and data analysis

Completly ramdomized design was employed in all of the executed experiments. Data analysis of variance was carried out according to **Stell and Torrie (1980).**

RESULTS AND DISSCUSION

Seeds disinfection

Disinfection of the seeds and subsequently germination is an important process to obtaine a success explants under the establishment stage and complet the following stages of tissue culture. Data in Table (2) referred that sterilization with Clorox 20% for 20 min resulted in highest percentage of free contamination (76.25%) compared to 10 min, which gave (47.50%). The highest percentage of survival and germinated seeds were recorded with 10 min (82.50%), however, sterilization for 20 min recorded the lowest percentage of survival (42.50%). These finding are in agreement with **Zobayed and Saxena (2005)** on *Echinacea purpurea* they found that using Clorox at different concentrations had a great effect on sterile seeds.

Callus induction

Data tabulated in Table (3) and Fig. (1) show the effect of supplementation of MS medium with 1 or 3 or 5 mg/l of BA in combination with 1mg/l NAA on percentage of callus formation from leaf, petiole and root explants cultured and incubated under light or dark condition. The highest observation of callus formation was remarket with leaves and petiole explants, respectively. The equal concentration of BA and NAA resulted the best results of callus formation compared with other concentration. However, increasing of BA concentration to 5mg/l stimulated of callus induction from root explants. The obtained results are in agreement with those obtained by **Koroch *et al.* (2002)** on *Echinacea purpurea* they found that incubation of leaf explants with MS basal medium supplemented with a suitable ratio of auxin/cytokinin achieved of callus formation after 4 weeks of incubation.

Shootlets formation

Data in Tables (4,5) and Fig. (2) show the effect of MS medium supplemented with different concentrations of BA on number of shootlets formation, length of shoot and number of leaves/shoot

The obtained results of Table (4) revealed that leaflet derived calli gave the maximum number of shootlets regeneration (1.85) compared to petiole (1.74) and root (0.23) calli respectively. Moreover, data on Table (4) demonstrated both the effect of different concentrations of BA and type of calli on the length of the regenerated shootlets. The longest shootlets (1.05 cm) was recorded with leaf calli as compared with petiole (0.84 cm) and root (0.53 cm) calli cultures respectively. These data are in agreement with those Koroch *et. al.,* (2002) which found that lower concentration of BA (0.44-8.88 μM) with leaf calli of *E. purpurea* showed adventitious shoots formation after 4 weeks. Lucchesini *et al.,* (2009) reported that CH basal medium supplemented with 0.5 mgL^{-1} BA gave the best results of shootlets formation from *Echinacea angustifolia*.

Table (2): Effect of some sterilization treatments on free contamination and survival seeds (%) from *Echinacea angustifolia* cultured *in vitro*.

Time / Clorox%	Free contamination %				Survival %			
	10 min	15 min	20 min	Mean B	10 min	15 min	20 min	Mean B
5%	10.0	45.0	60.0	38.33	100.0	45.0	70.0	71.67
10%	20.0	55.0	65.0	46.67	90.0	75.0	55.0	73.33
15%	70.0	80.0	85.0	78.33	85.0	85.0	35.0	68.33
20%	90.0	75.0	95.0	86.67	55.0	50.0	10.0	38.33
Mean A	47.50	63.75	76.25		82.50	63.75	42.50	

Table (3): Influence of MS medium supplemented with BA and NAA on percentage of callus formation from leaf, petiole and root explants of *E. angustifolia*. Cultures were incubated under light or dark condition at 26±1 °C for 4 weeks.

MS supplemented with mg/l		Explants					
		leaves		petiole		root	
BA	NAA	light	dark	light	dark	light	dark
0	0	-	-	-	-	-	-
1.0	1.0	+++	++	+++	+	-	-
3.0	1.0	++	-	++	+	-	-
5.0	1.0	++	+	-	-	++	-

Data are presented as coefficient (+ or -). (—) = No response
(+) = low, (++) = medium, (+++) = high

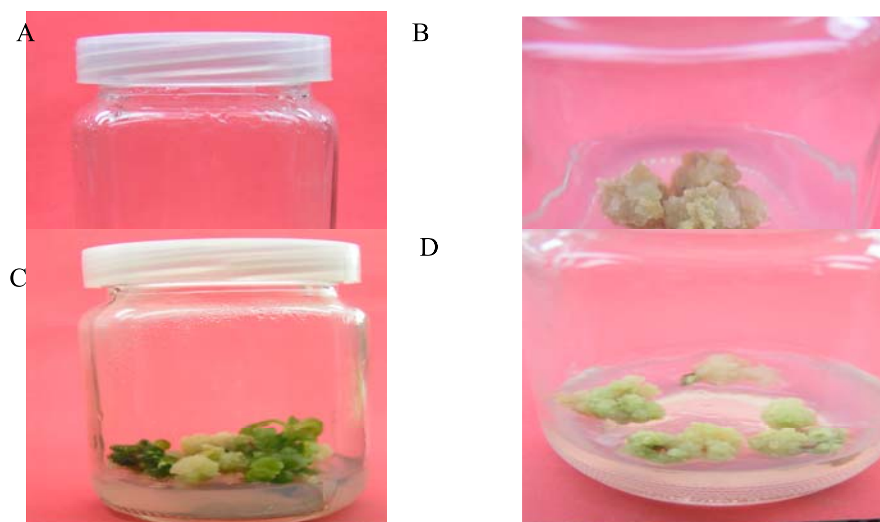

Fig.1. Callus formation from leaf explants under light (A), under dark (B); from petiole explants under light (C) and dark (D) conditions.

Table (4): Effect of MS medium supplemented with (0.2,0.4,0.6,0.8 and 1 mg/l) BA on number of shootlets and length of shootlets regenerated from leaf, petiole and root calli of *E. angustifolia*.

MS medium supplemented with:	Number of shootlets regenerated from callus of :			Length of shootlets (cm) regenerated from callus of :		
	Leaf	Petiole	Root	Leaf	Petiole	Root
Control	0.53±.07 (a)	0.50±.00 (a)	.50±.06 (a)	2.88±.15 (a)	1.13±.05 (a)	2.00±.25 (a)
0.2 mg/l BA	2.89±.08 (b)	3.87±.12 (b)	.90±.12 (b)	1.38±.10 (b)	1.06±.00 (a)	1.22±.05 (b)
0.4 mg/l BA	5.55±0.44 (c)	3.00±.11 (c)	.00±.00 (c)	1.15±.09 (b)	1.05±.03 (a)	.00±.00 (c)
0.6 mg/l BA	2.13±0.12(d)	2.10±.14 (d)	.00±.00 (c)	.98±.05 (b)	.86±.05 (a)	.00±.00 (c)
0.8mg/l BA	.00±.00 (a)	1.00±.15 (a)	.00±.00 (c)	.00±.00 (c)	.98±.07 (a)	.00±.00 (c)
1.0 mg/l BA	.00±.00 (a)	.00±.00 (e)	.00±.00 (c)	.00±.00 (c)	.00±.00 (b)	.00±.00 (c)
Mean	1.85	1.74	0.23	1.05	0.84	0.53
F_{ratio}	316.787	198.45	52.8	154.81	90.226	67.992
P_{value}	***	***	***	***	***	***

Means in a Column with similar letters are not significantly different according to LSD. *** = significant at P < 0.001.

Table (5): Effect of MS medium supplemented with (0.2,0.4,0.6,0.8 and 1 mg/l) BA on number of leaves from leaf, petiole and root calli of *E. angustifolia*.

MS medium supplemented with:	Number of leaves/ shootlets regenerated from callus of :		
	Leaf	Petiole	Root
Control	2.18±.10 (a)	2.00±.00 (a)	1.59±.08 (a)
0.2 mg/l BA	5.88±.09 (b)	6.93±.02 (b)	2.40±.08 (b)
0.4 mg/l BA	6.87±.053 (c)	6.10±.08 (c)	.00±.00 (c)
0.6 mg/l BA	5.05±.06 (d)	5.20±.04 (d)	.00±.00 (c)
0.8mg/l BA	.00±.00 (e)	3.60±.15 (e)	.00±.00 (c)
1.0 mg/l BA	.00±.00 (e)	0.00±0.00 (f)	.00±.00 (c)
Mean	3.32	3.97	0.66
F_{ratio}	2180.329	1334.666	522.638
P_{value}	***	***	***

Means in a Column with similar letters are not significantly different according to LSD.
*** = significant at $P < 0.001$.

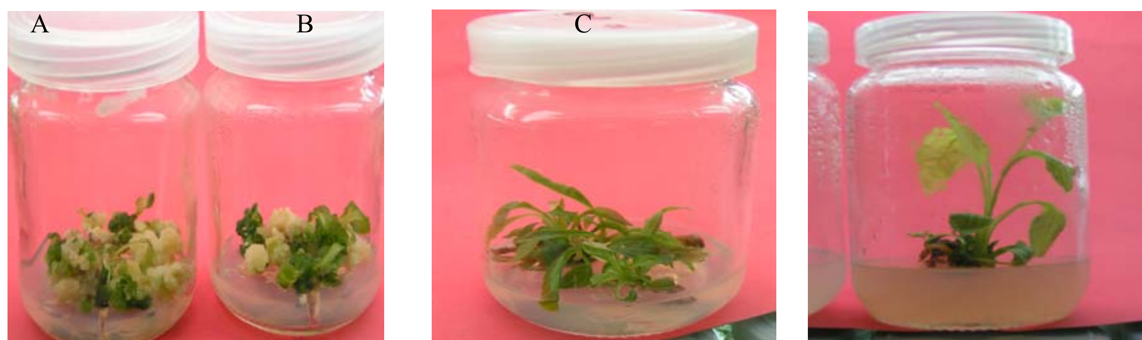

Fig (2). Effect of MS medium supplemented with 0.4 mg/l BA on shootlets regeneration from leaf (A), petiole (B) and root (C) calli culture of *E. angustifolia,* after 4 weeks of cultivation under light condition.

Moreover, data of Table (5) show the effect of different concentration of BA and the type of explants on number of leaves/shootlet formation. The highest number of leaves (3.97) was recorded with MS medium supplemented with 0.2 mgL^{-1} BA.

Roots formation

Data in Table (6) revealed that MS-medium with or without addition of 1 g/l activated charcoal (AC) and with or without 0.2mg/l IBA induced shootlet root formation in an equal rate (100%). Data Showed that the highest significant of roots number (1.8) and root length (5.21 cm/shoot) were recorded with MS + 1g/l AC+0.2mgL^{-1} IBA. However, the lowest significant of root number (0.8), and shortage of root length (3.54 cm/shoot) were recorded with MS medium free growth regulators. In close of the obtained results, George and Ravishankar (1997) on *Vanilla planifolia* found that the best rooting formation was observed in half-strength of MS-medium with or without activated charcoal.

Table (6): Effect of MS-medium supplemented with or without activated charcoal (1 g/l) and 0.2 mg/l IBA on formation roots of *E. angustifolia*.

Culture medium	Rooting %	Number of roots	Length of root (cm)
MS	100	0.8 ^{d}	3.54 ^{c}
MS+ 0.2mgL^{-1} IBA	100	1.2 ^{c}	4.65 $^{a b}$
MS + AC	100	1.4 $^{a b}$	3.66 ^{c}
MS + AC+0.2mgL^{-1} IB	100	1.8 ^{a}	5.21 ^{a}
Mean	NS	1.3	4.26

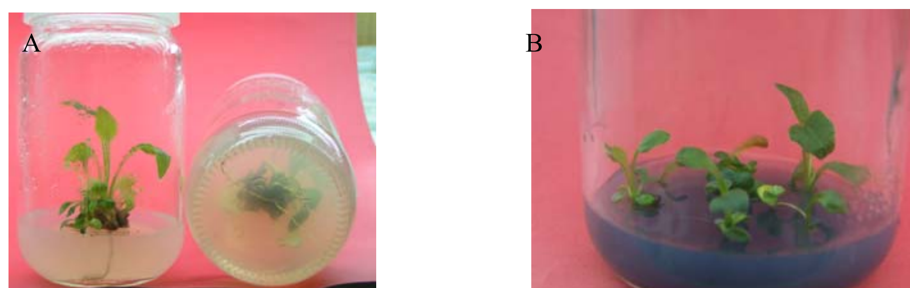

Fig. (3) Effect of MS medium supplemented with or without 1 g/l activated charcoal and 0.2 mg/l IBA. (A) MS with 0.2mgL^{-1} IBA (B) MS with 0.2mgL^{-1} IBA and activated charcoal.

RAPD analysis of *in vivo* and *in vitro E.angustifolia*.

Three random primers (AM1, AM2 and AM3) were screened in RAPD analysis for their ability to produce sufficient amplification products. The results of DNA fingerprints generated by PCR amplification using the three primers are presented in Figs (4,5 and 6) and Tables (7,8 and 9).The number of fragments generated per primer varied between 1-6, 1-4 and 1-3. The primer AM1 gave the highest number of bands (6) and the percentage of polymorphism were 89.5 and 86.9 with primer AM1 and AM2 respectively, the primer AM3 gave the highest percentage of polymorphism (100) and the number of bands was (3).

RAPD marker generated with primer (AM1) shown in Fig (4) and Table(7) showed that the same DNA amplification with the mother plant. The differences showed with three fragments with treatments 5 and 6, at 791, 475 and 368 bp, respectively between them and the mother plant. Also, presents data showed that there are five common bands in all treatments and the mother plant.

RAPD marker generated with primer (AM2) in Fig (5) and Table (8) showed minimal DNA amplification differences between the treatments and the mother plant. In treatment 5 there is one fragment at 791 bp, one band with treatments 2, 3, 4, 5, and 6 at 700 bp and tow bands with treatments 5 and 6 at 187 and 121 bp are not showed in the mother plant, and tow bands are common with all treatments and mother plant at 568 and 368 bp. Primer (AM3) in Fig. (6) and Table (9) showed that there are no differences between all treatments and the mother plant. However, these polymorphisms are considered to be primarily due to the variation in the primer annealing sites. RAPDs have been used for many purposes, ranging from studies at the individual levels (e.g. genetic identity) to studies involving closely related species. Due to their high genomic abundance, RAPDs have also been applied in gene mapping studies (Williams *et al.,* 1990). In this respect, RAPD markers have been able to access the genetic stability of micropropagated plants of *Deutzia scabra* (Sayed and Gabr, 2009) almonds (Martins *et al*., 2004), cassava (Angel *et al*., 1996), ginger (Rout *et al*., 1998), sweet potato (Dixit *et al*., 2003), turmeric (Tyagi *et al*., 2007), and yams (Ahuja *et al*., 2002). As RAPD markers amplify different regions of the genome, their simultaneous analyses give a better interpretation of the genetic stability of the *in vitro* regenerants (Martins *et al*., 2004). While, De Masi *et al.,* (2003) reported that, they used the RAPD analysis to distinguish the two more largely widespread fig cultivars in Italy (Bianco del Cilento and Dottato) and their clones. RAPD- PCR produced amplification patterns that did not significantly vary between the two cultivars. However, RAPD analysis allowed clone differentiate using a single primer.

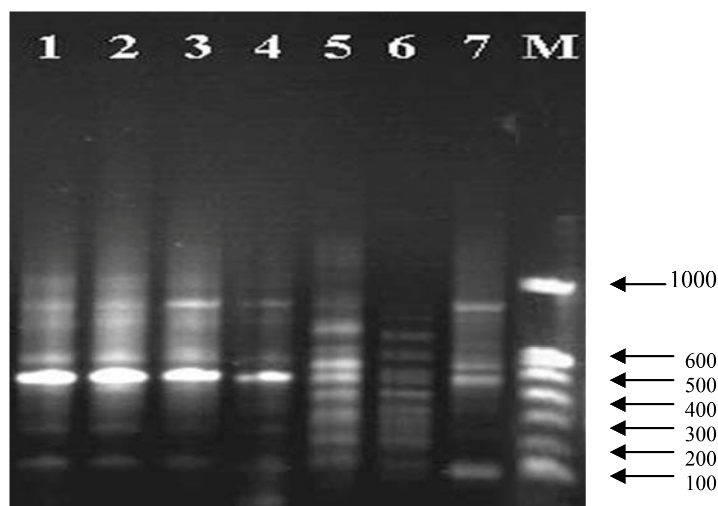

Fig (4). RAPD pattern of the *in vitro* treatments of *Echinacea angustifolia* with primer AM1.

Where: M. DNA marker (100 – 1000 pb). (1) Mother plant. (2) Callus from leaf in light. (3) Callus from petiole in light.(4) Callus from root in light (5) Callus from leaf in dark.(6) Callus from petiole in dark.(7)regeneration plant.

Table (7): RAPD-PCR analysis of *Echinacea angustifolia* using AM1 primer.

Band No.	M	1	2	3	4	5	6	7
1	1000	+	+	+	-	-	-	-
2	966	+	+	+	+	+	+	+
3	791	-	-	-	-	+	+	-
4	700	+	+	+	+	+	+	+
5	586	+	+	+	+	+	+	+
6	475	-	-	-	-	+	+	-
7	368	-	-	-	-	+	+	-
8	267	+	+	+	+	+	+	+
9	187	-	-	-	-	+	+	-
10	121	+	+	+	+	+	+	+

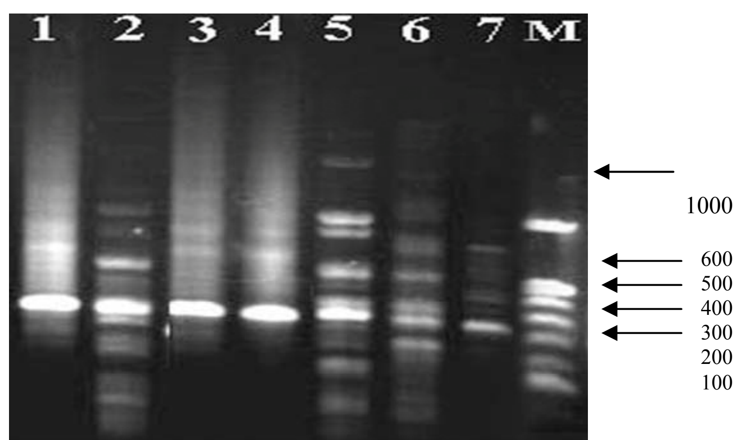

Fig (5). RAPD pattern of the *in vitro* treatments of *Echinacea angustifolia* with primerAM2.

Where: M. DNA marker (100 – 1000 pb). (1) Mother plant. (2) Callus from leaf in light. (3) Callus from petiole in light. (4) Callus from root in light. (5) Callus from leaf in dark. (6) Callus from petiole in dark. (7) regeneration plant.

Table (8): RAPD-PCR analysis of *E. angustifolia* using AM2 primer.

Band No.	M	1	2	3	4	5	6	7
1	791	-	-	-	-	+	-	-
2	700	-	+	+	+	+	+	-
3	586	+	+	+	+	+	+	+
4	475	+	+	-	-	-	-	+
5	368	+	+	+	+	+	+	+
6	267	+	+	-	-	+	+	+
7	187	-	-	-	-	+	+	-
8	121	-	-	-	-	+	+	-

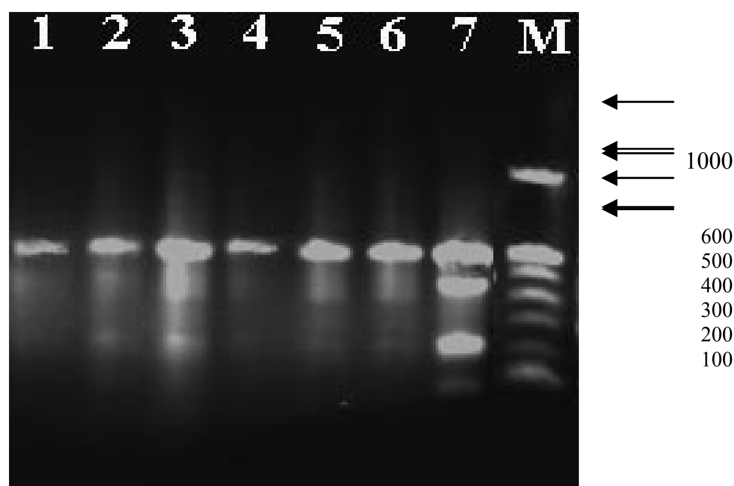

Fig (6). RAPD pattern of the *in vitro* treatments of *Echinacea angustifolia* with primer AM3.
Where M. DNA marker (100 – 1000 pb). (1) Mother plant. (2) Callus from leaf in light. (3) Callus from petiole in light.(4) Callus from root in light (5) Callus from leaf in dark.(6) Callus from petiole in dark.(7)regeneration plant.

Table (9): RAPD-PCR analysis of *Echinacea angustifolia* using AM3 primer.

Band No.	MW kbp	1	2	3	4	5	6	7
1	586	+	+	+	+	+	+	+
2	475	+	+	+	+	+	+	+
3	267	+	+	+	+	+	+	+

Table (10): Polymorphism percentage of treatments and mother plant based on RAPD product of AM1, AM2 and AM3 primers.

Primers	No. of polymorphism	Total No. of polymorphism	Polymorphism %
AM1	6	47/7= 7.8	6/7.8*100=89.5
AM2	4	32/7= 4.6	4/4.6*100= 86.9
AM3	3	21/7= 3	3/3*100= 100

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