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Regeneration via Direct Organogenesis from Leaf and Petiole Segments of Pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.]

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Abstract: A highly reproducible *in vitro* regeneration system in pyrethrum has been developed. Multiple shoots were induced *in vitro* from leaf and petiole explants through adventitious shoot bud regeneration. The ability of leaf segments to produce direct shoot buds varied depending upon their medium composition. Leaf segments were highly responsive than petiole cuttings and produced a maximum shoot regeneration (70%) on MS medium supplemented with 4 mg l^{-1} BA and 0.2 mg l^{-1} 2,4-D. The highest proliferation rate was observed on MS medium supplemented with 1.5 mg l^{-1} BA and 2 mg l^{-1} NAA. The highest means of shoot length were produced in all media without BA and using BA in culture media resulted in production of short shoots. The highest fresh weight of shoot was observed on MS medium when compared to B5 and SH. For rooting of the shoots, MS, B5 and SH medium supplemented with different concentrations of (alpha)-naphthalene acetic acid (NAA) were tried. The optimal result was observed on B5 medium supplemented with 2 mg l^{-1} NAA, on which 100% of the regenerated shoots developed roots with an average of 16 roots per shoot within 3 weeks. The *in vitro* raised plantlets were acclimatized and transferred to greenhouse with 80% success.

Key words:6-benzylaminopurine · Leaf explants · Petiole cuttings · Organogenesis · *Tanacetum cinerariaefolium*

INTRODUCTION

*Tanacetum cinerariaefolium i*s an important medicinal perennial herb belonging to the family Asteraceae. The genus *Tanacetum* encompasses many species found mainly in the temperate regions [1]. *Tanacetum cinerariaefolium* has been widely use in traditional medicine in Iran as well as in the insectiside industry because due mainly to their Pyrethrins production [2]. Pyrethrins are currently the most economically important natural insecticide of plant origin. They are a mixture of six compounds produced by acidification of two acids (chrysanthemic and pyretric acid) with three ketons-alcohols (pyrethrolone, jasmolone and cinerolone). Pyrethrins have some of the qualitities of an ideal peat control agent as they are very effective against a broad range of insects with little development of resistans strans and have knock-down and kill effects on insects. One of the major advantages of Pyrethrins over all other insecticide is their low toxicity to mammals and other warm blooded animals [3]. The vegetative propagation of this plant is generally achieved by either splits or shoot cutting. These propagation method results in low multiplication rate [4]. An additional problem is the high susceptibility of this plant to attack by root knot nematodes; the spiliting of infected stock plant result in a contaminated clone [5]. The great interest in Pyrethrins for insecticide use has led to the investigation of alternatives for the production of the biologically active plant constituents. Plant regeneration has been described from shoot tip and axillary bud explants [6], from callus [6-8]. In recent studies MS medium was dominant medium for multiplication The aim of this study was to establish a method for asexual multiplication of *Tanacetum cinerariaefolium* through direct organogenesis from leaf and petiole segments, which can be useful for obtaining phytomedicine stock of elite plant. Moreover, the perennial and highly heterozygous nature of this plant coupled with a prolonged flowering period (2-3 years) limits the speed of improvement using

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traditional methods. Therefore, application of biotechnological tools for genetic improvement of valerian attains greater significance, which in turn depends upon the availability of an efficient regeneration system. Also, efficient regeneration system producer can play an important role in genetic transformation experiments and industrial purposes of this medicinal plant.

MATERIALS AND METHODS

Plant Materials and Shoot Induction: Seeds of *Tanacetum cinerariaefolium* were cultivated in August 2004 in the greenhouse of Agriculture College, Boushehr University, Iran. Leaves of pyrethrum were collected from 4 month old and transferred to laboratory. The excised leaf and petiole explants were surface sterilized in 70% alcohol for 1 min followed by 15% sodium hypocloride for 20 min. They were rinsed four to five times in sterile distilled water with 5 min duration each. The sterilized explants were cultured in sterile culture bottle containing 15 ml of Murashige and Skoog basal medium [9] with 3% sucrose, 0.8% agar (Agar Powder, Extra pure, Merck, Darmstadt, Germany) and 100 mg l^{-1} myoinositol for induction of shoot organogenesis. The effect of BA (0, 2, 4 and 6 mg/l^{-1}), IAA (1, 2 and 3 mg/l^{-1}), NAA (1 and 2 mg/l^{-1}) and 2, 4-D (0, 0.2 and 0.4 mg/l^{-1}) and different combinations on induction of direct organogenesis from leaf and petiole explants was tested by supplementation into MS medium in varied concentrations. Also, organogenesis response of different size (5, 10 and 15 mm^2) of leaves explant was and their position (abaxial side and adaxial side) in contact with the medium were studied. This experiment was conducted as a complete randomized design in a factorial arrangement with four replicate and each replicate contain 20 explants. The appearance of shoot buds from leaves was taken into consideration for calculating the shoot organogenesis from leaves and petiols.

Multiplication of Shoots: Shoots of 1-1.5 cm derived from leaves were cultured in sterile culture bottle containing 25 ml of MS, B5 (Gamborg) [10] and SH (Schenk and Hildebrandt) [11] medium supplemented with NAA (0, 1, 2, 4 and 6 mg L^{-1}) and BA (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg L^{-1}) for induction of multiple shoots. The appearance of shoots from the base of the explant and also from the axils of leaves was taken into consideration for calculating the multiplication frequencies. The average number of shoots induced per explant was recorded after 28 days of culture. Also the mean length of shoot and total fresh weight were assessed after 4 weeks. Total fresh weight was determined and growth value(GV) was measured according to the following equation [12].

$$\text{f.wt} = \frac{\text{final weight-initial weight}}{\text{initial weight}} \times 100$$

$$\text{G.V} = \frac{\text{Final fresh weight - Initial fresh weight}}{\text{Initial fresh weight}} \times 100$$

Root Induction from Shoots: Healthy shoots (3-4 cm in length) derived from leaves or multiple shoots were transferred to MS, B5 and SH medium supplemented with different concentrations of naphthalene acetic acid (NAA) at 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 mg l^{-1}. The number of shoots that formed roots, the number of root per explant and root length was recorded after 3 weeks. For all the experiments on shoot induction, shoot multiplication and root induction from shoots, the cultures were kept at 25±3°C under a 16-h photoperiod with a photosynthetic photon flux density of 40 ìmol m^{-2} s^{-1} provided by white fluorescent lamps.

Acclimatization of Regenerated Plants and Data Analysis: Regenerated plants having well developed roots were removed from culture bottles and washed free of agar. They were transferred to small pots containing 1/3 Vermimiculite, 1/3perlite, 1/3 sand (V.V). The pots placed under transparency box and maintained under 25±3°C temperature and 70% relative humidity. After an adaptation period (4 weeks), acclimatized plantlets were transferred to greenhouse. This experiment was conducted as a complete randomized design in a factorial arrangement with four replicate and each replicate contain 12 explants. Duncan's multiple range test (DNMRT) was used for comparison among treatment means.

RESULTS

Induction of Direct Shoot Organogenesis: Plant regeneration via direct shoot organogenesis has been achieved from the cultured of leaf (Fig. 1a) and petiole (Fig. 1b) explants. Leaf segments was responsive than prtiol cuttings. Between different auxins that were tried in this study 2,4-D was effective than IAA and NAA. Between different concentration of 2,4-D 0.2 mg L^{-1} gave high frequency of direct organogenesis in combination with 4 mgL^{-1} BA. High number of direct shoot sorganogenesis was achieved with a frequency of

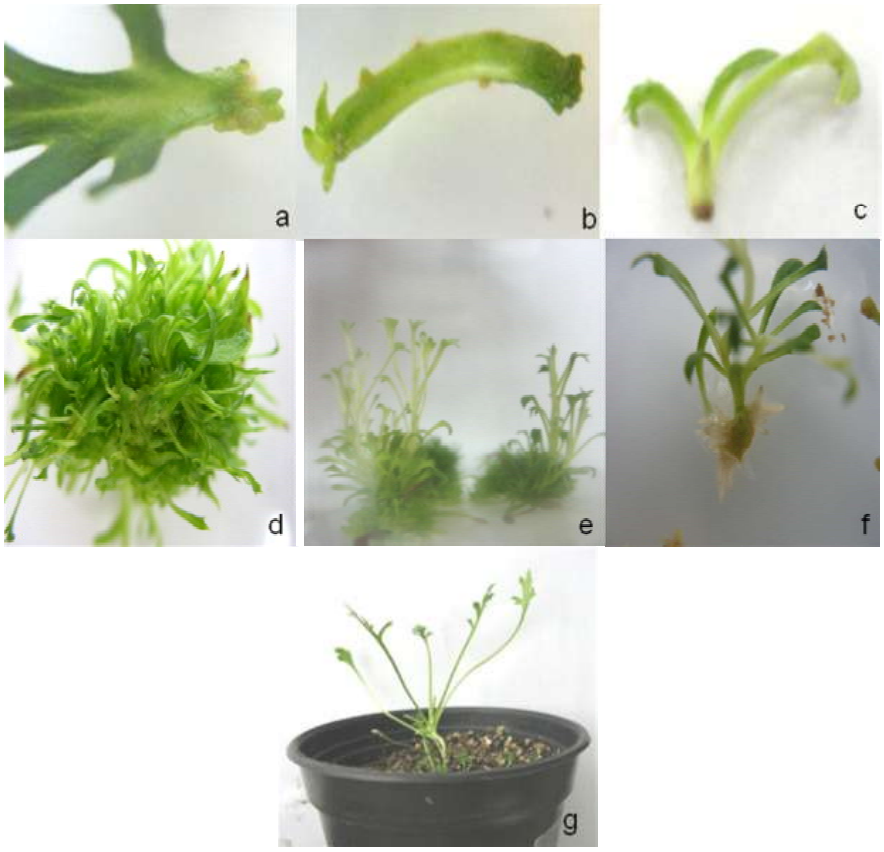

Fig. 1: Micropropagation of Pyretrum, (a) Direct adventitious shoot bud induction from cultured leave after 4 weeks. (b) Direct adventitious shoot bud induction from cultured petiole after 4 weeks. (c) Single regenerated shoot from direct shoot bud from leaf explant. (d) The proliferation of shoot on MS medium contaning 1.5 mg l^{-1} BA and 2 mg l^{-1} NAA. (e) Increasing shoot length in BA free medium. (f) Rooting of a regenerated shoot in B5 medium supplemented with 2 mg l^{-1} NAA 21 days after culture. (g) two-week-old acclimatized plants growing in greenhouse

70 % (medium containing with 4 mg l^{-1} BA and 0.2 mg l^{-1} 2,4-D) and 18% (medium containing with 2 mg l^{-1} BA and 2 mg l^{-1} IAA) from leaves and petiole on MS medium, respectively (Table 1). Also, the maximum average length of shoots was higher in treatments that showed single shoot regeneration (Fig. 1c).

Multiplication of the Shoots: Between different medium that used in this study, MS medium showed highest number of shoot per explant, maximum average length of shoots (mm) and fresh weight of shoot (%) comparing than other medium. Percent of shoot induction in all medium was 100% (Table 2).

Shoot Proliferation: Multiple shoots were induced from shoot differentiated from leaves at a high frequency (Table 3). The emergence of multiple shoots occurred in 12-15 days. BA at 1.5 mg l^{-1} and NAA at 2 mg l^{-1} in MS medium (Fig. 1d) supported induction of 21.3 shoot, shoot length of 64.2 mm. evaluation between different medium on shoot production showed that MS medium more proliferation than B5 and SH culture medium. The number of shoot multiplication and average shoot length decreased with the increase in the concentration of BA (from 1.5 to 3 mg l^{-1}) and NAA (from 2 to 6 mg l^{-1}). The highest average shoot lengths were observed in BA free medium (Fig. 1e). Application of BA with each concentration of NAA in all medium decreased average shoot length. Between media that used in this study, MS medium showed highest average shoot length compared to other medium (Table 4). Also, the same result obtained about growth value as shoot fresh weight (Table 5).

Table 1: Effect of different concentration of BA and different auxins (2, 4-D, NAA and IAA) on growth value of direct shoot organogenesis from leaves and petiole segments of *Tanacetum cinerariaefolium*

		(Auxins) mg l^{-1}							
		2,4-D			NAA		IAA		
Type of explant	BA (mg l^{-1})	0	0.2	0.4	1	2	1	2	3
Leaf	0	0g	0g	0g	0g	0g	0g	0g	0g
	2	0g	35c	0g	0g	8e	0g	12e	3f
	4	0g	70a	3f	48b	36c	2f	18d	12e
	6	0g	0g	0g	0g	0g	0g	2f	0g
Petiole	0	0g	0g	0g	0g	0g	0g	0g	0g
	2	0g	12e	0g	0g	4f	0g	d18	2f
	4	0g	2f	0g	4f	2f	8e	12e	2f
	6	0g	0g	0g	0g	0g	0g	2f	0g

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by New Duncan Multiple Range Test (DNMRT)

Table 2: Effect of different medium on average shoot length, Shoot induction (%), number of shoot per explant and Total fresh weight of shoot (%) in *Tanacetum cinerariaefolium*, 28 days after culture

Media	Shoot induction (%)	No. of shoots / explants	Average length of shoots (mm)	Growth value
MS	100a†	8.34a	59.02a	76.99a
B5	100a	4.38b	38.97b	72.92b
SH	100a	7.39a	42.15b	38.87b

† Means followed by the same letter in a column are not significantly different ($p < 0.05$) by New Duncan Multiple Range Test (DNMRT)

Table 3: Interaction between different medium and different concentration of BA and NAA on multiple shoot induction from shoots regenerated from leaves in *Tanacetum cinerariaefolium*, 28 days after culture

		NAA (mg l^{-1})				
Medium	BA^{-1}	0	1	2	4	6
MS	0	0.3g†	1.3g	6ef	6.7def	12bcd
	0.5	2.7fg	4fg	6.3def	7.3def	7.3def
	1	2.6 fg	12.7bcd	14bc	14.7bc	8de
	1.5	4fg	15ab	21.3a	17.7ab	14.3bc
	2	2g	8.3de	20a	14.7bc	10.3cde
	2.5	1.7g	5.3efg	19a	5efg	3fg
	3	1g	5.3efg	11.3cd	4fg	2.7fg
B5	0	2.3fg	3.6fg	2fg	1.3g	1.3g
	0.5	11cd	14.7bc	5.3efg	5efg	2fg
	1	7.7def	12.7bcd	5.3efg	3.3fg	2.3fg
	1.5	6ef	12.7bcd	5efg	3.3fg	2.3fg
	2	4.3fg	8.7de	2.7fg	2.7fg	1.7g
	2.5	3.3fg	5.7ef	2.3fg	2.3fg	1.3g
	3	2.3fg	2.3fg	2.3fg	1.3g	1g
SH	0	3fg	3.3fg	3.3fg	3fg	2.3fg
	0.5	10cde	10.3ede	16ab	13.3bcd	7.3def
	1	7def	10.3cde	15ab	10cde	5.7ef
	1.5	8.7de	9cde	14bc	8.3de	6ef
	2	6.3ef	7.7def	11cd	5.7ef	5efg
	2.5	5efg	5.7ef	9.3cde	6ef	5.3efg
	3	4.7efg	5.7ef	9cde	4fg	2.3fg

†Means followed by the same letter in a column are not significantly different ($p < 0.05$) by New Duncan Multiple Range Test (DNMRT)

Table 4: Interaction between different medium and different concentration of BA and NAA on average shoot length (mm)in *Tanacetum cinerariaefolium*, 28 days after culture

		NAA (mg l^{-1})				
Medium	BA^{-1}	0	1	2	4	6
MS	0	62.7abc†	71.7a	75.4a	77.2a	77.7a
	0.5	49.9cd	51.6cd	60.8bc	61.8abc	62.2abc
	1	57bc	62.2abc	68.2ab	68.1ab	64.6ab
	1.5	57.6bc	61.6abc	64.2ab	61.4abc	63.1ab
	2	57.8bc	60.2bc	62.83ab	55.1bcd	60.2bc
	2.5	53.1bcd	59.7bc	55.2cd	57.4bcd	52.2bcd
	3	44.1cde	53.6bcd	59.4bc	33.8ef	23.9fg
B5	0	57.3bc	66.9ab	62.9ab	60.9bc	50.1cd
	0.5	55.8bc	59.3bc	44.8cde	33.9ef	38.5def
	1	51.5cd	50.7cd	31.1ef	44.2cde	33.6ef
	1.5	46.4cde	45.7cde	33.9ef	21.9fg	25fg
	2	39.8de	42.5de	32.4ef	27.1fg	19.6g
	2.5	37.8def	39.7de	26.7fg	27.7fg	23.5fg
	3	36.7def	37.5def	27.5fg	18.7g	12.3h
SH	0	58.2bc	60.1bc	64.9ab	63.2ab	62.8abc
	0.5	47.1cd	55.9bc	60.1bc	54.7bcd	49.3cd
	1	46.9cde	45.4cde	52.6bcd	46.9cde	45.3cde
	1.5	45.8cde	41.3de	49.8cd	42.1de	26.3fg
	2	44.4cde	32.5ef	50.6cd	27.6fg	34.6ef
	2.5	25.9fg	29.9efg	37.8def	29efg	20.6g
	3	21.7fg	22.9fg	30.5efg	28.6efg	19.7g

†Means followed by the same letter in a column are not significantly different ($p < 0.05$) by New Duncan Multiple Range Test (DNMRT)

Table 5: Effect of different concentration from BA and NAA on growth value of shoot fresh weight in *Tanacetum cinerariaefolium*, 28 days after culture

		NAA (mg l^{-1})				
Medium	BA^{-1}	0	1	2	4	6
MS	0	68.1 ef†	66.8 efg	72.7cde	78.5 abc	80.1 ab
	0.5	68.2 ef	70.1 def	68.4 ef	79.4 abc	80.5 ab
	1	72.4 cde	82.8 a	83.5 a	82.3 a	82.5 a
	1.5	76.1 bcd	84.4 a	84.4 a	84.5 a	83.9 a
	2	82.3 a	84.6 a	85.1 a	84.9 a	84.4 a
	2.5	77.8 bc	79.7 abc	80.2 ab	74.3 cd	68.9 def
	3	70.7 de	73.6 cd	73.7 cd	63.5 fgh	63.7 fgh
B5	0	66.7 efg	77.1 bc	67.5 efg	65.4 fg	60.2 ghi
	0.5	79.2 abc	81.6 ab	79.3 abc	74.5 cd	73.1 cde
	1	82.5 a	82.1 ab	80.3 ab	73.7 cd	69.1 def
	1.5	79.3 abc	80.9 ab	77.5 bc	71.2 de	68.6 ef
	2	75.7 bcd	80.3 ab	70.7 de	70.7 de	66.7 efg
	2.5	75.5 bcd	79.4abc	70.6 de	63.2 fgh	65.2 fg
	3	76.3 bcd	77.7bc	64.8 fg	63.2 fgh	61.9 gh
SH	0	63.5 fgh	68.7 ef	76.9 bc	78.8 abc	78.3 abc
	0.5	68.7 ef	76.9 bc	81.1 ab	79.8 abc	74.6 cd
	1	71.7 de	81.8 ab	78.3 abc	78 abc	74.4 cd
	1.5	79.6 abc	80.1 ab	75.1 bcd	76.1 bcd	71.8 cde
	2	72.8 cde	76.6 bc	74.7 cd	76.1 bcd	70.8 de
	2.5	71.8 cde	76.9 bc	73.7 cd	75.4 bcd	70.6 de
	3	71.5 de	73.3 cde	71.8 cde	68.2 ef	68.7 ef

†Means followed by the same letter in a column are not significantly different ($p < 0.05$) by New Duncan Multiple Range Test (DNMRT)

Root Induction from the Shoots: Isolated shoots were transferred to different medium supplemented with various concentrations of NAA for rooting. All of shoots were rooted in various medium and in various concentration of NAA. The optimal medium for rooting was B5 contained 2 mg l^{-1} NAA, on which 100% of the regenerated shoots developed roots with an average number of 16 roots per shoots within 21 days (Fig. 1f). Root induction was quick in all the auxin tried and root primordia were observed in 10-14 days. Roots induced by a high concentration of NAA were thicker and shorter, without branches; thus, the survival rate of transplanted plantlets was lower. Also, the highest number of roots per shoot and the minimum root length were observed in plant growth regulator free medium (Table 6). The rooted plantlets were hardened and after 4 weeks of hardening, the plants were transferred to greenhouse. We have observed no phenotypic variations in the regenerated acclimatized plants and they behaved normally (Fig. 1g).

DISCUSSION

Adventitious shoot formation is a reliable technique for clonal propagation as it prevents somaclonal variations in the cultures. The type of tissue or explant used for clonal multiplication also influences the chances of genetic variation. Because of the non-uniform nature of callus tissue ([13], genetic mutations are more frequent in shoots regenerated from callus, particularly with prolonged subculturing, than from other types of tissues [14]. Adventitious shoot regeneration is most preferred if *Agrobacterium*-mediated gene transfer

Table 6*: In vitro* rooting of *Tanacetum cinerariaefolium* shoots on different medium fortified with different concentrations of NAA

	No. of roots/shoot			Mean root length (mm)		
NAA (mg l^{-1})	MS	B5	SH	MS	B5	SH
0	38.3ab†	44a	44.3a	1.7e	1.3e	1.7e
0.5	28.7cd	35.3bc	33.7bc	4.7cd	5.3cd	5.3cd
1	24de	16.3efg	15.7efg	9.3bcd	9bcd	10bc
1.5	23.7de	15efg	11.3gh	10.3abc	10bc	12.7ab
2	19ef	15.7efg	13.3fgh	14.3a	16a	13.3ab
2.5	12.3fgh	9.7ghi	10.3ghi	11.3abc	10.3abc	10.7abc
3	9ghi	8.7hi	10ghi	10bc	8.3bcd	10.3abc
3.5	5.3i	4.3i	5.3i	9.3bcd	5.7cd	9.7bc

†Culture period 21 days. Means within a column followed by the same letters are not significantly different by new Duncan's multiple range test ($P > 0.05$)

is to be achieved and leaf explants are best suited for both adventitious shoot formation and *Agrobacterium*-mediated gene transfer experiments. Leaf explants taken from greenhouse grown plants exhibited shoot bud induction within 28 days. Leaves have been most widely studied for adventitious shoot formation [15, 16]. In these studies, leaves obtained the highest induction (70% with 7.4 shoots per explant). The best response of shoot organogenesis was observed from leaves of 10 mm^2 that were cultured with the abaxial side in contact with the medium. Yamanouchi *et al*. [17] reported stable regeneration of plantlets from immature leaves isolated from winter buds of field-grown mulberry. It is inferred from these studies that the size of the leaf plays an important role in its endogenous potential to regenerate adventitious buds. Our results also confirm observations by Mhatre *et al*. [18] who concluded that the upright position of leaf explants is favourable for maximum induction of shoot buds in *Morus indica* L. In this study 2, 4-D was more effective than IAA and NAA in bud induction. Variation in the activity of different auxins can be explained by their differential uptake rate reported in different genomes [19], varied translocation rates to meristematic regions and metabolic processes, in which the auxins may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as reported by Tran Thanh Van and Trinh [20] and Kaminek [21]. Our result showed that 1.5 mg l^{-1} BA and 2 mg l^{-1} NAA in MS medium is a combination suitable for multiplication. This combination produce vigorous shoots in yield of 21.3 per explant in four weeks. MS medium was better than SH and B5 medium in shoot multiplication. The same result obtained by Misava [22] that reported MS medium showed better response than other medium in Catharanthus plant. Also, study on *Denderanthema grandiflora* showed MS medium was better than other medium for proliferation. MS medium have been used successfully for study of *pyrethrum* species [3, 23-25]. Probably it is related to MS composition and best response of pyrethrum species. Result showed that the combination of cytokin and auxin is nessery for best proliferation. Similar result reported by Hitmi *et al*. [4] and Pal and Dhar [8]. In our experiment the high shoot length observed in BA free medium and addition BA to medium decreased average shoot length. It can be due to effect of BA on induction more shoot that resulted low shoot length. Our results also confirm observation of Keskitalo *et al*. [26] similar results reported about Tanacetum *vulgare*. There are numerous reports about using auxin and plant growth regulator free medium for rooting of pyrethrum [8, 24, 27 and 28]. The elongated shoots produced roots in MS medium fortified with NAA in all levels. Similar result reported by Keskitalo *et al*. [24] Pal and Dhar, [8] and Hitmi *et al*. [27]. The rooted plants were hardened and after 4 weeks of hardening, the plants were transferred to greenhouse. We have observed no phenotypic variations in the regenerated acclimatized plants and they be haved normally.

CONCLUSION

In conclusion we describe an effectiive and reproducible procedure of *Tanacetum cinerariaefolium* micropropagation via direct organogenesis and a successful adaptation of plants to greenhouse condition. This producer can play an important role in genetic transformation experiments and industrial purposes of this multipurpose medicinal plant.

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