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Regeneration of plants from leaf derived callus of *Turnera Subulata Sm.*- an Important Medicinal Herb

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ABSTRACT

A highly stable, cost-effective, efficient and successful protocol for indirect regeneration of a medicinal plant *Turnera subulata* through the leaf segment by using Murashige and Skoog (MS) nutrient medium supplemented with varied concentrations and combinations of growth hormones was established. The maximum organogenic callus initiation frequency (92%) was observed in MS medium with BAP and IAA at 12.32 and 1.8 µM/L respectively. The highest shoot initiation rate was observed in MS medium with BAP and IAA at 2.22+0.90 µM/L. The maximum shoot multiplication (82 %) was noticed on the MS medium enriched with BAP and IAA at (2.22 and 0.90µM/L) respectively. The high amount of multiple shoot (4 shoots/explant) was observed on the same combination of growth regulators. The *in-vitro* regenerated shoots were rooted successfully on MS medium supplemented with IAA alone at 2.86 µM/L. The well-developed healthy plantlets were effectively acclimatized after successive hardening. The higher survival percentage 92% of the healthy plantlets was under greenhouse condition in the hardening medium encompassed with decomposed coir waste, garden soil and vermiculate.

Keywords: *Turnera subulata*, Leaf, BAP, IAA,

1. INTRODUCTION

Medicinal plants are the very important traditional sources for chemicals used as pharmaceutical products, food colors, fragrances and flavors. Plants with high medicinal value are oldest friend for humans and medicinal plants with high therapeutic values are the backbone of renowned folk medicine [1]. Almost 80% of the world population's primary - health care need are fulfilled by plant based phyto medicines. More than 25 % of the drugs are plants based phyto-compounds and 87% of human cancer therapy treatments depend on plant based products [2].

Plant tissue culture plays a major role in the development and commercial production of pathogen-free plants [3], and helps in the germplasm conservation of rare and endangered species [4]. The biotechnology applications like tissue culture plays an important role in the propagation of selected genotypes [5]. The seed borne pathogens may continue over the next generation, to overcome that problem plant tissue culture offers an efficient protocol to achieve disease free plantlets and germplasm conservation. The necessity and efficiency of tissue culture in plant breeding has been extensively recognized and used for crop improvement program. A single cell can be regenerated into an individual plant [6]. The plant regeneration through tissue culture is required for genetic manipulation of commercially important plants.

Turnera subulata belongs to the passion flower family, recently moved to Turneraceae family, which is one among the 28 angiosperm family displays heterostyly [7]. *Turnera* is one among the most important genera of the family Turneraceae which encompasses more than 100 species, which are grouped into nine small series [8]. *Turnera* are most extensively distributed in the tropical and subtropical region of Asia and Africa [9]. *T. subulata* is a polymorphic polyploid complex of perennial weeds commonly called 'Butter cup, sulphur alder and white alder'. This variety of plants is a compact, thick herb with dark green foliage and light yellow or white flower with dark base. *Turnera* is most widely adopted as an ornamental garden plant [10]. In folk medicine *Turnera* is taken as tea decoction for the treatment of disease related mainly to gastric dysfunction, skin infection, respiratory ailments and many research data indicating that the plant extract has a important anti-ulcerogenic effect [11]. In this view, the present study was to designed, a simple, disease free and cost-effective protocol for *in-vitro* regeneration of *T. subulata* through callus culture, which would be very much useful in the efficient propagation of this important medicinal plant.

2. MATERIALS AND METHODS

Study species were collected near Maruthamalai hills, Coimbatore. Young, healthy, and disease free portion of the branches was selected and used as explants. Healthy leaves were selected as explant. The selected leaves were washed thoroughly under running tap water for 15 minutes to wash off all the dust, dirt's and microbes present on the surface of the leaf sample. The leaf sample was separated and washed with a detergent solution (Tween 20) for 10 minutes. Followed by, the leaves were thoroughly washed under running tap water until all the traces of detergent solution were completely removed. All other steps of surface sterilization were carried out under sterile conditions in the laminar air flow chamber. The leaves were then subjected to 70% ethanol treatment for 30 seconds and then rinse with sterilized double distilled water for at least three times, followed with mercuric chloride (0.12%w/v $HgCl_2$) solution treatment for 3 minutes and rinsed with sterile double distilled water for three times. Surface sterilized leaf explants were properly trimmed on a sterile seed germination sheet. The surface sterilized and trimmed leaf explants were inoculated on MS medium with (BAP, IAA, 2,4-D) different concentration and combination of growth regulator for callus induction.

2.1. Shoot initiation and multiplication

Callus from primary cultures were transferred to MS medium containing different concentration of BAP, IAA for shoot initiation. The shoots initiated from callus was subcultured on MS medium supplemented with varied concentration of BAP and IAA for multiple shoot induction. Twenty explants were used for each culture. The number of explants responding for shoot formation were documented after 5 weeks. In the succeeding subcultures, the multiple shoots induced from the leaf callus derived *in-vitro* produced shoots were recorded.

2.2. Rooting of in vitro produced shoots

The well-developed multiple shoot after reaching 5-6 cm height were cut down from the base part were separated in to an individual shoot. The separated shoots were transferred to the rooting medium containing different concentration of Kn, IAA and IBA. All the subculture bottles were incubated in appropriate culture condition with proper temperature and lighting, until complete root formation from the micro shoots. Finally, the percentage of rooting were documented.

2.3. Acclimatization of plantlets

The healthy *in-vitro* propagated plantlets were carefully removed from the culture bottles, washed thoroughly and carefully with tap water to remove agar and dipped in fungicide for a 3-4 minutes. Then the plantlets were transferred to a net pot contains different type of harding media and survivability rate of the plantlets were determinate after 20 days of step-wise hardening process. The survival frequency of the plantlets was recorded after 60 days.

3. RESULTS AND DISCUSSION

The morphogenic response of leaf explants was observed on MS medium [12] containing different concentration and combination of BAP and IAA. MS medium without any growth hormones showed no callus initiation. The callus initiation and proliferation rate was wide in MS medium supplemented with different contraction and combination of growth regulators like BAP, IAA and 2,4-D (Table-1; Figure-1A). The range of callus induction response varied with different concentration of growth regulators, while all the concentration and combination showed satisfactory response (Figure-B, C). The highest percent of callus induction (92%) was achieved in MS medium enriched with BAP and IAA at 12.32 and 1.8 M /L respectively after 20 days of inoculation (Figure-F). A similar kind of result was reported for the species, *Tylophora indica* [13] and *Withanian somnifera* [14].

The sub culturing experiments were conducted by using callus as the secondary explants for shoot proliferation on MS basal medium containing different concentration and combination of growth regulators, BAP, IAA. Shoot initiation was observed after 3 to 4 weeks of sub culturing leaf derived callus. The highest amount (85.50%) of shoot initiation was observed on MS medium supplemented with BAP and IAA at 2.22 and 0.90 µM/L respectively (Table-2). Follows that 81.33% of response was observed on MS medium containing BAP and IAA at 4.44 and 0.90 µM/L respectively. The other concentration BAP, IAA produced satisfactory results. Further, the increasing concentration of IAA in MS medium registered decreasing culture response. The result reported that, the different explant of the study species induced shoots at low concentration of growth regulators in the MS medium [20] (Table 2: Figure-G) .

The *in-vitro* produced shoots was subcultured in MS medium enriched with different concentration of BAP and IAA for shoot multiplication. The highest (82.33%) shoot multiplication was observed on the medium containing, BAP and IAA at 2.22 and 0.90 µM/L respectively. Whereas the highest number (4.33 shoots) of shoots/ explant and higher shoot length (4.86 cm) was observed in the MS medium enriched with BAP and IAA at 2.22 and 0.90 µM/L respectively (Table.3; Figure-H). However, the MS medium containing high concentration of growth regulator registered low percentage shoot multiplication. The varying response of explant for shoot multiplication depends on endogenous levels of growth hormones. The systematic effect of growth regulators (auxin and cytokinin) has been demonstrated in several medicinal plants viz., *Petasites hybridus* [15], Dieffenbachia [16] and *Hypericum perforatum* [17]. The most interesting thing is the main activity of cytokinin's is not only the small cell population maintenance but also it enhances the further growth of cells into tissues and organs. The high auxin also plays a major role in redirecting the cytokinin stirred growth into new organ formation [18]. *In-vitro* produced and multiple shoots were transferred to MS medium supplemented with different concentration of Kn, IAA and IBA for root induction. Root initiation from the base end of the regenerated shoots were observed after one week, without any callus formation. The highest rooting percentage (87.34%) with greatest number of roots per shoot and increased root length (5.66 cm) was observed in the MS medium supplemented with IAA alone at 2.86 µM/L (Table-4). It indicates that the growth regulator, auxin is most essential for rooting attribute. Similar kind of results was reported in medicinal plants like *Disporum leschenaultianum* [19]. The well-developed healthy plantlets raised through indirect regeneration were transferred to pots containing different combination of hardening substrate (Table-5). The hardening medium composed of decomposed coir waste, garden soil and vermiculite in the ratio of (1:1:1) was observed with higher survival percentage (92%) (Figure-I). The hardening is recommended for acclimation before transferring the plantlets to the natural communities.

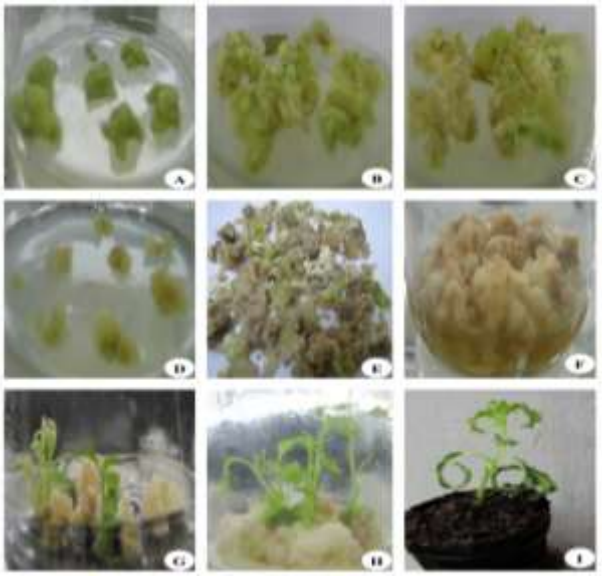

Figure 1. *In- vitro* regeneration of *Turnera subulata* through leaf explants. A-Callus formation; B & C - development of callus; D-Subcultured callus; E & F- Mass production of callus; G – Shoot formation from callus; H – Shoot development; I - Hardening

Table 1. Effect of various concentration of growth regulators on callus induction from leaf explants of the species, *Turnera subulata*

Growth Regulators (µM/L)			Days required for callus formation after inoculation	Callus formation (%)
BAP	**IAA**	**2, 4-D**		
2.22	0.90	-	6	21±0.83
4.44	0.90	-	12	33±0.57
6.66	0.90	-	15	40±0.62
8.88	0.90	-	16	41±0.36
10.10	0.90	-	17	49±0.54
12.32	0.90	-	19	71±0.14
2.22	1.8	-	24	41±0.92
4.44	1.8	-	25	53±1.12
6.66	1.8	-	23	61±0.93
8.88	1.8	-	22	72±0.82
10.10	1.8	-	20	84±0.76
12.32	1.8	-	20	92±0.84
2.22	-	0.3	13	27±0.37
4.44	-	0.5	14	30±0.74
6.66	-	1.0	15	47±0.63
8.88	-	1.5	17	62±0.55
10.10	-	2.0	10	72±0.28
12.32	-	2.5	09	74±0.56

Table 2. Effect of different concentration of growth hormones added to the MS medium on shoot initiation from leaf explant derived callus of *Turnera subulata*.

Growth Regulators (µM/L)		Days required for shoot initiation	Shoot Initiation (%)
BAP	**IAA**		
2.22	0.90	32	85.50±0.81
4.44	0.90	23	81.33±0.53
6.66	0.90	21	71.33±0.51
8.88	0.90	19	53.66±0.42
10.10	0.90	17	37.66±0.61
12.32	0.90	9	10.83±0.46
2.22	1.8	25	70.16±0.04
4.44	1.8	25	67.50±0.54
6.66	1.8	23	51.66±0.51
8.88	1.8	22	38.33±0.75
10.10	1.8	20	21.16±0.42
12.32	1.8	19	15.33±0.46
2.22	2.7	22	52.16±1.04
4.44	2.7	21	41.16±0.56
6.66	2.7	20	30.50±0.39
8.88	2.7	19	28.83±0.63
10.10	2.7	17	18.50±0.75
12.32	2.7	15	10.16±0.81

Table 3: Effect of different concentration of growth regulators on percent of shoot multiplication, shoot number and shoot length after sub culturing of *in vitro* leaf callus derived shoots of *Turnera subulata*.

Growth Regulators (µM/L)		Percent explants with multiple shoots	No. of shoots/ explant	Shoot length (cm)
BAP	IAA			
2.22	0.90	82.33±1.36	4.33±0.51	4.86±0.51
4.44	0.90	80.00±0.89	3.66±0.81	1.50±0.54
6.66	0.90	67.83±0.75	2.33±1.03	4.83±0.75
8.88	0.90	50.66±0.42	2.83±0.75	4.33±1.03
10.10	0.90	39.55±0.54	2.16±0.75	3.33±0.81
12.32	0.90	20.33±0.33	1.66±0.33	2.50±0.54
2.22	1.8	60.00±0.63	2.00±0.44	0.91±0.04
4.44	1.8	58.66±0.51	2.33±0.33	1.50±0.54
6.66	1.8	50.50±0.54	2.66±1.03	2.00±0.81
8.88	1.8	39.50±0.54	3.33±0.51	3.00±0.63
10.10	1.8	18.33±0.51	3.00±0.63	2.66±0.81
12.32	1.8	10.16±0.40	3.16±0.30	2.33±0.51
2.22	2.7	45.83±0.75	0.85±0.71	0.83±0.75
4.44	2.7	40.66±0.81	1.00±0.63	1.51±0.03
6.66	2.7	29.66±0.49	1.33±0.51	1.50±0.54
8.88	2.7	26.33±0.33	1.50±0.54	1.83±0.75
10.10	2.7	19.33±0.51	2.00±0.57	2.83±0.75
12.32	2.7	16.00±0.57	2.00±0.63	2.50±0.54

Table 4: Effect of different concentration of growth regulators on rooting percentage, root number and root length after subculturing of shoots of *Turnera subulata*.

Growth Regulators (µM/L)			Percent Shoots rooted	Number of roots/shoot	Root length (cm)
Kn	IAA	IBA			
5.35	-	-	46.53±0.33	3.66±0.47	2.85±0.69
10.74	-	-	34.82±0.04	2.33±0.04	2.00±0.51
15.05	-	-	23.62±0.30	2.00±0.42	2.00±0.69
20.40	-	-	21.43±0.63	1.16±0.36	1.00±0.63
25.75	-	-	13.16±0.42	0.80±0.13	0.85±0.75
-	2.86	-	87.34±0.75	6.00±0.51	5.66±0. 75
-	5.72	-	84.83±1.03	5.5±0.61	4.83±0.63
-	8.58	-	44.66±0.81	4.33±0.63	3.26±0.63
-	11.44	-	24.16±0.03	2.83±0.75	2.42±0.89
-	14.30	-	10.16±0.63	1.50±0.83	1.53±0.69
-	-	2.46	70.33±0.52	4.16±.63	3.85±0. 81
-	-	4.92	63.83±0.81	3.26±0.54	3.66±0.75
-	-	6.38	51.66±0.75	2.50±0.51	2.85±0.04
-	-	8.84	24.16±0.32	2.00±0.75	3.00±053
-	-	10.30	50.16±0.63	1.16±0.83	1.16±0.02

Table 5: Effect of different composition of hardening medium on survivability of plantlets of *Turnera subulata.*

Hardening medium composition (v/v)	No. of plants under hardening	No. of plants survived	Percentage of survivability
Garden soil	50	26	52
Vermiculite	50	34	68
Decomposed coir waste	50	42	84
Decomposed coir waste: garden soil: vermiculite (1:1:1)	50	46	92

4. CONCLUSION

The present investigation we have formulated a rapid, disease free, efficient and cost-effective protocol for indirect regeneration of *T.subulata.* Moreover, the initiation of callus in to a healthy plantlet was completed within 18 weeks.

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Conflicts of Interest

There are no conflicts of interest.

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