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In vitro callus induction and plants from leaf explants of *Wattakaka volubilis* L. (Staf. Arn)- An vulnerable medicinal plant

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Abstract
Callus regeneration from leaf explants of *Wattakaka volubilis* an important medicinal plant inoculated on MS medium supplemented with 0.6mg/l BAP and 0.2mg/l NAA, produced numerous (26.4±0.48). Shootlets with an average length of 9.6±0.04 cm. Small shootlets were transferred to shoot elongation medium supplemented with 0.2mg/l BAP plus 0.4mg/l GA_3. Elongated shoots produced roots within 6 weeks in half strength MS basal medium supplemented with 0.6mg/l IBA and 3% (w/v) sucrose (12.2±0.40). Rooted plantlets were acclimatized with a survival rate of 95% and transferred to the green house and thereafter successfully transferred to the field with 90% survival rate.

Keywords: *Micropropagation, callus regeneration, plant growth regulators and Wattakaka volubilis.*

Introduction
Medicinal plants are important source of traditional and synthetic medicines containing different types of organic compounds having therapeutic properties. Approximately 80% of people in developing countries still rely on traditional medicine for their primary health care. This usually involves the use plant extracts [23]. Many medicinal plant species are disappearing at an alarming rate, as a result of rapid agricultural and urban development, deforestation and indiscriminate collection. The tissue culture technique has been proved very efficient in rapid mass propagation and conservation of these rare and endangered medicinal plants [1, 13, 4].
The medicinal plant, *Wattakaka volubilis* is an important member of the family Asclepiadaceae. It is a fleshy, large climber with green flowers in drooping umbels and found throughout the plains. This plant is used in the treatment of various ailments since ancient time [11]. The literature survey revealed that among the various saponins obtained from the stem and flowers of this plant, two compounds are active against Enrlich's ascites carcinoma [27, 14].
The roots contain a glucoside, which lowers carotid blood pressure in mice and dogs when administered intravenously. The extract from the root is also applied to care snake bites and given to women to care headache after child birth. The leaves are common ingredients of many folk and herbal medicines [8, 19]. Leaf extract has been reported to possess pharmacological activity including anti-inflammatory activity [9]. *W.volubilis* is used for treating rheumatic pain cough, fever and severe cold. [15]. Earlier *in vitro* propagation of *W.volubilis* was attempted through organogenesis from different explants such shoot tip and nodal [3, 25]. There is no published information on the callus regeneration of *W.volubilis*. This report established methodology for organogenesis and callus regeneration of *W.volubilis* from leaf explants.

Materials and Methods
Plant Material
The plants of *W.volubilis* were collected from Cauvery river basin in Tiruchirappalli district of Tamil Nadu, India and maintained in earthen pots in the glass house of The Rapinat Herbarium, St. Joseph's College Tiruchirappalli under controlled conditions (Temp 26±2°C and RH 70-75%). Explants were collected from these plants after stabilization.

Surface sterilization
Leaf explants (2-3cm length) were washed under running tap water for 30min and surface sterilized with 70% ethanol for 25s, rinsed with sterile distilled water and treated with Tween 20 (15 drops per 100ml of sterilized distilled water) for 10min with a final rinse in sterile distilled water thrice for 5min. These sterilized explants were inoculated on culture medium.

Culture medium and conditions

Murashige and Skoog [6] medium containing 3 % (w/v) sucrose, 0.8% agar (Hi-media, Mumbai) was supplemented with different growth regulators alone or in combinations as specified below. The pH of the medium was adjusted to 5.6±0.2 and dispensed into tissue culture flasks (100ml) or tubes (25 X 150 mm) and autoclaved at 121°C for 15 min. following inoculation all the culture were incubated at 24±2°C under 16h photoperiod at 80% relative humidity.

Callus induction

For callus induction explants were placed on MS medium supplemented with 2,4-D (0.2-1.2mg/l), KIN (0.2-1.2mg/l) BAP (0.2-1.2mg/l), NAA (0.5-2.5mg/l) and IAA (0.5-2.5mg/l). Callus cultures were subcultured at 3 week intervals on respective media.

Shoot induction and multiplication

For shoot induction, green nodular calli (approximately 500mg fresh weight per piece) were selected and cultured on MS medium supplemented with BAP (0.2-1.2mg/l), KIN (0.2-1.2mg/l), BAP with NAA (0.6+0.1-0.6mg/l) and KIN with NAA (0.4+0.1-0.6mg/l) alone or combination. After 4 weeks of culture, the frequency of calli producing shoots was recorded and all the cultures were subcultured on fresh medium as before.

Well-developed multiple shoots were transferred to MS medium supplemented with different concentrations and combinations of GA_3 (0.1-1.6mg/l), BAP (0.1-0.6mg/l) and KIN (0.1-0.6mg/l) for shoot elongation.

Rooting and Acclimatization

For root induction separated shoots were transferred to half strength MS basal medium supplemented with different concentration of IBA 0.2-1.2mg/l, IAA 0.2-1.2mg/l and NAA 0.1-0.6mg/l and 2% (w/v) sucrose. Rooted plantlets were thoroughly washed to remove the adhering gel and planted in specially made paper cups containing sand, garden soil and farmyard manure (1:2:1) and kept in the green house for acclimatization.

Results and Discussion

W.volubilis leaf explants were cultured on MS medium supplemented with different concentrations of NAA (0.5-2.5mg/l), BAP (0.2-1.2mg/l), IAA (0.5-2.5mg/l), 2,4-D (0.2-1.2mg/l) and KIN (0.2-1.2mg/l) individually. Leaf explants were enlarged in size and thickened within a week time. Callus was initiated from cut region of the explants after one week (Figure 1a). 2,4-D containing MS medium induced yellowish with less compact callus. Different concentration KIN, NAA, BAP and IAA containing MS medium induced brown with less compact, brown yellowish with compact and pale yellow or green with less compact respectively (Table1; Figure 1b,c). Significant callus formation was observed with 15-20 days at the cut ends of the explants. Among the different concentration of growth regulators (Auxin & Cytokine) used for callus induction. 1.0mg/l 2,4-D, 2.0mg/l KIN, 1.5mg/l BAP, 1.0mg/l NAA and 2.0mg/ IAA were found to be optimum, while increasing their growth hormons (Auxin & Cytokine) above this level reduced callus proliferation. Vinothkumar *et al*., [24], Sundar and Jawhar [21] observed similar results in *Psudarthria viscida* and *Solanum xanthocarpum*. Muthukumar and Arockiasamy [7] reported that the 2.0mg/l BAP induced high amount callus in *Datura metal*. Pawar *et al*., [10] observed that BAP and KIN were more effective for induction of organogenic callus form leaf explants of *S. surattense*.

Table 1: Effect of different concentrations of Auxins (2,4-D, NAA and IAA) and Cytokinins (BAP and KIN) on callus induction, color and compactness from leaf explants on MS medium.

Plant growth regulators	Callus Induction, Color and Compactness		
2,4-D	X	Y	Z
0.2	Y	LC	4
0.4	Y	LC	5
0.6	Y	LC	5
0.8	Y	LC	5
1.0	Y	LC	6
1.2	Y	LC	5
KIN			
0.2	B	LC	1
0.4	B	LC	1
0.6	B	LC	1
0.8	B	LC	2
1.0	B	LC	1
1.2	B	LC	1
BAP			
0.2	YG	LC	4
0.4	YG	LC	4
0.6	YG	LC	6
0.8	YG	LC	3
1.0	YG	LC	3
1.2	YG	LC	3
NAA			
0.5	YB	C	3
1.0	YB	C	4
1.5	YB	C	3
2.0	YB	C	2
2.5	YB	C	2
IAA			
0.5	PY	LC	2
1.0	G	LC	2
1.5	PY	LC	3
2.0	G	LC	5
2.5	PY	LC	3

X- Color of the callus: Y-Yellow, G-Green, B-Brown, YG-Yellowish Green, YB-Yellowish Brown, PY-Pale Yellow. Y- Nature of Callus: C-Compact, LC-Less Compact.

Z- Mass of the Callus scored in a scale of 1-6 in which 1 indicated less and 6 more.

Multiple shoots and regeneration

For shoot multiplication and regeneration light green compact supplemented with different concentration and combination of BAP (0.2-1.2mg/l) KIN (0.2-1.2mg/l) and NAA (0.1-0.6mg/l). Initially two or three shoot buds per callus emerged 15days after inoculation and gradually the number of shoot buds per callus increased up to 25-27 (Figure1d; Table 2). Among the three different treatment combination, after four weeks of incubation higher number of multiple shoots (26.4±0.40) along with 85 survival rate was observed in MS medium supplemented with 0.6mg/l BAP combination of 0.4mg/l NAA (Figure1e;Table 2). Many authors have reported that a combination of BAP and NAA are required for shoot multiplication in *Gentiana kurroo* [16], *Pinellia ternate* [22], *Pseudarthira viscida* [24, 26], *Wattakaka volubilis* [25] and *Momordica charantea* [20]. When treated with high concentration of KIN, the callus did not produce multiple shoots; hence colour was changed from light green

to brown colour. In the same concentration of KIN the callus produced very low multiple shoots [18, 24]. The healthy shoots (3-5 cm long) were transferred to MS medium supplemented with different concentrations and combinations of GA_3 (0.1-0.6mg/l), BAP (0.1-0.6mg/l) and KIN (0.1-0.6mg/l). Among the three different concentrations, GA_3 along with BAP showed excellent shoot elongation. In this combination, the highest success rate (94%) with maximum shoot length (9.6±0.04) was observed with 0.4mg/lGA_3 and 0.2mg/l BAP after four weeks of inoculation (Table 2; Figure1f).

Table 2: Effect of different concentrations of BAP, KIN, BAP+NAA, KIN+NAA on callus induction in leaf explants of *W.volubilis.*

Plant Growth regulators (mg/l)	Percentage of Responding cultures	Mean No. of shoots/ explants	Mean shoot/length (cm)
BAP			
0.2	65	6.0±0.59	-
0.4	78	7.0±0.48	-
0.6	81	8.6±0.72	-
0.8	76	7.2±0.65	-
1.0	68	5.8±0.53	-
1.2	59	4.4± 0.51	-
KIN			
0.2	57	4.9±0.62	-
0.4	61	5.6±0.80	-
0.6	54	4.5±0.75	-
0.8	47	4.0±0.48	-
1.0	44	2.9±0.40	-
1.2	38	2.4±0.40	-
BAP+NAA			
0.6+0.1	82	22.3±0.48	-
0.6+0.2	92	26.4±0.40	-
0.6+0.3	81	21.1±0.40	-
0.6+0.4	73	17.9±0.11	-
0.6+0.5	65	16.2±0.60	-
0.6+0.6	60	10.2±0.17	-
KIN+NAA			
0.4+0.1	62	9.5±0.12	-
0.4+0.2	69	13.0±0.12	-
0.4+0.3	75	6.5±0.32	-
0.4+0.4	81	19.5±0.74	-
0.4+0.5	72	12.2±0.64	-
0.4+0.6	64	8.4±0.82	-
GA_3			
0.1	68	-	6.8±0.07
0.2	73	-	7.5±0.22
0.3	82	-	7.8±0.07
0.4	91	-	8.8±0.13
0.5	74	-	7.1±0.19
0.6	67	-	6.9±0.08
GA_3+BAP			
0.4+0.1	84	-	8.2±0.10
0.4+0.2	94	-	9.6±0.04
0.4+0.3	75	-	8.8±0.14
0.4+0.4	72	-	8.3±0.10
0.4+0.5	71	-	7.9±0.04
0.4+0.6	64	-	7.0±0.13
GA_3+KIN			
0.4+0.1	66	-	1.6±0.14
0.4+0.2	73	-	1.8±0.11
0.4+0.3	78	-	3.0±0.89
0.4+0.4	88	-	3.9±0.60
0.4+0.5	80	-	3.5±0.17
0.4+0.6	60	-	2.8±0.13

Well-developed shoot elongations were excised from culture tubes and cultured on half strength MS containing different concentrations of IBA (0.2-1.2mg/l), IAA (0.2-1.2mg/l) and NAA (0.2-1.2mg/l). The percentage of root frequency, number of roots per shoot and length of roots were recorded after 4-5 weeks of culture. The rooting response to different auxins treatments is shown in Table 3. Of the three types of auxins, IBA was found to be comparatively more effective than the other two auxins (IAA and NAA). IBA (0.6mg/l) was found to be the best concentration of auxin for proper rooting where 95% of the shoots rooted with 6 weeks of culture. The highest average number of roots was (12.2±0.40) with root length (13.5±0.13cm) (Table 3;Figure1g). Many authors have reported IBA as the best rooting auxins for *Anethum graveolens* [17], *Thapsia garganica* [5], *Anisochilus carnosus* [2], *Annona sequamosa* [12], *Pseudarthira viscida* [24, 26] and *Wattakaka volubilis* [25]. At higher concentrations of auxins profuse callus was produced at the basal end of microshoots which inhibited the growth and elongation of roots.

Table 3: Effect of different concentrations of IBA, IAA and NAA on rooting

Plant growth Regulators (mg/l)	Percentage of Responding cultures	Mean No. of Roots / explant	Average Root length (cm)
IBA			
0.2	60	9.3±0.47	10.0±0.12
0.4	90	11.6±0.80	11.4±0.28
0.6	95	12.2±0.40	13.5±0.13
0.8	80	10.4±0.48	11.0±0.19
1.0	75	9.4±0.48	10.2±0.25
1.2	50	8.0±0.63	9.4±0.37
IAA			
0.2	50	3.4±0.48	2.3±0.07
0.4	60	3.8±0.68	3.1±0.16
0.6	65	4.0±0.63	3.6±0.14
0.8	80	4.8±0.74	3.7±0.14
1.0	55	3.0±0.63	3.0±0.14
1.2	45	2.2±0.40	2.6±0.13
NAA			
0.2	60	1.8±0.74	2.8±0.10
0.4	75	2.4±0.48	3.1±0.13
0.6	55	2.2±0.40	2.9±0.10
0.8	40	1.8±0.74	2.7±0.04
1.0	30	1.4±0.48	2.4±0.06
1.2	25	1.2±0.40	2.0±0.13

For plantlets were transferred to *in vivo* conditions. The plantlets were carefully taken out and then washed with tap water to remove the traces of agar stick into the roots. Care was taken not to injure the roots while washing. The plants were transferred to paper cups containing sand, garden soil and farmyard manure in the ratio of 1:2:1 (Figure1h) and covered with plastic bag for one month in culture room, half or quarter strength MS medium was added twice a week. After one month plants were transferred to plastic bags and maintained to room temperature for 10 days and later moved to the green house.

Fig 1: *in vitro* multiplication of *Wattakaka volubilis* through indirect regeneration. a,b and c) induction from stem explants in MS+BAP (0.6mg/l); d and e) Shoot multiplication in MS+BAP+NAA (0.6mg/l+0.2mg/l); f) MS+GA_3+BAP (0.4mg/l+0.2mg/l); g) Root development in half MS+IBA (0.6mg/l); h) Hardening (sand + garden soil + farmyard manure, 1:2:1)

Conclusion

We have developed an efficient method for shoot regeneration from callus derived from leaf explants of *W.volubilis*. Development of regeneration protocols for medicinally important species will facilitate access to natural and induced variations.

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