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IN VITRO MULTIPLICATION OF CLINACANTHUS NUTANS (BURM.F) LINDAU. - A MEDICINAL PLANT

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Abstract

The objective of this study was to develop a rapid system for generation of the important thai medicinal plant, Clinacanthus nutans from nodal explant. Single nodal explants were inoculated on basal MS medium containing 3% (W/V) sucrose, supplemented with different concentration and combinations of 6- benzyl aminopurine (BAP), Indole-3-acetic acid (IAA) and indole -3 butyreric acid (IBA) for direct plant regeneration. Maximum number of shoots was observed on the medium containing 0.5 mg/L BAP after four weeks of culture. Regenerated shoots were separated and rooted on same half strength MS medium of supplemented with 1.5 mg/L alone for three weeks. Rooted plantlets were planted in sterile soil mix and gradually acclimatized to the green house environmental conditions. Hardened plantlets were maintained in the green house for the field transplantation.

Keywords: *Clinacanthus nutants; MS medium; In vitro; Thai*

Introduction

The Genus Clinacanthus belongs to family Acanthaceae and consists of two species of herbs widely distributed in areas of South China to Malesiz [1]. C*linacantus nutans* (Burm.F) Lindau has been reported to be used by Local ethnic community. Tender parts of this plant cure snake bite [2]. Fresh leaves are used externally for treatment of skin rash, especially genital lesions caused by herpes simplex (HSV) and varicella zoster (VZV) viruses. In support, leaf extracts have shown significant inhibitory activity. In *vitro* virucidal models and several clinical trials have reported to have a cream containing *C. nutans* extract for the treatment of genital herpes in patents [3]. In nature, propagation of this plant is by seeds, but the problem is very poor flower setting. So far, there is no report on *in vitro* method of propagation for this plant in order to improve its cultivation. Our objective was to develop a tissue culture protocol for Thai plant using nodal explants from mature plants. Therefore this first report on *in vitro* multiplication of *C.nutans* through direct plant regeneration technique offers an effective alternative method of propagation for this important multipurpose medicinal plant. Such a micropropagation process involving bud multiplication from shoot tips, axillary buds or nodes possesses less probability of somoclonal variation among regenerates in comparison with callus mediated regeneration pathway. According to our knowledge however there is no such protocol available for clonal propagation of *C. nutans*. In this present study a standard protocol for micropropgation has been raised by the use of explant.

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Materials and Methods

Explants were collected from two years old field grown mature plants, cut into to 1.0 to 2.0cm nodal segments and used for induction of multiple shoots. Explants were washed thoroughly under running tap water for 30 min and treated with a surfactant, Tween 20 (10 drops per 100mL of sterilized distilled water). Later these explants were surface sterilized with 0.1% Mercuric Chloride (w/v) for 5 min and washed thrice using sterilized distilled water. Under aseptic conditions, explants were inoculated on basal MS 4 medium and combinations of 6 benzylaminopurine (BAP: 0.5, 1.0, 1.5 and 2.0 mg/L), Kinetin (KIN: 0.5, 1.0, 1.5 and 2.0 mg/L), Indole-3 acetic acid (IAA) and Indole -3-butyric acid (IBA), (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) for direct plant regeneration and root induction. The pH was adjusted to 5.8 prior to the addition of 3% agar and autoclaved at 121º C for 15 min. Cultures was then incubated at 26 ± 2ºC with at 16h photoperiod by cool white fluorescent tubes and 70-75% relative humidity. After 45 days, shoot buds were transferred to hormone free media for shoot growth and elongation. The isolated plantlets were planted on MS Media containing 0.5 to 2.0 µM IAA and 0.5 -1.5 µM IBA for rooting. Rooted plantlets were thoroughly washed to remove the adhering gel and planted specially made plastic cup containing soil rite and kept in the green house for acclimatization. Twenty cultures were used per treatment and each experiment was repeated at lease three times. Percentage of success was scored four weeks after culture. Data collected were statically analyzed and resulted presented in the tables 1 and 2.

Table 1. Effect of Cytokinins on shoot production from the nodal segments of *Clinacanthus nutans* (Burm.F)Lindau. on MS medium

Sl. No.	BAP concentration (mg/L) BAP Kin	Percentage of shooting	No. of shoots/node±SD (after 5 weeks of culture)	Mean length of shoots (cm)
1.	0.50	95	12.0 ±0.5	2.36±0.185
2.	1.00	90	11.9± 0.5	1.62±0.189
3.	2.00	70	11.0 ± 0.4	1.95±0.404
4.	0.5	50	6.2 ± 1.6	2.20±0.496
5.	1.0	65	7.0 ± 0.4	2.03±0.536
6.	1.5	70	8.0 ± 0.5	2.07 ±0.500
7.	2.0	70	7.9 ±0.5	2.00±0.489

Table 2. Effects of auxins on rooting of *in vitro* shoots of *Clinacanthus nutans* (Burm.F) Lindau. in half strength MS medium

Sl. No.	Concentration (µM/L) IAA IBA	Percentage of rooting response	Mean no. of roots / shoot lets±S.D.	Mean length of rootlets (cm)
1.	0.5	80	1.5 ± 0.09	3.2 ± 0.30
2.	1.0	90	2.5 ± 0.31	3.6± 0.62
3.	1.5	95	2.0 ± 0.29	3.5 ± 0.27
4.	2.0	90	1.8 ± 0.15	3.1 ± 0.50
5.	0.5	75	1.5 ± 0.10	2.4± 0.29
6.	1.0	70	1.6 ± 0.19	3.1 ± 0.42
7.	1.5	75	2.0± 0.28	2.25 ± 0.26

Each experiment was performed with 10 replicates and was repeated thrice.

Results and Discussions

The nodal explants of *C. nutans* were collected and inoculated on MS media fortified with different concentration (BAP and KIN 0.5 - 2.0 mg/L). The responses of nodal segements are presented in Table 1. MS basal medium was not effective in inducing shoot buds.

Incorporation of BAP into the medium caused the production of 95% shoots per culture (Fig. 1). BAP is considered to be one of the most useful cytokinins for achieving the multiplication and microprogation of the plants. *A.M.S. Pereira* et al. [5] and *A. Dewan et al.* [6] observed the important role of BAP in stimulating multiple shoot formation in other species. Lack of BAP mostly produced a single shoot, but the addition of 0.5 mg BAP significantly stimulated shoot multiplication. Maximum shoot number, 12 shoot per plant was obtained on a medium containing 0.5mg/L. High shoot number was obtained with 0.5 mg/L BAP, regardless of kinetin concentration.

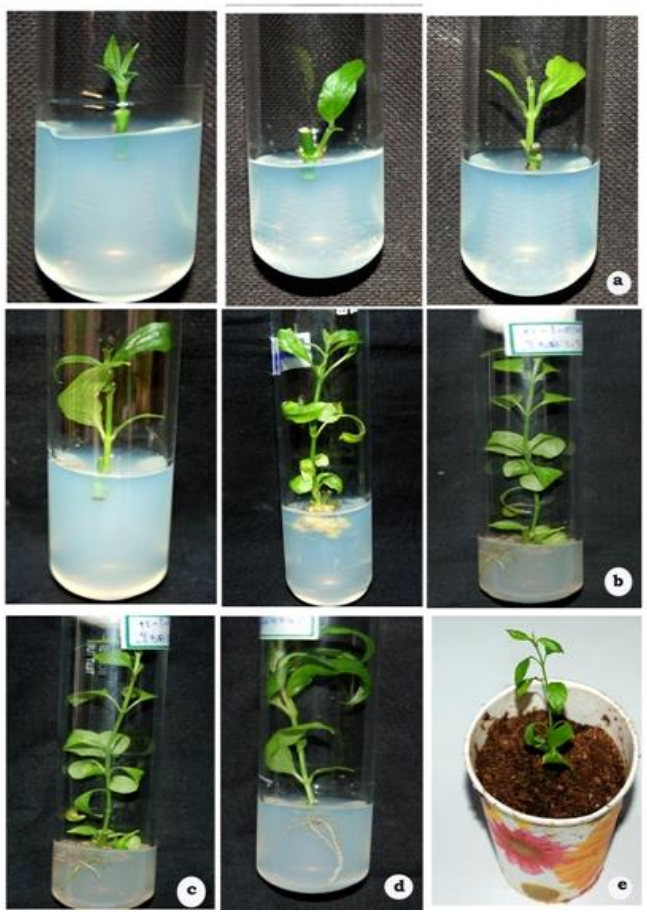

Fig.1. Micropropagation stages of *Clinacanthus nutans* (Burm.F) Lindau
a. Shoot initiation from nodal segment of *Clinacanthus nutans* on M.S Medium containing BAP and Kinetin;
b. Shoot elongation from nodal Segement; c & d. Rooting of shoots on MS Medium Containing IBA;
e. Hardened plantlets of *Clinacanthus nutans*

Kinetin was less effective than BAP in inducing shooting buds (Table 1). The superiority of BAP over KIN has also been reported in *Tecomella undulate* and *Albizzia lebbeck*. There was no improvement in the number of shoots and their quality on BAP + KIN containing medium. The best response was obtained on MS medium supplemented with BAP in which an average of 95% shoots per culture was produced in 16 days. At higher concentration BAP caused the production of few shoots with stunted growth. Transfer of shoots (< 3cm long) to MS medium without growth hormones resulted in continued elongation of shoots (< 0.5cm in

7 days. The number of nodes / shootlets and length of the shoots proliferated from the cultures were used as a better source of explant for further multiplication.

The stimulatory effect of a singular supplement BAP on bud break and multiple shoot formation in *C. nutans* was similar to that reported earlier in other medicinal species including *Ceropegia bulbosa* [7], *Saussurea involucrata* [8], *Cunila galioides* [9], *Plantago major* [10], *Piper longum* [11], *Phyllanthus caroliniensis* [12], *Centella asiatica* [13], *Calendula officinalis* [14], *Ruscus aculeatus* [15], *Lilium nepalense* [16], *Phyllanthus urinaria* [17], *Lippia alba* [18], *Adenophora triphylla* [19], *Wedelia chinensis* [20], *Holestemma annulare* [21], *Plumbago zeylanica* [22].

For induction of rooting, the shoots from MS basal medium were transferred to MS + IBA and IAA. In the preliminary rooting experiment MS salts at full, ½ and 1/10 strength were tested. Average number of roots obtained on these media was 0.5, 1.0, 1.5 and 2.0 respectively. Since ½ strength gave better response, only this concentration of roots in shoots has been reported in many systems. IAA in the concentration range of 0.5 – 2.0 mg/L induced rooting (Table 2.) beyond 2.0 mg/L IAA did not improve rooting but induced callusing at the cut ends of shoots (Fig. 1.) IBA was not much effective in inducing rooting. IBA at 0.5 - 2.0 mg/L caused 60-70% of the shoot to develop roots. But when compare to IAA, IBA was taken 30 to 45 days time developing roots. The results obtained with different concentration of mineral salts in the medium plays an important role in root initiation. The primitive effect of IBA has been reported for the *in vitro* rooting on many herbs such as *Decalepis hamiltonii* [23] and *Psoralea corylifolia* [24]. In MS medium fortified with IBA (0.4 mg/L) and NAA (0.1 mg/L) maximum rooting was observed. In the combination of NAA and BAP roots were not formed but 80% of basal callus were formed. The plantlets that regenerated were thoroughly washed in tap water to remove agar medium from roots and transferred to 6 – 7.5 diameter plastic cups containing acid-washed sand. The cup was then covered with a polythene bag to ensure high humidity and placed in the culture room. Plants were irrigated thrice a day with 1/10 strength Hoagland's solution. After 30 days, the plantlets were transplanted to mini pots (10 diameter) containing sand soil and farmyard manure (1:1) and placed in the green house. Survival rate of plantlets transferred to soil after acclimatization was 95 % as a total of 57 plantlets survived out of 60 transferred to soil over a period of two month. The plants developed from *in vitro* cultures were morphologically identical to normal vegetative propagated plants. The plants raised through *in vitro* system are established in the green house with 70 % survivability.

Conclusion

The literature review reveals that this plant has immense medicinal and economic uses in Thai medicinal practice system and the plant been included in 'Thai Herbal National Essential Drug List' and promoted for the treatment of herpes simplex, simplex, herpes zoster, and skin pruritis in the primary health care programmes. The plant extracts are reported to possess potential anticancer, antioxidant, antidiabetic, immunomodulatory, wound healing, anti-inflammatory and analgesic activities [3]. The medicinal significance of this plant has leaded in reduction of its population in the nature. To overcome the present scenario, we have developed a facile system for the regeneration of *C. nutans* plants from stem segments of mother plant. In light of our results it would appear that only low concentration of BAP (0.1, 0.2 and 0.3mg /L) is required for the induction of multiple shoot lets and the combination of IBA (0.4mg/L) with NAA (0.25mg/L) the best combination for the induction of roots from the shoot lets of *C.nutans*. Thus the established protocol standardized through this study demonstrates the possibility of developing an efficient *in vitro* propagation system for successful mass propagation of *C. nutans* at need and also for the conservation of this valuable species.

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