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Micropropagation of *Rhinocanthus nasuta* (L) Kurz. – An Important Medicinal Plant.

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Article

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ABSTRACT

A rapid micropropagation protocol by using leaf, node and intermodal explants for callus induction, organogensis and successful plantlet survivability through hardening was established for *Rhinocanthus nasuta*, (L.), Kurz., an important medicinal plant species which is having antitumor, antidiabetic and hepatoprotective properties. It was observed that among the three explants attempted, the leaf part responded well for callus formation. Therefore, further studies on organogenesis and hardening were carried out by using leaf derived calli. The study revealed that the MS medium containing the growth hormones, like 2,4-D and TDZ at 0.1mg/Land 0.5mg/L respectively was found to be optimum for high degree of callus induction. Rapid proliferation of shoots (15.33/callus) was achieved by subculturing the *in vitro* leaf derived callus onto the MS medium supplemented with BAP, IAA and Kn each at 0.5mg/l. Higher number of roots (12.66 roots/ shoot) was produced in MS medium containing BAP and IAA each at 0.5mg/l while sub culturing. The *in vitro* regenerated plantlets were successfully acclimatized (90% of survivability rate) in the hardening medium encomposing garden soil, farmyard manure and sand in the ratio of 2:1:1 by volume.

INTRODUCTION

Rhinacanthus nasutus (Linn.) Kurz. belongs to the family, Acanthaceae is an undershrub, widely distributed throughout India, Taiwan, Thailand, South China, Ceylon and Madagascar. It is extensively used in traditional medical practice to treat skin and liver diseases and for the ulcer treatment also. Further it is reported to have antitumor, hepatoprotective and antiinflamative[1]. In indigenous system of medicine, the root and leaves of *R. nasutus* are used to treat herpes and other viral infections [2, 3]. In recent decades, this species seems to appear seldom in its habitats like serves jungles and deciduous forests in the low hills of Nilgiris, the Western Ghats due to anthropogenic disturbance by tribels and traditional healers for its medicinal uses. Hence to enhance its population, modern method of propagation, the *in vitro* regeneration studies were attempted by using leaf, node and internodes explants.

MATERIALS AND METHODS

Healthy and immature leaf, node and internodal segments of *Rhinacanthus nasutus* were collected from three months old plant grown under greenhouse condition and used as explants. These segments were washed under running tap water followed by treatment with a surfactant, tween 20 (5% w/v) for 5 minutes. After repeated washes in double distilled water, to eliminate the fungal contamination, the explants were treated with Carbendazim (50% w/v) fungicide (10%) also for 15 minutes and rinsed with double distilled water 2 or 3 times. To eliminate bacterial contamination, the explants were treated with 5% antibiotics (Ampicillin and Rifampicin) for 30 minutes followed by three rinses in sterile double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 minutes followed by 3-4 rinses in sterilized double distilled water inside the Laminor air flow chamber.

These explants were inoculated separately in tissue culture bottles containing MS[4] medium fortified with various combinations and concentrations of different growth regulators *viz.,* BAP, NAA, 2,4-D, Kn and TDZ for callus induction. The pH of the medium was adjusted to 5.6 - 5.8 before autoclaving at 121°C for 20 min. The culture was incubated at a constant temperature of 25±2°C with 14h photoperiod (3000 lux) and 8h darkness. Calli from these primary cultures were transferred to MS medium containing different concentrations of BAP, NAA and IAA for shoot induction. After the origin of multiple shoots, elongated shoots of 2 cm long were excised from the culture and transferred to MS medium supplemented with different concentrations of IBA, IAA and NAA for root initiation. After two weeks, the percentage of shoot forming roots, roots per shoot and root length were noted. Rooted shoots were thoroughly washed to remove the adhering gel and planted in polythene bags containing different hardening media and kept in greenhouse for acclimatization. The pots were watered at one day interval and supplied with ½ strength MS salts, twice a week by spraying. The survival rate of plantlets was recorded one month after transfer to polythene bags. Triplicates were maintained for all experiments.

RESULTS AND DISCUSSION

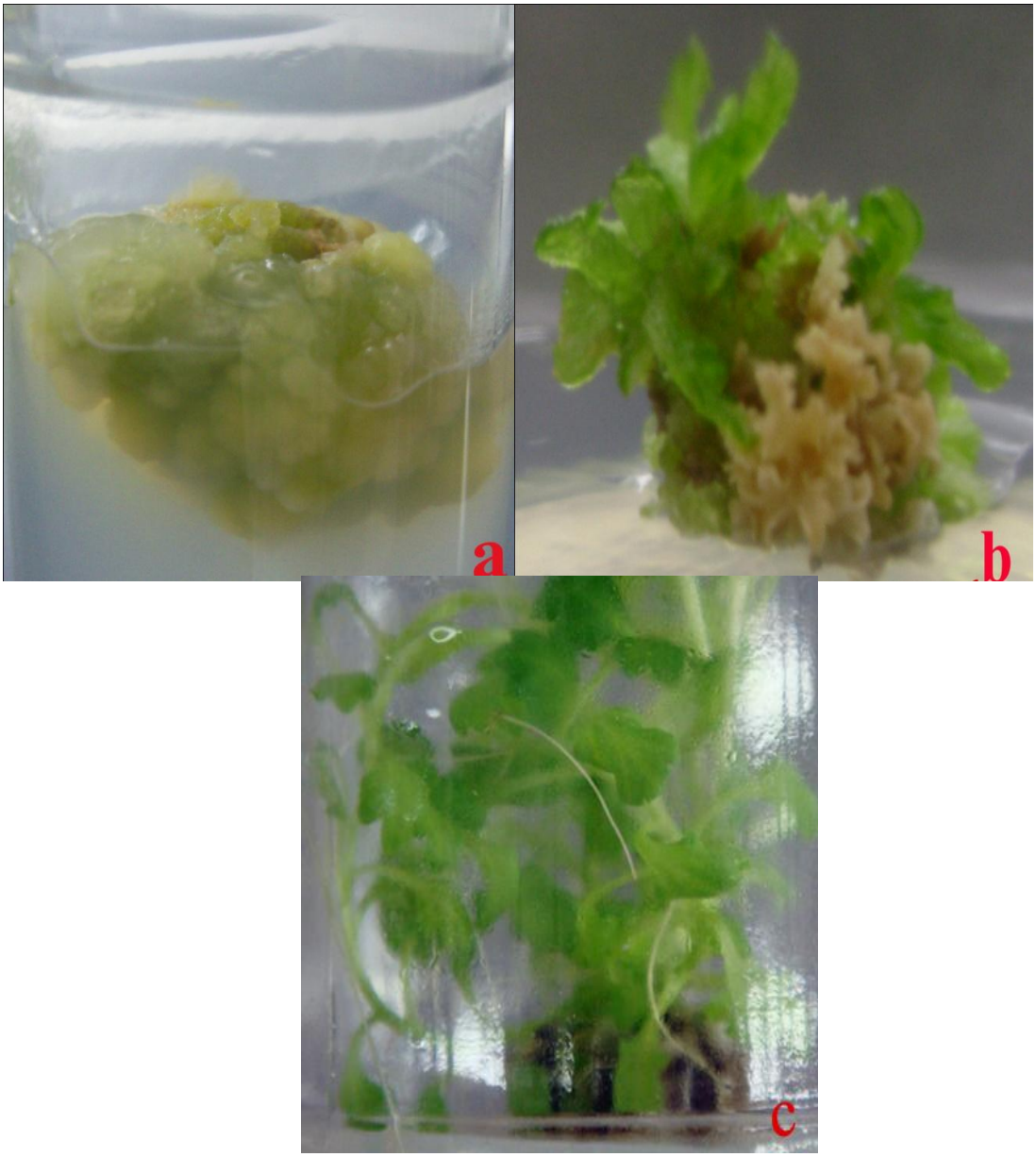
The number of days required for callus induction from the leaf explants of *R. nasuta* is noted to be varied from 10 to 30 days according to the combinations and concentrations of the growth regulators *viz.,* 2, 4-D, NAA and TDZ supplemented in the MS medium (Table 1). It may be explained that the specific growth hormones at appropriate concentrations can play major role to induce callus besides the other factors [5,6] also reported the requirement of longer period of more than 35 days for effective callus formation in many species of *Acacia* and pointed out that several factors including the culture environments and hormonal and non- hormonal regulators act synergistically in determining the proper induction, proliferation of calli and regeneration into plants. The amount of leaf explant responding for callus formation was ranging between 20 and 96% (Table 1). MS medium fortified with 2, 4-D and TDZ at 0.1 mg/L and 0.5mg/L respectively initiated 96% of leaf explants for callus formation (Fig.1a) followed by 2, 4-D at 0.1 mg/L and TDZ 0.4 mg/L which initiated 85% of leaf explants for callusing and 2, 4-D at 0.5 mg/L and NAA at 0.1 mg/L in which 65% of nodal explants produced callus. The other combinations and concentrations of growth hormones in the medium initiated only around 20 to 60% of explants for callus formation[7], explained that the differential response of same or different explants for callus formation could be due to the nature of tissue, degree of totipotency and composition of medium with respect to micronutrients and hormones. Further, it is explained that the variation in response of discs in terms of callus formation may be due to the variation in distribution of endogenous level of growth regulators as observed in many other[8,9]. It was noted that the auxin, 2, 4-D in combination with TDZ generally have the efficiency of initiation of high frequency of callus (>50%) from the leaf explant in many species [10,11, 12, 13, 14]. Ganesan and Paulsamy reported that then higher concentration of 2,4-D with cytokinins like TDZ induced higher amount of callus from the leaf discs of *Lobelia nicotianaefoliae.* Callus potential has been reported to vary from species to species and often differs in varieties of same species [15, 16, 17, 18]. Karappusamy and Pullaiah (2007) for the species, *Bupleurum distichophyllum* and [19] for the species, *Ageratum conyzoides* have also reported effective callus formation from the leaf explant in the medium containing high quantity of auxin, NAA. Mariani *et al.,*[20] reported the requirement of the cytokinin like compounds, TDZ for effective callus formation in the ornamental plant, *Aglaonema* sp. Generally, high number of leaf discs produced callus than the other two explants do. Therefore further studies on organogenesis were carried out by attempting the leaf derived callus only.

The results of the subculturing experiments by using the secondary explant, leaf derived callus showed that the cytokinin, BAP in combination with the auxin, IAA and Kinetin enhanced the response of calli for shoot formation by 95 and 83% respectively (Table 2) (Fig.1b). In addition, greater number of 15 shoots/callus was produced while subculturing onto MS medium with BAP and IAA at 0.1 and 0.5 mg/l respectively (Table 2) (Fig.1c). However, the production of higher shoot length of 10cm was achieved in the MS medium fortified with IAA and Kn at 1.0 mg/l and 0.1 mg/l respectively (Table 2). All these facts indicate that the cytokinin and auxin are the most essential growth regulator for effective shooting of the study species, *Rhinacanthus nasutus*. It is of common fact that cytokinin is the major growth hormone involved in shoot formation in many plant species [21, 22, 23, 24, 25].

The performance for rooting attributes of *R. nasutus* from the secondary explant, leaf callus derived shoots was well appreciated in the MS medium supplemented with the cytokinin and auxin (Table 3). The BAP at 0.1 mg/L and IBA at 0.5mg/L initiated 95% shoots for root formation followed by 0.1 mg/L of BAP and 0.4mg/L of IBA initiated 90% and 0.1 mg/L of BAP and 0.3mg/L of IBA initiated 80% of shoots for root formation. The number of roots per shoot was observed to be higher (12 roots/shoot) in the MS medium containing the IAA and BAP at 0.5 mg/L for *R. nasutus.* Similarly, the root length was greater (7.6 cm) during the subculturing of *in vitro* cultured shoots for roots on MS medium with 0.5 mg/l IAA and BAP. All these facts showed that the auxin , IBA is the most required growth regulator for shooting characters of the study species, *R. nasutus*. It agrees the concept that auxins are the plant hormones endogenously or exogenously inducing root formation in majority of plant species[26]. Similar kind of findings of effective root formation by the influence of various types of auxins in many plant species have been reported elsewhere [27, 28, 29, 30, 31, 32].

The hardening experiments showed that high degree of acclimatization was achieved by performing 90% of plantlet survivability in the hardening medium encomposed by garden soil, farmyard manure and sand in the ratio of 2:1:1 by volume. Hence, before transplanting the plantlets, hardening can be done in this prescribed encomposed medium for higher survivability rate of plantlets. However, field observations must be made after transplantation to know the rate of survivability in the open environmental conditions.

The present paper describes a standardized protocol for large scale production of elite plantlets of *Rhinacanthus nasutus* through leaf discs and hence the mass production of the species. It will be useful for the enhancement of its population and hence to meet the demand as well.



a) Leaf disc derived callus in MS medium supplemented with 2,4-D at 0.1mg/L and TDZ at 0.5 mg/L
b) Effective shoot formation from the derived callus in MS medium containing BAP, IAA and Kn at 0.5 mg/L each.
c) High degree of multiple shoot formation from the leaf derived callus in MS medium with BAP and IAA at 0.1mg/L and 0.5 mg/L respectively.

Table 1. Effect of different concentrations of growth regulators on per cent callus induction from leaf, nodal and internodal explant of the species, *Rhinacanthus nasutus*.

Growth regulators(mg/L)			Days required for callus formation after inoculation			Callus formation (%)		
NAA	2,4D	TDZ	Leaf explant	Nodal explant	Internodal explant	Leaf explant	Nodal explant	Internodal explant
0.0	0.1	0.1	10	-	-	20.00±0.81	-	-
0.0	0.1	0.2	12	-	22	40.00±0.81	-	12.00±0.81
0.0	0.1	0.3	15	-	21	60.00±0.81	-	11.00±1.63
0.0	0.1	0.4	20	17	23	85.00±1.24	07.00±0.81	13.33±1.24
0.0	0.1	0.5	21	19	-	96.00±2.05	09.01±1.63	-
0.1	0.0	0.1	16	23	-	34.00±1.24	13.00±1.63	-
0.2	0.0	0.2	23	21	22	37.00± 0.81	11.00± 1.63	22.01±0.81
0.3	0.0	0.3	17	18	21	45.00±1.24	18.66±1.69	21.66±2.49
0.4	0.0	0.4	22	19	19	48.00±1.24	18.33±1.69	26.66±2.49
0.5	0.0	0.5	23	21	30	50.00±1.24	21.00±1.63	40.33±1.69
0.1	0.1	0.0	21	15	18	28.00±0.81	24.66±2.05	14.00±1.63
0.1	0.2	0.0	25	24	23	30.00±1.63	35.00±2.10	13.00±1.63
0.1	0.3	0.0	17	17	17	35.00±1.69	46.00±2.10	07.66±1.69
0.1	0.4	0.0	24	22	23	48.00±0.81	54.33±2.05	13.00±3.26
0.1	0.5	0.0	15	12	25	55.00±2.86	65.01±0.81	15.33±2.86

Table 2. Effect of different concentrations of growth regulators on shoot initiation, shoot number and shoot length after subculturing of leaf derived callus of the species, *Rhinacanthus nasutus*

Growth regulator (mg/l)			Culture response (%)	Number of shoot callus	Shoot length(cm)
BAP	IAA	Kn			
0.1	0.1	0.0	54.00±0.81	09.33±1.24	02.96±1.24
0.1	0.2	0.0	59.00±0.81	11.66±1.69	03.66±1.24
0.1	0.3	0.0	64.00±2.94	12.33±1.24	03.33±1.27
0.1	0.4	0.0	73.33±1.24	13.01±0.81	04.66±1.92
0.1	0.5	0.0	62.66±2.05	15.33±1.24	05.00±0.81
0.1	0.0	0.1	70.66±1.69	07.00±2.05	05.00±0.81
0.2	0.0	0.2	71.33±1.24	07.06±1.63	05.00±0.81
0.3	0.0	0.3	76.00±2.94	09.00±0.81	05.00±0.81
0.4	0.0	0.4	83.33±1.24	06.21±0.81	05.00±0.81
0.5	0.5	0.5	95.66±2.62	06.11±1.63	06.06±1.99
0.0	0.6	0.1	47.66±1.24	06.98±0.81	06.23 ±1.47
0.0	0.7	0.1	52.33±2.05	04.83±1.69	07.83±1.27
0.0	0.8	0.1	46.33±2.05	05.21±2.44	08.30±0.81
0.0	0.9	0.1	56.33± 3.09	05.56±1.96	08.90±0.81
0.0	1.0	0.1	31.00±1.63	05.66±1.27	10.00±0.86

Table 3. Effect of different concentrations of growth regulators on root number, rooting percentage and root length after subculturing of leaf calli derived shoots of the species, *Rhinacanthus nasutus.*

Growth regulator (mg/L)			Shoots rooted (%)	No. of roots/shoot	Root length (cm)
BAP	IBA	IAA			
0.1	0.1	0.0	71.33±1.24	03.53±2.62	03.00±1.08
0.1	0.2	0.0	76.66±1.47	03.82±5.62	03.53±0.85
0.1	0.3	0.0	80.00±8.16	04.00±1.69	02.83±0.53
0.1	0.4	0.0	90.00±0.00	04.66±1.63	02.86±1.02
0.1	0.5	0.0	95.33±2.05	05.33±1.24	03.33±0.84
0.1	0.0	0.1	22.66±3.39	06.33±1.24	03.23±0.61
0.2	0.0	0.2	27.33±2.49	08.93±1.24	03.76±1.07
0.3	0.0	0.3	33.33±0.18	09.01±1.63	03.46±0.53
0.4	0.0	0.4	38.33±2.47	10.66±2.49	03.96±0.55
0.5	0.0	0.5	46.66± 1.23	12.66±2.49	04.93±1.02
0.1	0.0	0.5	52.33±0.27	06.33±1.24	03.93±1.38
0.2	0.0	0.5	55.66±1.33	05.33±1.24	04.90±0.86
0.3	0.0	0.5	58.66 ±0.23	06.66±0.94	05.30±0.72
0.4	0.0	0.5	59.66±0.27	05.58±1.63	06.10±0.57
0.5	0.0	0.5	50.66±1.47	05.33±2.05	07.60±0.58

Table 4. Effect of different composition of hardening medium on survivability rate of plantlets of the species, *Rhinacanthus nasutus.*

Hardening medium composition (V/V)	No. of plantlets under hardening	No. of plantlets survived	Survivability rate (%)
Red soil + Sand (1:1)	20	12.02±0.81	60
Vermiculate + Coir waste+ Forest litter (1:1:1)	20	13.06±1.63	65
Garden soil + Farmyard manure + Sand (2:1:1)	20	18.01±1.24	90
Vermiculate + Garden soil (3:1)	20	15.00±2.41	75
Red soil + Coir waste +Vermicompost (1:1:1)	20	09.04±0.94	45

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