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EFFECTIVENESS OF THIDIAZURON AND NAPHTHALENEACETIC ACID DURING ADVENTITIOUS SHOOT BUD INDUCTION AND PLANT REGENERATION FROM LEAF EXPLANTS OF *RHINACANTHUS NASUTUS* (L.) KURZ

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ABSTRACT

An efficient adventitious shoot bud induction and regeneration protocol from leaf explants was established for *Rhinacanthus nasutus* within a short period 35-40 days. Leaf explants were cultured on Murashige and Skoog (MS) medium was supplemented with 30 g/l sucrose and different concentrations of cytokinin and auxin viz., Thidiazouron (TDZ) (1.0 – 5.0 mg/l), 6- Benzylaminopurine (BAP) (0.5 – 5.0 mg/l) , Kinetin (Kn) (0.5 – 5.0 mg/l), and Combination of Napthelene acetic acid (NAA) 0.5 - 2.0 mg/l. The maximum number of adventitious shoot buds (42.0) per explants observed at Thidiazouron (TDZ) 1.0 mg/l and Naphthalene acetic acid (NAA) 0.5 mg/l. The maximum number of rootlets (11.0) and length (4.2 cm) response were obtained with IBA (0.5 mg/l). The highest adventitious shoot bud proliferation efficiency (42 adventitious shoot buds per explant) was obtained in the present study through primary callus on the primordial region. The influence of TDZ and NAA culture medium had a major role effect on adventitious shoot bud induction and consequent plant regeneration. Optimum adventitious shoot bud induction (95 %) was achieved on MS medium supplemented with TDZ (1.0 mg/l) and NAA (0.5 mg/l). This protocol resulted here for *R. nasutus* is an efficient method and reproducible could be used for agrobacterium-mediated genetic transformation experiments.

KEYWORDS: **Leaf segment, Adventitious shoots bud induction, Plant regeneration, *Rhinacanthus nasutus***

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INTRODUCTION

The tradition of medicinal plants is increasing worldwide. According to the World Health Organization (WHO), approximately 80% of the world's Population presently uses herbal medicines directly as teas, decocts or extracts with easily available liquids such as water, milk, or alcohol[1]. Medicinal plants have been used for thousands of years as vital sources of cures for human Disease[2]. More recently, drug discovery techniques have been useful to the standardization of herbal medicines, to interpret analytical marker[3]. Natural products play on significant part in drug up grading in the pharmaceutical industry[4]. Plant regeneration during tissue culture provides an alternative method for development of plant genetic resources and its bioactive compounds as well as protection of that species[5]. *Rhinacanthus nasutus* (L.) Kurz., is highly valuable important multi-purpose ethnomedicinal plant shrub belonging to the family Acanthaceae, it is distributed in Southeast china, Thailand and India[6]. This plant leaves and stems are normally used for the treatment of septic ulcer and hypertension[7] and confirmed to treat with antidiabetic in rats[8]. In Philippines, traditionally in this plant parts leaves and roots were used for decoction[9]. In India kurumba tribals used as a *R. nasutus* plant leaf paste apply externally to treat wounds and mixed with this leaf juice in cow's milk to treatment for cough and fever[10]. Whole plant parts being a rich source of therapeutics has been used to remedy different types of diseases such as diabetes, hepatitis, pulmonary tuberculosis, eczema and skin diseases[11-12]. Various advantageous secondary metabolites were isolated from *R.nasutus* plant. It includes flavonoids, anthraquinones, triterpenes, sterols and naphthoquinones had been isolated named as rhinacanthin (RC) A–D and G–Q of which RC-C, RC-D and RC-N are the major role for various valuable biological active compounds and this three compound Rhinacanthin C, D and N are concluded its prove antiviral activity against, cytomegalovirus, antioxidant and anti inflammatory properties[13-14]. This plant leaves and roots were collected from natural habitat during the year. It contains high therapeutic value of this shrub, over harvesting from wild populations are destruction has exhausted the nature of this species at an existing rate. Wild propagation of *R. nasutus* is by cuttings, roots and seeds are very inclined to climatic factors. Due to the geographical factors, rate of germination seeds are very low viability[15]. Thus, conventional propagation through seeds is not dependable and vegetative cuttings are insufficient to meet require of large scale quality planting materials. Therefore, to meet the highly demand and ensure easy supply of this medicinal important elite plant material, there is a need to develop mass propagation protocol of this plant an essential to conserve the wild population. *In vitro* micropropagation method is major tool for the propagation of important medicinal and crop plants[16-17]. Moreover, *In vitro* regenerate plantlets could give opportunities for development of transgenic and expressing trait of genetic transformation[18]. Micropropagation is a quite novel tools and application of new technique have propagation of elite species conservation and large-scale propagation of medicinally important endangered plants. *In vitro* growth and improvement is significantly influenced by a number of factors like genotype, phenotype and different growth conditions of mother plant. The production of virus free plant and propagation of healthy plants are ideal. Other valuable that agree by use this method more number of plants are micropropagate in a short time period of this method include large number of plant production in shorter time period[19-21]. The objective of this study was to develop adventitious shoot bud regeneration method from leaf explants of *R. nasutus* for subsequent use for in mass multiplication and genetic development.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL AND CULTURE ESTABLISHMENT

Plant material and culture establishment

Mother plants of *R. nasutus* were collected from a wild population from Ooty, India. Identification and authentification of *R.nasutus* was done by Dr. R. Elangomathavan, Scientist, Division of Plant Biotechnology, PRIST University, Thanjavur, Tamilnadu.Young leaf measuring about 2–3 cm length and 10–15-days-old immature leaves were used as explants. The Plants were collected from Yercaud, India and established in the institute herbal garden. They were washed first under running tap water, consequently rinsed with fungicide 20 mg/100 ml of sterile distilled water for 6 min followed by 1 % (w/v) sodium hypochlorite (NaOCl) for 2 min and finally washed well with sterile double distilled water. Explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min inside laminar airflow chamber and washed four times with sterile distilled water. The surface sterilized explants were cut into small pieces 1-2 cm in height prior to inoculation on MS (Murashige and Skoog) medium was supplemented with 30 g/l sucrose and different concentrations of cytokinin and auxin viz., Thidiazuron (TDZ) (1.0 – 5.0 mg/l), 6-Benzylaminopurine (BAP) (0.5 – 5.0 mg/l) , Kinetin (Kn) (0.5 – 5.0 mg/l), and combination of Napthalene acetic acid (NAA) 0.5 - 2.0 mg/l, For adventitious shoot bud regeneration. The medium was adjusted to pH 5.8 and gelled with 0.8% agar before autoclaved at 121^0C for 20 min. The culture tubes were incubated at 25 ± 2 ◦C under 16 hr photoperiod with a photosynthetic photon flux density of 60 µ mol m^2/s supplied by Philips TL 40 W fluorescent tubes. The data were collected from the cultures grown for about 35 days. After 35 days induction of adventitious shoot bud from the leaf segment. The regenerated shootlets were cultured in the optimal medium composition for mass propagation.

Rooting and acclimatization

The regenerated shootlets for root induction (3–4 cm in height) were transferred on to half MS medium supplemented with Indole-3- acetic acid (IAA – Filter sterilized), Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) at different concentrations (0.5 - 3.0 mg/l). The cultures were incubated under the same conditions as above. Rooting percentage, number of roots, and length of root were recorded after 4-5 weeks of culture period. Healthy plantlets with well-developed shoots and roots were removed from culture vessels, washed gently under running tap water, and transferred to plastic cups containing sterile soil and sand mixture

under diffuse light (16 h/8 h photoperiod) conditions. Plantlets were covered with transparent perforated polythene foil to ensure maintain humidity and were watered every 3 days with 1/8-strength MS basal salt solution devoid of sucrose and myo-inositol. The potted plantlets were initially maintained in the controlled environment for two weeks and sub-sequently they were shifted to the greenhouse. After three weeks, the plantlets were successfully established in the field.

RESULTS

Adventitious shoot bud induction and regeneration

The leaf explants of *R. nasutus* were cultured on MS Medium supplemented with various concentrations of TDZ, BAP and Kn with combination of NAA produced high response in terms of adventitious shoot bud induction. Adventitious shoot bud formation was observed from the peripheral region of the leaf explants were enlarged and induced regenerative green color shoot buds without intervening callus within 15-20 days, subsequent incubation leads to the development of shoot bud regeneration and presence of intermediary callus occurred after adventitious shoot bud induction within 35 days. The cytokinin alone at various concentrations did not induce adventitious shoot bud proliferations. While the addition of NAA (0.5mg/l), it showed significantly leaf proliferation and induction on light green color adventitious shoot bud regeneration with primary callus. The optimum frequency of adventitious shoot bud regeneration and produced more number of adventitious shoot bud (42.0) per explants subsequent elongation length (0.8 cm) was observed on MS medium supplemented with (TDZ 1.0 mg/l and NAA 0.5 mg/l) after 35days culture (Table 1;Fig 1c&d). Among the various concentrations of TDZ (1.0 - 0.5 mg/l) and NAA (0.5 mg/l) employed gave optimum frequency of adventitious shoot bud developed (42.0) per explants even if, the mean shoot number did not high in different concentrations of TDZ and NAA. The optimum number of adventitious shoot bud (20.1) per explants was observed on MS medium supplemented with BAP (2.0 mg/l) with NAA (0.5 mg/l). The maximum number of shoot elongation in height (1.5 cm) was observed on MS medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l), though increased only BAP concentration frequency of response and mean number of shoots reduce. A further optimum rate number of multiple shoots (12.1) was observed on MS medium supplemented with Kinetin (2.0 mg/l) and NAA (0.5 mg/l). The maximum mean number of shoot elongation in height (1.3cm) was observed on MS medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l) other the different concentration of kinetin alone did not raise the micro shoot bud. Both cytokinin and auxin BAP, TDZ, Kn and IAA reduced the regeneration of adventitious shoot bud their higher concentrations above 5.0 mg/l and only stunted shootlets with low number of micro shoot buds poorly developed leaves were observed. The adventitious shoot bud response is declined with an enhancement in cytokinin and auxin concentration beyond the optimum level. This was considered as an optimal growth regulator combinations of adventitious shoot bud regeneration in *R. nasutus* among all the treatments.

Rooting of shootlets and established into soil

The healthy elongated shoots were excised measuring a length of above 2.0 - 3.0 cm were cultured on half strength MS medium used for root induction. While the full strength MS produced basal callusing at the basal cut end of shoots. Rootlets were initiated from the basal cut end of the shoots implanted on IBA supplemented medium after 2 weeks of the time period without any basal callusing. The maximum mean number of rootlets (11.0) was obtained while culturing the shoots on half strength MS with IBA (0.5 mg/l) containing medium, followed by half strength MS with IBA (1.0 mg/l) induced (8.1) numbers of rootlets (Table 2; Fig 1g). In the case of IAA, maximum number of rootlets (9.0) were observed in cultures grown on half strength MS with IAA (0.5 mg/l) followed by 1.0 mg/l IAA induced (4.7) numbers of rootlets followed by NAA (0.5 mg/l) induced (5.0) number of rootlets. It was obvious that higher the concentration of auxin the lesser the number of rootlets formation along with slight basal callus. Consequently, the plantlets growth was also stunted. There was no rooting from the shootlets planted on basal medium. The rooted healthy plantlets were successfully transferred into soil after acclimatized (Fig 1I). The acclimatized plantlets were implanted into field environmental conditions after 3 to 4months.

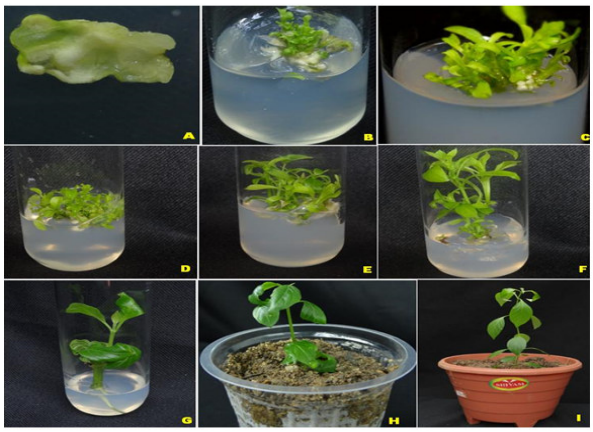

Figure 1

Adventitious shoot bud regeneration of Rhinacanthus nasutus from leaf explants: (A) Leaf explants on MS medium supplemented with TDZ and NAA (1.0 +0.5 mg/l) (B) Adventitious shoot bud induction with primary callus (C,D&E) Emerging of multiple shoots (F) Shoots elongation medium contain BAP (0.5 mg/l) (G) Root induction of shootlets (H&I) Healthy rooted plants transfer in to poly cups containing bio-compost nutrients.

Table 1
Effect of adventitious shoot bud induction and regeneration of R. nasutus leaf explants cultured on MS medium containing Cytokinin and auxin

Concentration of Plant Growth Regulators (mg/l)		Shooting Response %)	Mean No. of adventitious shoot bud /explants ± SD	Mean height of Shootlet (cm) ± SD
Control	0.0	0.0	0.0	0.0
TDZ + NAA	0.5 + 0.5	80	22.0 ± 2.8	2.1 ± 0.5
	1.0 + 0.5	95	42.0 ± 3.3	1.9 ± 0.4
	2.0 + 0.5	75	26.0 ± 1.8	1.7 ± 0.3
	3.0 + 0.5	70	17.0 ± 2.0	1.5 ± 0.2
	4.0 + 0.5	55	5.0 ± 1.3	1.4 ± 0.2
	5.0 + 0.5	50	2.2 ± 0.1	0.8 ± 0.2
Kn + NAA	0.5 + 0.5	60	12.0 ± 1.5	3.3 ± 1.0
	1.0 + 0.5	75	18.3 ± 1.8	3.5 ± 0.7
	2.0 + 0.5	85	22.0 ± 2.8	3.6 ± 0.5
	3.0 + 0.5	55	25.7 ± 1.5	3.8 ± 0.6
	4.0 + 0.5	45	10.5 ± 1.7	2.0 ± 0.5
	5.0 + 0.5	40	5.5 ± 1.1	1.7 ± 0.3
BAP+ NAA	0.5 + 0.5	55	6.3 ± 1.3	2.5 ± 0.3
	1.0 + 0.5	65	7.2 ± 2.5	4.1 ± 0.2
	2.0 + 0.5	80	11.3 ± 1.6	4.5 ± 0.3
	3.0 + 0.5	70	15.0 ± 2.5	3.3 ± 0.3
	4.0 + 0.5	45	8.0 ± 2.4	1.8 ± 0.3
	5.0 + 0.5	40	4.3 ± 1.0	1.5 ± 0.3

The values correspond to means ± SD (Standard deviation) of three replicates (10 explants each replicates) cultures were used for all replicates.

Table 2
Rooting of R. nasutus shootlets cultured in ½ MS medium containing auxins

Concentration of Plant Growth Regulators (mg/l)		Rooting response (%)	Number of rootlets /explants ± SD	Root length (cm) ± SD
Control	0.0	0.0	0.0	0.0
IBA	0.5	90	11.0 ± 1.8	4.2 ± 0.6
	1.0	70	8.1 ± 1.4	2.8 ± 0.6
	2.0	55	6.8 ± 0.8	2.2 ± 0.1
	3.0	50	2.2 ± 0.1	1.4 ± 0.4
IAA	0.5	75	9.0 ± 3.3	3.5 ± 0.2
	1.0	60	4.7 ± 0.7	2.3 ± 0.1
	2.0	55	2.3 ± 0.3	2.0 ± 0.3
	3.0	50	1.5 ± 0.3	1.2 ± 0.1
NAA	0.5	65	5.0 ± 0.8	2.1 ± 0.5
	1.0	50	3.0 ± 1.0	1.8 ± 0.6
	2.0	45	2.0 ± 0.8	1.5 ± 0.4
	3.0	50	1.3 ± 0.2	1.2 ± 0.1

The values correspond to means ± SD (standard deviation) of three replicates (10 explants each replicates)cultures were used for all replicates.

DISCUSSION

Adventitious shoot bud induction is an efficient method for clonal propagation as it averts in the somoclonal variations in the cultures. The various type of explants used for the clonal propagation and multiplication of cultures also effects may get occur genetic dissimilarities. For that reason of the non-uniform proliferations of callus cells[22], developed multiple shoots proliferation from callus cultures occur genetic mutations formed, mainly continuously subculturing of callus culture and other type of various explants tissues[23-24]. These efficiency adventitious shoot bud regeneration is more supported for agrobacterium-mediated gene transformation using the leaf explants are most apt for developed adventitious shoot regeneration method for genetic transformation methods. In these studies, adventitious shoot bud regeneration from leaf segment produced more number of adventitious shoot induction (95% with 42.0 adventitious shoot buds per explants). In the present study, in abundance induction of regenerative primary callus phase growing of adventitious shoot bud regeneration was obtained from leaf explants treated on all media combinations used. This work shows that TDZ and combinations of NAA played an essential role in reproducible protocol was developed for *R. nasutus*, by high frequency adventitious shoot bud proliferation from primary callusing leaf segment. Various types and concentration of plant growth regulators were used for inducing adventitious shoot bud plant regeneration from leaf. Proliferation of adventitious shoot bud regeneration from leaf has been reported in some systems like[25-29] In earlier reports recorded on the *R. nasutus* plant indicates that most of the cases organogenesis from five days old leaves callus induction and multiple shoot regeneration with combination of higher the concentration of kinetin (3.0 mg/l) with IAA (0.5 mg/l) produced (36 multiple shoot per explants) was resulted multiple shoot regeneration[30]. It was understood that leaf segment proliferation callus and developed multiple shoots with sub-culturing developed multiple shoots grown on MS medium. In the present communications, it is emphasized that leaf segment were able to produce adventitious shoot bud regeneration from leaf tissue by undergoing with initial callusing adventitious shoot bud regeneration cultured on TDZ (1.0 mg/l) with combination of NAA (0.5 mg/l). The major difference

from the existing reports is that high number (42 adventitious shoot bud per explant) regeneration was produced in the single step procedure. Our results are consistent with these facts, as TDZ and NAA has played an important role in proliferation of adventitious shoot bud regeneration from the whole leaf segment with better than that of the other auxin and cytokinin combinations. Effectiveness of thidiazuron is capable of fulfilling both the cytokinin and auxin requirements of various regeneration responses and organogenic differentiation has been reported in several medicinal plants[31-33]. The adventitious shoot bud formation on various media were multiplied and maintained on MS medium supplemented with TDZ (1.0 mg/l and NAA 0.5 mg/l) for adventitious shoot bud regeneration from leaf explants, various concentrations (0.5 - 5.0 mg/l) of BAP and Kinetin, (1.0 - 5.0 mg/l) TDZ and combination with NAA (0.5 mg/l) were used. The higher concentrations of BAP, Kinetin and TDZ with combination of NAA in the medium reduce percent response and number of adventitious shoot bud regeneration response of leaf explants are stunted in growth. There was no adventitious shoot bud proliferations occur when cytokinin used alone. Most of the shoot regeneration developed from the different type of explants with the combinations of cytokinin and auxin. It is well reported in several plant systems[34-36]. We achieved more number of adventitious shoot buds (42.0) induction and regeneration from young plant leaves using TDZ with combination of NAA.The healthy shootlets for about 3-4 cm in height were excised from the elongated shoots culture on ½ MS with various concentrations of IAA, IBA and NAA. Data were recorded on percentage of rooting, number and length of rootlets after 4 weeks of culture. Rootlets were initiated from the basal cut end of the shootlets implanted on auxin medium after 2 weeks of time period. The maximum number of rootlets (11.0) and length (4.2) was obtained while culturing the shoots on ½ MS with (0.5 mg/l) IBA followed by ½ MS with IAA (1.0 mg/l) induced (9.0) number of rootlets (Table 2; Fig. 1c). In the case of NAA, maximum number of rootlets (5.0) was observed in cultures grown on ½ MS with NAA (0.5 mg/l). It was obvious that higher the concentration of auxin the lesser the number of rootlets formation along with slight basal callus. Consequently, the plantlets growth also stunted. Maximum root length (4.2 cm) was observed in shootlets grown in ½ MS medium augmented with IBA (0.5 mg/l). There was no rooting from the shootlets planted on basal medium. Utility of half strength MS medium for root induction has been well documented in species like[37]. The promotive role of IBA in root induction has been well documented in this species of *R. nasutus*[38] was observed. The most critical step is the acclimatization in micropropagation protocol, it is a process to screen the plantlets capable to tolerate in field condition for successful establishment. Plantlets were carefully transferred to sterilized soil with compost in small plastic cups and covered with polythene bags to maintain humidity level and watered alternative days with 1/16 MS salt solution for two weeks. *In vitro* acclimatized regenerated plants have been reported in many plants[39,-41]. After four weeks, acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse under natural sun light. After 2 months the plants were transferred to field containing bio-compost soil. The plants showed good growth and developed new leaves and lateral branches after 3 months of establishment.

CONCLUSION

From this study it is obvious that in *R. nasutus* leaf explants could be exploited for proliferation of adventitious shoot buds induction as well as healthy plant regeneration while culturing in MS medium fortified with appropriate concentration and combinations of TDZ and NAA within short period of time. The given protocol may be used for the mass propagation of *R. nasutus* an important medicinal plant.

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CONFLICT OF INTEREST

Conflict of interest declared none

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