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High frequency plant regeneration through rhizome derived callus in *Curculigo orchioides* Gaertn – An endangered Medicinal herb

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Abstract
Curculigo orchioides Gaertn is an important medicinal plant with anticancer properties. The rhizome and tuberous roots of the plant have been used extensively in India in indigenous medicine. Due to its multiple uses, the demand for *Curculigo orchioides* is constantly on the rise; however, the supply is rather erratic and inadequate. Destructive harvesting, combined with habitat destruction in the form of deforestation has aggravated the problem. The plant is now considered 'endangered' in its natural habitat. Therefore, the need for *in vitro* propagation of this plant is crucial. Here, we describe a successful protocol for effective plant regeneration through shoot regeneration was achieved using rhizome derived callus in *Curculigo orchioides*. Three weeks old nodular callus from medium containing NAA (0.5-2.5 mg/l) and BAP (0.5-4 mg/l), or NAA (0.5-2.5 mg/l) and Kn (0.5-4 mg/l), when sub cultured on MS medium with low levels of NAA (0.1-0.5 mg/l) and high levels of BAP (1-5mg/l) or Kn (1-5 mg/l) the regeneration of shoot buds were observed. Among different concentrations and combinations tried, maximum number of shoot buds induction were noticed in the presence of BAP (3 mg/l) and combined with 0.3 mg/l of NAA and 90% response within 4 weeks of incubation. Effect of different levels of sucrose (0, 2, 3, 4, 5%, w/v) for shoot bud regeneration was tested, medium fortified with 3% sucrose was found to be optimum concentration resulting in maximum number of shoot buds regeneration. Half strength MS liquid medium with IBA or NAA (0.5-2 mg/l) tested for root induction. Maximum of 18 - 19 roots were produced at 1 mg/l of IBA on MS half -strength liquid medium with an average root length of 6 - 8 cm after 20 days. The rooted plantlets were successfully transferred to soil with 85-90% survival, and the plants showed normal morphological characteristics.

Keywords: *Cuculigo orchioides;* Embryogenic callus; Plant regeneration; Somatic embryogenesis

Introduction
Medicinal plants have vast genetic diversity, which is a valuable source of agronomic gene/s of interest for the future. The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines. Also, extensive destruction of the plant-rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc. are other factors. Hence there is a strong need for proactive understanding in the conservation, cultivation, and sustainable usage of important medicinal plant species for future use.

Curculigo orchioides Gaertn. (Hypoxidaceae) is one such important monotypic taxa of India, popularly known as black Musali. The rhizome as well as tuberous roots of the plant has been extensively used in indigenous system of medicine in India, Pakistan and China, for the treatment of various diseases including jaundice, asthma and Diarthrosis. The juice extracted from the rhizome has also been used as a tonic to overcome impotency (Chopra *et al.* 1956) [7], to prevent bone loss (Cao *et al.* 2008) [5] for antidiabetic activity (Madhavan *et al.* 2007) [17], antitumor activity (Singh *et al.* 2008) [29] and antibacterial activity (Nagesh and Shanthamma, 2009) [11].

C. orchioides is a small geophilous, perennial herb with long cylindrical rhizome. The plant is found from near sea level to 2300 m especially on moist laterite soil. The active principles that have been reported are flavones, glycosides, steroids, saponins, triterpenoids (Misra *et al.* 1984; Misra *et al.* 1990; Xu *et al.* 1992; Wu *et al.*, 2005; Dall' Acqua *et al.*, 2009; Zuo *et al.*, 2010) [21, 19, 50, 46, 8, 53]. The plant is propagated itself in nature through seeds and grows only during rainy season. Poor seed setting and poor seed germination restricts the natural multiplication.

Associated with these, over-exploitation has lead to the present endangered status of this plant (Ansari 1993; Anonymous 2000) [3, 1]. Plant tissue culture has been useful as a tool for the conservation and rapid micro propagation of rare and endangered medicinal plants.

Direct somatic embryogenesis and direct shoot formation from rhizome and leaf culture has been reported in *C. orchioides* (Augustine and Souza, 1997; Suri *et al.* 1999; Nagesh *et al.*, 2008). Somatic embryogenesis was reported using rhizome derived callus (Nagesh *et al.*, 2010) [4, 39, 27, 12] and another culture. However, there are no reports on regeneration of shoot buds from rhizome derived callus.

Thus the present investigation was undertaken with the objectives to improve and standardize rapid and efficient *in vitro* techniques for shoot regeneration from rhizome derived callus culture of *C. orchioides*.

Materials and methods

Explant source

Mature plants approximately 12 cm lengths were collected during the monsoon season from Biligiri Rangana Hills (altitude about 600-1300m) (Karnataka). Rhizome (approximately 10 cm length) were collected and washed with neutral detergent, teepol (10% v/v) for 10 min. Explants were surface disinfected using a mixture of certified (0.25% w/v) and ampicillin (0.15% w/v) for 10 min followed by surface sterilization with mercuric chloride (0.1% w/v). The disinfected explants were washed with sterile distilled water to remove traces of storylands after each treatment. Rhizome were sliced into number disc of 1 cm thickness for inoculation.

Determination of potentiality of rhizome disc from proximal to distal end of shoot axis for callus induction

In the preliminary experiment, to determine potential explant source, rhizomes were sliced into ten discs of 1 cm thickness each from proximal to distal end and were tested for callus induction on MS+2,4-D (1mg/l). Since the proximal rhizome disc (PRD) produced large callus, further experiments were carried out with PRD only.

Culture media and conditions

The basal medium consisted of Murashige and Skoog mineral nutrients and vitamins with 30 g/1 sucrose and 0.8% agar. Plant growth regulators and their combinations were added to the medium. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC at 24+2ºC in a culture cool-white fluorescent lamps and a 16-h photoperiod, except that explants for inducing callus were incubated at 24+2ºC in the dark.

Effect of different concentrations and combinations of growth regulators on callus induction from rhizome disc explant

Proximal rhizome discs were cultured on MS medium with different auxins such as 2,4-D (0.5-4 mg/l), NAA (0.5-4 mg/l) and IAA (0.5-4 mg/l) individually and also in combination of 0.5-2.5 mg/l of auxins (NAA and IAA) with cytokinins such as BAP (0.5-4 mg/l) or Kn (0.5-4 mg/l) for callus induction.

Effect of sub-culturing on shoot regeneration

The calli obtained from media containing NAA (0.5-2.5 mg/l) and BAP (0.5-4 mg/l) or Kn (0.5-4 mg/l) was tested on medium fortified with NAA (0.1-0.5 mg/l) and high concentrations of BAP (0.5-4 mg/l) or Kn (0.5-4 mg/l) for shoot buds regeneration.

Root induction

The *in vitro* derived shoot-lets were transferred to MS half strength liquid medium with NAA (0.5-2 mg/l) or IBA (0.5-2 mg/l) for root induction.

Effect of different levels of sucrose on regeneration of shoots

For this, all conditions like basal medium, carbon source, pH, gelling agent and its concentrations were maintained same as employed in earlier experiments. However, the concentrations of sucrose in the medium varied (0, 2, 3, 4, 5%) to test for regeneration / multiplication of shoot buds on medium supplemented with NAA (0.3 mg/l) and BAP (2 mg/l) and results were recorded after 4 weeks.

Acclimatization

The well rooted plantlets were transferred to sand: soil: humus (1:1:1) in plastic pots and covered with polythene bags having small holes and maintained under culture room temperature 22±2^{0}C with 80-90% of relative humidity for one week. The pots were irrigated with quarter strength of MS basal salt solution and when plant lets produced 1-2 new leaves they were transferred to green house and eventually to field conditions. Survival percentage was recorded for four weeks.

Statistical analysis

All the experiments were carried out in triplicates each consisting of 25 explants. The data were statistically analyzed by ANOVA and subjected to Duncan's Multiple Range Test.

Results

Responses of rhizome disc, cultured on MS medium fortified with different concentrations of auxins alone and also in combinations with cytokinins were represented in Fig.1 and Table 1. Effect of 2, 4-D.

The rhizome disc explant exhibited better proliferation of callus on MS medium containing 0.5-4 mg/l of 2, 4-D. 90% of the explants showed luxuriant callus induction on MS medium with 2, 4-D (2 mg/l). As the concentration of 2, 4-D increased, the allogeneic potency of the explant decreased. This was confirmed quantitatively by growth rate experiments. The callus induced on different concentrations of 2, 4-D showed morphologically whitish, unorganized, compact and hard in nature.

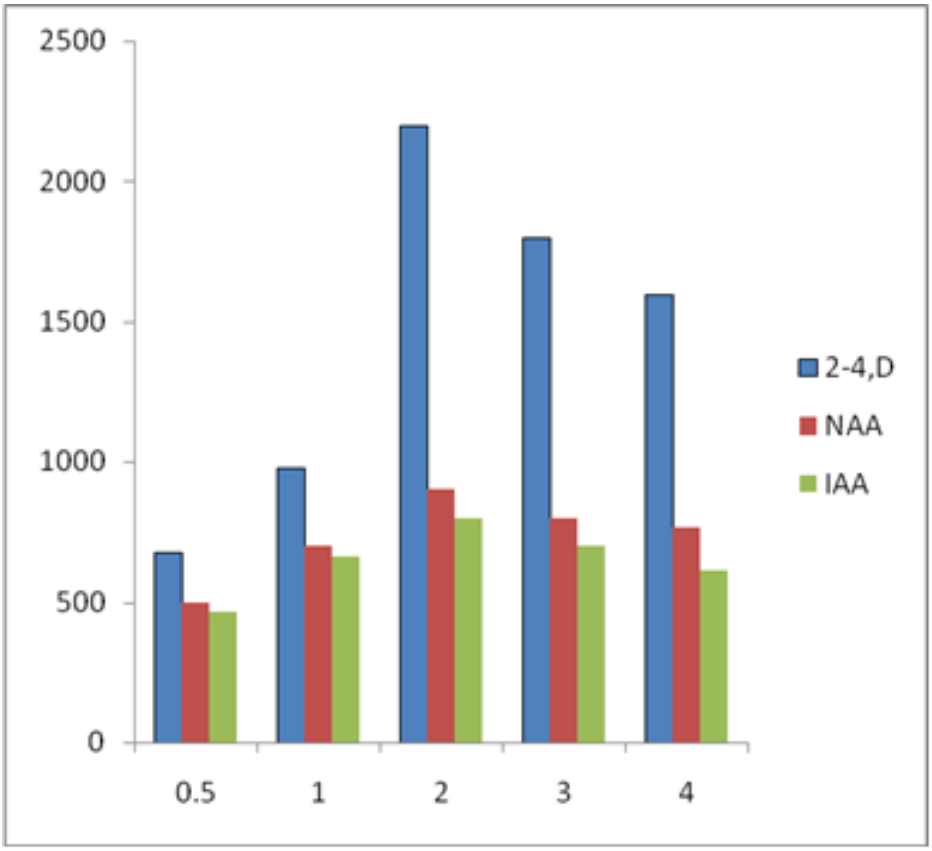

Fig 1: Effect of different concentrations of Auxins on callus induction

Effect of NAA
Medium with various concentrations of NAA (0.5-4 mg/), rhizome disc, showed different type of response. At lower concentrations of NAA (0.5-2 mg/l) induction of roots were noticed. However, at higher concentrations of NAA (3-4 mg/l), scanty amount of callus was noticed. The percent of explant responding towards the induction of callus was also very low. The callus was whitish and hard in nature. Later the callus turned into brown within 5-6 weeks of incubation, even on transferring to fresh medium.

Effect of IAA
Medium containing IAA (0.5-4mg/l), the rhizome disc produced callus. Maximum amount of callus induction was noticed on medium with 3 mg/l of IAA. The amount of callus was very less irrespective of decrease or increase in the concentrations of IAA, the callus was compact whitish and unorganized in nature.

Effect of auxins and cytokinin
The results of the above experiments indicated that the different concentrations of auxins are less effective in stimulating the callogenic response and the callus produced was totally unorganized in nature. Therefore, the experiments were designed to know the combined effect of auxins and cytokinins on induction of callus for further morphogenesis.

Effect of NAA and cytokinins
Medium with different levels of NAA (0.5-2.5 mg/l) and BAP (0.5-4 mg/l), rhizome disc produced nodular callus after 2 weeks of incubation. Maximum amount of nodular callus formation was noticed on medium supplemented with BAP (2 mg/l) and NAA (1.5 mg/l) (Table.1), this callus found to be suitable for organogenesis.
However, the rhizome disc explants when incubated on different concentrations of NAA (0.5- 4 mg/l) and Kn (0.5-2.5mg/l) produced whitish, soft watery callus without any organized structure even after 5 weeks of incubation was noticed. The callus was found unsuitable for further organo genetic studies.

Effect of IAA and cytokinin
On various concentrations of IAA (0.5-2.5 mg/l) and BAP (0.5-4 mg/l) produced, unorganized watery callus, which was not found to be suitable for organogenesis. Whereas the rhizome disc when cultured on MS medium with IAA (0.5-2.5 mg/l) and Kn (0.5-4 mg/l) scanty amount of callus was obtained. After 4 weeks of incubation, the callus turned into brown and necrosed.

Morph genic response on subculture
Three weeks old nodular callus from medium containing NAA (0.5-2.5 mg/l) and BAP (0.5-4 mg/l), when sub cultured on MS medium with low levels NAA (0.1-0.5 mg/l) and high levels of BAP (1- 5 mg/l) or Kn (1-5 mg/l) the regeneration of shoot buds were observed. The data recorded after 30 days and is presented in Table 2. The whitish nodular calli, on medium supplemented with NAA (0.1-0.5 mg/l) and BAP (1-5 mg/l), turned into greenish opaque structure within 4 weeks of incubation (Fig. 2A). These greenish structures further grown and developed into shoot buds within 6 weeks of incubation. Maximum number (43.3±0.37) of shoot buds induction were noticed in the presence of BAP (3 mg/l) and combined with 0.3 mg/l of NAA and 90% response within 4 weeks of incubation (Fig. 2B). Shoot buds were stout and well developed with 3-4 leaves within 8 weeks of incubation. As the levels of BAP and NAA increased, the number of shoot buds decreased. On medium with NAA (0.1-0.5 mg/l) and Kn (1-5 mg/l), also regeneration of shoot bud was noticed, however the number of shoot buds was lesser than that of

medium containing BAP and also shoot buds were weak. Maximum numbers of shoot buds were regenerated on medium with Kn (2 mg/l) and NAA (0.2 mg/l) (Table 2). However, decrease or increase beyond the optimal levels of Kn (2 mg/l) and NAA (0.2 mg/l) decreased the regeneration of number of shoot buds. The results of the present investigation indicated that low concentration of NAA and higher concentration of cytokinin (BAP or Kn), influenced positively on regeneration of multiple shoot buds, however on higher concentrations of NAA roots were induced along with low number of shoot buds.

Effect of different levels of sucrose on shoot bud regeneration

The morph genic callus, when sub cultured on shoot regeneration medium containing with NAA (0.3 mg/l) and BAP (3 mg/l) fortified with different concentrations of sucrose (0, 2, 3, 4, 5%, w/v), it was observed that, 3% sucrose was found to be optimum concentration resulting in maximum number of shoot buds regeneration. Beyond the level, the percent of response and also the number of shoot buds retarded. (Table 3).

Table 1: Effect of different concentrations and combinations of auxins and cytokinins on callus induction and morphology of callus

Growth regulators (mg/l)		NAA	IAA	Amount of Callus (mg/explants) M±S.E	Morphology
Basal media		0	0	-	-
BAP	0.5	0.5	-	909.80 ± 0.54^{e}	Yellow Whitish nodular callus
	1	1.0	-	1801.0 ± 0.28^{b}	
	2	1.5	-	2002.0 ± 0.18^{a}	
	3	2.0	-	1707.0 ± 0.65^{c}	
	4	2.5	-	1654.0 ± 0.65^{d}	
KN	0.5	0.5	-	401.30 ± 0.16^{e}	Yellow Whitish nodular organized callus
	1	1.0	-	901.0 ± 0.17^{d}	
	2	1.5	-	1809.0 ± 0.40^{a}	
	3	2.0	-	1714.0 ± 0.15^{b}	
	4	2.5	-	1612.0 ± 0.60^{c}	
BAP	0.5	-	0.5	501.35 ± 0.48^{e}	White watery Unorganized callus
	1	-	1.0	801.50 ± 0.13^{b}	
	2	-	1.5	887.00 ± 0.29^{a}	
	3	-	2.0	712.40 ± 0.68^{c}	
	4	-	2.5	664.90 ± 0.14^{d}	
KN	0.5	-	0.5	401.40 ± 0.15^{e}	White watery Unorganized callus
	1	-	1.0	600.85 ± 0.20^{c}	
	2	-	1.5	622.80 ± 0.20^{b}	
	3	-	2.0	713.20 ± 0.15^{a}	
	4	-	2.5	597.00 ± 0.34^{d}	

Data given are Mean of triplicates ± standard error (n=25)

Values followed by superscript letters through columns differs significantly at ($p < 0.001$) 1% level when subjected DMRT

Table 2: Effect of different concentrations of NAA and Cytokinins on shoot regeneration from rhizome derived callus of *Curculigo orchioides*

BAP	NAA	Number of Shoots/culture (M±S.E)
0	0	-
1	0.1	12.0 ± 0.22^{e}
2	0.2	29.65 ± 0.22^{b}
3	0.3	43.30 ± 0.37^{a}
4	0.4	22.95 ± 0.45^{c}
5	0.5	20.0 ± 0.11^{d}
Kn		
1	0.1	9.0 ± 0.21^{e}
2	0.2	20.0 ± 0.35^{b}
3	0.3	18.15 ± 0.18^{a}
4	0.4	14.0 ± 0.11^{c}
5	0.5	20.0 ± 0.23^{d}

Data given are Mean of triplicates ± standard error (n=25)

Values followed by superscript letters through columns differs significantly at ($p < 0.001$) 1% level when subjected DMRT

Table-3: Effect of different concentrations of Sucrose on shoot buds regeneration and somatic embryoids from rhizome derived callus

Concentrations of sucrose W/V (%)	Number of shoot/culture (M±S.E)
0%	00.00 ± 0.00^{e}
2%	12.40 ± 0.16^{d}
3%	45.65 ± 0.10^{a}
4%	29.55 ± 0.15^{b}
5%	23.45 ± 0.38^{c}

Data given are Mean of triplicates ± standard error (n=25)

Values followed by superscript letters through columns differs significantly at ($p < 0.001$) 1% level when subjected DMRT

Root induction

Auxins like IBA and NAA are well known for their ability to induce rooting *in vitro*. On half strength MS liquid medium with IBA (0.5-2mg/l) produced healthy and sturdy roots after 20 days. Maximum of 18 - 19 roots were produced at 1 mg/l of IBA on MS half-strength liquid medium with an average root length of 6-8 cm after 20 days (Fig. 2C and 3), while increase or decrease in the concentrations of IBA reduced the number of roots and also root length. However medium with NAA (0.5-2 mg/l) only few weak roots were formed.

When well-established plantlets were transferred to pot containing sterile sand: soil: humus (1:1), with high relative humidity (80-90%) and culture room temperature of 25±2ºC, plantlets showed the better adaptability. Such plantlets periodically provided with quarter strength of MS salt solution, for early stages of the plant growth and vigor. Survival rate was 100%, when transferred to green house and eventually to the field conditions.

Fig 2A: Regeneration of shoots from rhizome derived callus on MS medium with NAA (0.3mg/l) and BAP (3 mg/l) within 3 weeks of incubation

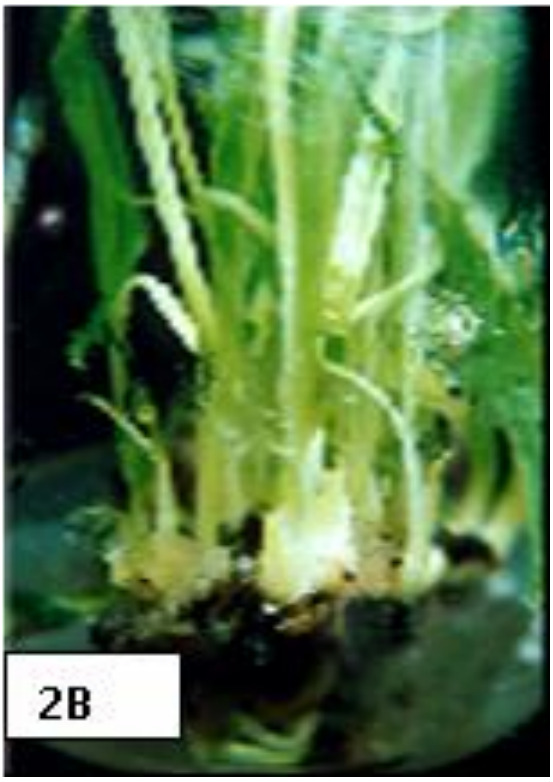

Fig 2B: Maximum number of well-developed shoots on MS medium with NAA (0.3mg/l) and BAP (3 mg/l) within 6 weeks of incubation

Fig 2C: Maximum number of sturdy roots developed on MS half-strength liquid medium with IBA (1 mg/l)

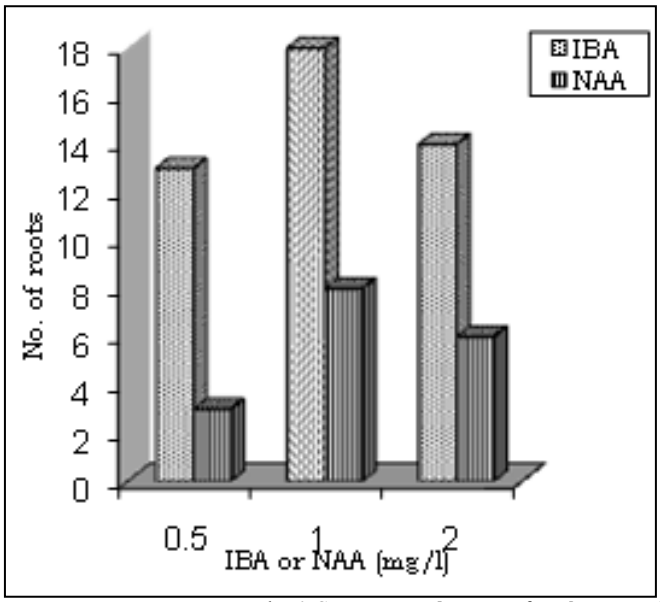

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Fig 3: Effect of IBA or NAA on induction of roots

Discussion

In India the Ayurvedic system of medicines has been in use for over three thousand years. Charaka and Susruta developed smites based on herbal sources and is still esteemed even in this day as treasure of indigenous medicines. These indigenous medicines are preferred over allopathic medicine,

since the latter cause's lot of side effects due to its synthetic nature. The choice of today's therapy is therefore exploration of plant drugs. However, due to the overexploitation of the medicinal plants, many of them have become endangered. Plant biotechnology has played an important role in the mass multiplication for conservation and also to procure innumerable content of drugs. *Curculigo orchioides* one such important endangered medicinal plant, due to constraints in natural propagation methods, there is shortage of plant source for commercial propagation. This could be over come by *in vitro* techniques. Thus, the present investigation revealed a reproducible protocol for *de novo* shoot regeneration, multiplication using rhizome derived callus of *C. orchioides*.

Excised explants mostly require an exogenous supply of plant growth regulators for inducing morph genic response in culture. An exogenous supply of growth regulators is often recommend to initiate callus formation. However, the exogenous growth regulators requirement depends strongly on the genotype and endogenous hormone content.

In number of experiments, various auxins and cytokinins have successfully been utilized for inducing morphogenetic response in cultured plant tissues. The present investigation also revealed that the explants required an exogenous supply of growth regulators for the induction of callus and shoot regeneration. Proximal rhizome discs were most suitable explant material for callus induction and shoot regeneration on MS medium with cytokinins and auxins. These results clearly indicated that, the explants from different position of the stem axis behave differently under a particular set of culture conditions. Similar type of responses has been reported in *Pennisetum americanum* and *Lilium longiflorum*.

In the present study, callus was successfully induced in Proximal rhizome discs using auxins and cytokinins. Though it is believed that strong auxins such as 2, 4-D alone is used frequently for morph genic callus induction, its further proliferation in most of the plant tissue culture techniques [George, 1993]. Interestingly, in the present study, auxins combined with cytokinins, produced morph genic callus as observed in Brahmi.

In most of the plant the decisive factor, controlling organogenesis in the tissue culture was the balance of cytokinins and auxins ratio. In the present study also auxin and cytokinin interaction provoke the direction of morphogenesis. The interaction of BAP at higher concentration and NAA at lower levels induced shoot bud differentiation from the rhizome disc explant calli, when concentration of NAA was increased, the shoot inducing capacity of BAP or Kn was suppressed. This showed that cytokinin was causative substance for shoot bud initiation.

The effects of different combinations of NAA and BA concentrations on shoot organogenesis, indicated that maximum of shoots on MS medium containing high concentrations of BAP (3 mg/l) and low concentrations of NAA (0.3 mg/l), shoot multiplication rate generally increased with increased BA and NAA concentrations. It appeared that BA and NAA have synergistic effects on shoot multiplication. The synergistic effect of BA in combination with an auxin has been reported for many medicinal plants such as Holostemma annulare, Hemidesmus indicus and Ceropegia candelabrum.

Root Induction

Auxins like IBA and NAA are well known for their ability to induce rooting *in vitro*. Suri *et al.*, (1999) [39] reported in *C. orchioides* where *in vitro* derived shoots were rooted on basal medium, however in the present investigation, half strength MS liquid medium with IBA (0.5-2 mg/l) produced healthy and sturdy roots after 20 days. Maximum of 18-19 roots were produced at 1 mg/l of IBA on MS half -strength liquid medium with an average root length of 6-8 cm after 20 days. Whereas increase or decrease in the concentrations of IBA reduced the number of roots and also root length. However medium with NAA (0.5-2 mg/l) only few weak roots were formed. The study showed the use of IBA was more effective than NAA for *in vitro* induction of roots, as reported in *Ixora Singaporenesis* and *Gloriosa superba*.

This *in vitro* protocol could be useful for not only preventing further depletion of their population in nature and also large-scale production of plant source throughout the season for the extraction of drugs.

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