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High Frequency Multiple Shoot Induction of *Curculigo orchioides* Gaertn.: Shoot Tip V/S Rhizome Disc

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ABSTRACT: *Curculigo orchioides* Gaertn. is an endangered 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 conservation of this plant is crucial. Here, we describe a successful protocol for multiple shoot induction of *C. orchioides* using shoot tip and rhizome disc. We find that proximal rhizome discs are optimal for high frequency shoot bud formation than shoot tip and distal rhizome disc. We observed a synergistic effect between 6-benzylaminopurine (BAP) and kinetin (KN) (each at 1 mg/L) on the regeneration of shoot buds from proximal rhizome disc than shoot tip explant. Optimum root induction was achieved on half-strength MS liquid medium supplemented with 1 mg/L of indole-3-butyric acid (IBA). The *in vitro* raised plantlets were acclimatized in green house and successfully transplanted to natural condition with 90% survival.

KEY WORDS: Proximal rhizome disc, multiple shoot induction, *Curculigo orchioides*.

INTRODUCTION

Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. Greater demand for these plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (Boro et al., 1998).

Curculigo orchioides Gaertn. (Hypoxydiaceae) is an important, endangered, medicinal plant popularly known in India as black musali. The rhizome and tuberous roots of the plant have been used extensively in indigenous medicinal practices in India, Pakistan, and China for the treatment of various diseases, including cancer, jaundice, asthma, and diarthrosis (Dhar et al., 1968). The juice extracted from the rhizome has also been used as a tonic to overcome impotence (Chopra et al., 1956).

C. orchioides is a small, geophilous, perennial herb with a long cylindrical rhizome. The plant is found near sea level up to an altitude of 2300 m, and in particular on moist laterite soil. The active compounds in this plant have been reported to include flavones, glycosides, steroids, saponins, triterpenoids (Misra et al., 1984; Misra et al., 1990; Xu et al., 1992). Conventionally, the plant propagates through seeds and grows only during rainy season. Poor seed setting and germination restrict its abundance in nature, and overexploitation has led to the plant's current endangered status (Augustine and Souza 1997; Shrivastava et al., 1998). Low-frequency regeneration of shoot buds has been reported from shoot tip culture in *C. orchioides* (Augustine and Souza, 1997). However, clonal propagation of this plant for commercial purposes requires a simple, economical, reproducible, and rapid multiplication protocol that overcomes the constraints on the plant's propagation in nature (Anonymous, 2000). Thus, the current study was undertaken to improve and standardize rapid and efficient *in vitro* techniques for clonal propagation using shoot tip and rhizome disc explant.

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MATERIALS AND METHODS

Explant source

Mature plants approximately 12 cm in length were collected during the monsoon season from Biligiri Rangana Hills (Karnataka, altitude of 600-1300 m). Shoot tips 0.4 cm in length and the remaining portion of the rhizome (approximately 10 cm in length) were

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collected and washed for 10 min with the neutral detergent teepol (10%, v/v). Explants were surface-disinfected for 10 min using a mixture of cetrimide (0.25%, w/v) and ampicillin (0.15%, w/v), followed by surface sterilization with mercuric chloride (0.1%, w/v). The disinfected explants were washed with sterile distilled water to remove traces of sterilizing agents after each treatment.

Culture media

MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar was used for all experiments. The medium was supplemented with different concentrations of plant growth regulators, either individually or in combination: BAP and KN for shoot multiplication and somatic embryogenesis; IBA and NAA for root induction; and 2,4-D for callus induction. The pH of the medium was adjusted to 5.7 prior to addition of the gelling agent. Aliquots of the medium were transferred to 100-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. Cultures were incubated at 26 ± 2°C under cool white fluorescent light for a daily photoperiod of 16 h.

Determination of the potential for multiple shoot bud induction from rhizome discs cut from the proximal to the distal end

In preliminary experiments, rhizomes were sliced from the proximal to the distal end into 10 discs 1 cm thick. Each disc was tested for *de novo* shoot bud regeneration on MS containing 1 mg/L BAP. Since the proximal rhizome disc (PRD) produced a greater number of shoot buds, subsequent experiments were carried out with the PRD only.

Shoot bud multiplication: shoot tip vs. PRD

To induce shoot bud multiplication, shoot tips and proximal rhizome discs (PRD) were cultured on MS medium with different concentrations of BAP (0.5-4 mg/L), KN (0.5-4 mg/L), or both. *In vitro*-derived shoots were transferred to half-strength MS liquid medium containing different concentrations of IBA (0.5-2 mg/L) or NAA (0.5-2 mg/L) for root induction.

Rooting

In vitro-derived shoots were transferred to half-strength MS liquid medium containing different concentrations of IBA (0.5-2 mg/L) or NAA (0.5-2 mg/L) for root induction. One set of cultures was inoculated in basal MS medium without the addition of auxins and kept as control.

Acclimatization and field experiment

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water to remove the adhering medium, plantlets were transferred to plastic cups (10 cm diameter) containing growing medium (autoclaved garden soil, farmyard soil and sand mixture (2:1:1)). Each was irrigated with distilled water every 2 d for 3 weeks followed by tap water for 2 weeks. The potted plantlets were initially maintained inside the culture room conditions (5 weeks) and later transferred to regular laboratory (33 ± 1°C) conditions (4 weeks). After 65 d, the plantlets were then transplanted to the field in the Botanical Evaluation Garden of University of Mysore, India and grown for 5 months. The morphological and growth characteristics were examined.

Data recorded at 42nd day of culture. Treatment means followed by different letters in their superscript are significantly different from each other ($p < 0.05$) according to Duncan's multiple range test.

RESULTS AND DISCUSSION

Determination of potential for *de novo* shoot bud regeneration of rhizome discs cut from the proximal to the distal end

In the present work, we have successfully developed a protocol for shoot multiplication of *C. orchioides*. Observation of rhizome discs cut from the proximal to the distal end revealed that, on MS basal medium fortified with 1 mg/L BAP, greater number of buds (11) were regenerated from proximal end rhizome discs than discs from the distal end of the shoot axis (Fig. 1). These results clearly indicate that the explants from different positions on the stem axis behave differently under a particular set of culture conditions, as in the case of *Narcissus pseudonarcissus* (Sage et al., 2000). Further experiments were therefore carried out using only proximal rhizome discs for multiple shoot induction.

Shoot bud multiplication: shoot tip vs. PRD

Shoot tips (Figs. 2A-D) and proximal rhizome discs (PRD) cultured on MS medium with different concentrations and combinations of BAP and KN were tested for shoot multiplication and the results are presented in Table 1 BAP alone was efficient in shoot bud induction, which was highest at 1 mg/L, while further increases in the BAP level progressively decreased shoot bud numbers in both shoot tip and rhizome disc explants (Table 1).

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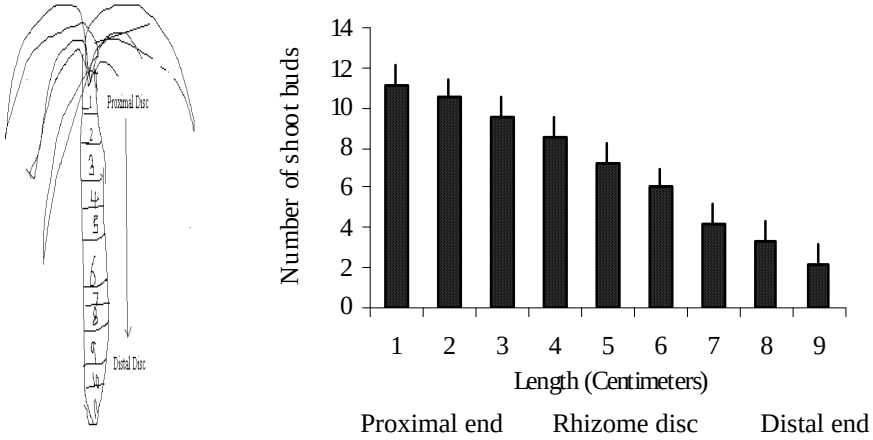


Fig. 1. Role of polarity in *de novo* shoot bud formation: The number of shoot buds produced from proximal to distal end of the shoot axis. (Each disc was about 0.5 cm thick)

Table 1. Shoot tip and proximal rhizome discs on MS medium containing different concentrations and combinations of growth regulators for shoot multiplication.

MS + Growth regulators (mg/L)	Shoot multiplication from shoot tip explant (M ± S.E)	Shoot multiplication from shoot tip explant (M ± S.E)
Control	$1.10 \pm 0.0.6^{i}$	00.00 ± 0.00^{l}
BAP		
0.5	2.25 ± 0.05^{g}	08.00 ± 0.12^{f}
1	4.80 ± 0.40^{d}	11.35 ± 0.13^{b}
2	5.90 ± 0.03^{b}	09.50 ± 0.12^{d}
3	3.30 ± 0.15^{f}	07.50 ± 0.11^{g}
4	3.35 ± 0.10^{f}	05.55 ± 0.11^{h}
KN		
0.5	1.55 ± 0.11^{h}	1.90 ± 0.23^{k}
1	2.00 ± 0.00^{g}	2.30 ± 0.11^{j}
2	1.50 ± 0.12^{hi}	3.10 ± 0.25^{i}
3	1.40 ± 0.12^{hi}	1.50 ± 0.42^{k}
4	1.40 ± 0.23^{h}	1.50 ± 0.16^{k}
BAP+KN		
0.5 + 0.5	3.20 ± 0.16^{f}	08.50 ± 0.45^{e}
1 + 1	9.96 ± 0.19^{a}	13.50 ± 0.48^{a}
2 + 2	5.25 ± 0.12^{c}	10.40 ± 0.56^{c}
3 + 3	3.70 ± 0.23^{e}	09.20 ± 0.26^{d}
4 + 4	3.25 ± 0.52^{f}	07.40 ± 0.24^{g}

Data given are mean ± standard deviation (n = 25). Values followed by superscript letters differ significantly at $p < 0.001$ (1% level) when subjected to DMRT.

Kinetin, also a cytokinin, was less efficient than BAP in bud induction, with a meager number of one bud per explant, as has been reported in other systems such as *Centella* asiatica (Suchitra et al., 1999). In contrast to earlier report on *Curculigo orchioides*, (Augustine et al., 1997) where, inadequate number of buds were induced on medium containing BAP or KN alone. However, in the present investigation, when both cytokinins (BAP and KN) were supplied in combination, there was a significant enhancement of shoot bud induction (13) was observed from rhizome disc (Figs. 3A & B) compared to shoot tip. Maximum of shoot induction probably due to the synergistic activity of BAP and KN as observed in *Pisonia alba* (Jagadishchandra et al., 1999). However, increased levels of these growth regulators in combination (BAP + KN) reduced the number of shoot buds.

Thus the present investigation clearly demonstrated that, rhizome disc explants were more suitable for mass multiplication than shoot tip culture, since continuous excision of the meristematic region, may threaten the existence of the mother plant, which has been reported in *Dendrobium moschatum* (Kanjilal et al., 1999). And also combination of cytokinins was most suitable for high frequency of shoot indction.

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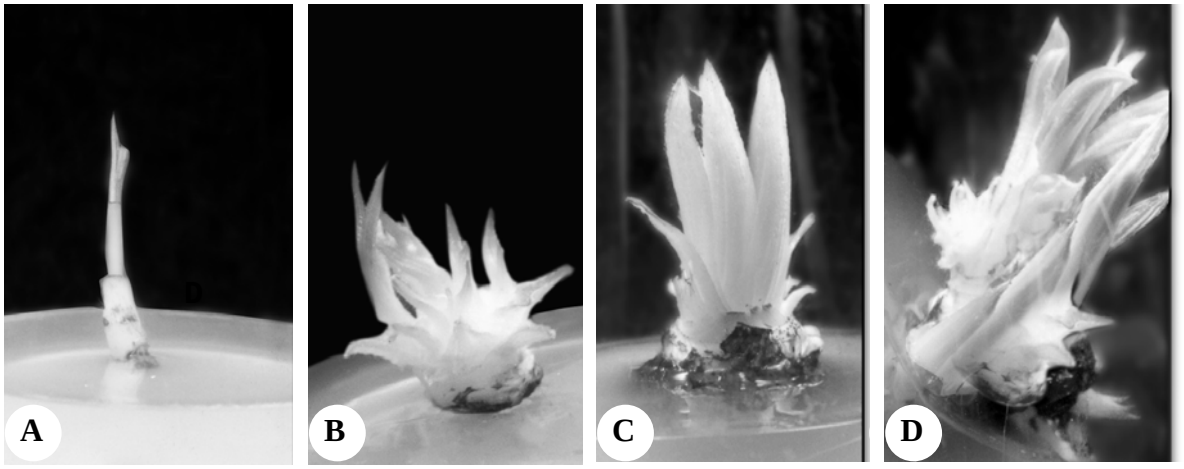


Fig. 2. Multiple shoot induction from shoot tip culture. A: Single shoot emerged on Basal medium. B: Maximum number of shoot buds induction on MS+ BAP (1mg/L). C: Low frequency of shoot bud induction on MS + KN (1 mg/L). D: High frequency of shoot bud induction on MS + BAP (1 mg/L) and KN (1 mg/L).

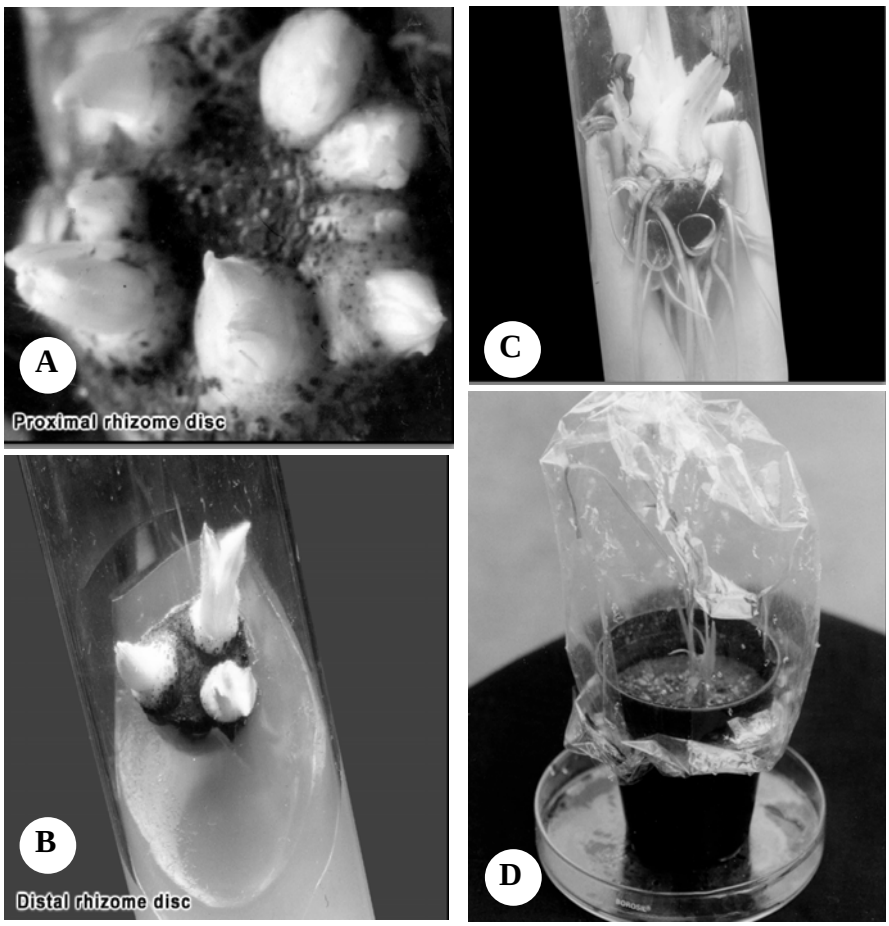


Fig. 3. Multiple shoot induction from rhizome disc. A: High frequency of shoot bud induction on MS + BAP (1mg/L) and KN (1mg/L) from proximal rhizome disc. B: Low frequency of shoot bud regeneration on MS + BAP (1mg/L) and KN (1mg/L) from distal rhizome disc. C: Maximum number of root induction on MS+ IBA (1mg/L). D: Well acclimatized plant on autoclaved garden soil, farmyard soil and sand mixture 2:1:1.

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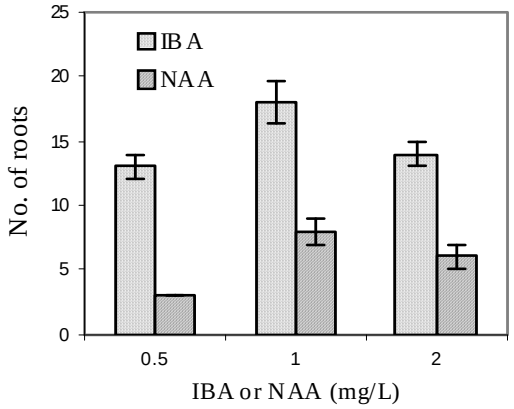


Fig. 4. Effect of IBA and NAA on induction of roots: *In vitro*-derived shoots were rooted on MS liquid medium containing different concentrations of IBA (0.5-2 mg/L) or NAA (0.5-2 mg/L).

Root Induction

In vitro-derived shoots (5 cms) that were rooted on half-strength MS liquid medium containing 1 mg/L IBA produced a greater number of healthy and sturdy roots than those cultured with NAA (Figs. 3C & 4) in contrast to an earlier report on *Ixora singaporenesis* (Lalit and Tewari, 1998).

Acclimatization of rooted plantlets and examination of morphological characteristics

A crucial aspect of *in vitro* propagation is to acquire regenerated plants that are capable of surviving outside the sterile and protected *in vitro* environment. A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light septic environment that are stressful to micropropagated plants compared to *in vitro* conditions. The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro* (Hazarika, 2003). In the present study, the successfully rooted plantlets were transferred to plastic cups containing growing medium for hardening. Plantlets were acclimatized without growth chamber facilities. Plantlets were maintained in the culture room (25 ± 1°C) conditions initially for 5 weeks and after transferred to normal laboratory conditions and maintained for about 4 weeks. Finallythe plantlets were transferred to Botanical Evaluation Garden and maintained. 100% of plantlet survival was seen after hardening on growing medium for 6 weeks. However, the survival ability decreased to 98% and 88 % after 10 and 20 weeks of acclimatization, respectively. The initial growth rates of plant height were 12.3±0.30 cm during first 2 weeks of acclimatization. However, in the following 3-20 weeks, substantial increase in plant height was observed (Fig. 3D). There was no detectable variation among the acclimatized plants with respect to morphological, growth characteristics. All the micropropagated plants were free from external defects. The data generated here is of great use in establishing improved protocols for mass propagation of this endangered plant.

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Curculigo orchioides 的多芽體誘導：莖頂 vs. 地下莖圓片

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摘　　要

Curculigo orchioides 是瀕臨絕種的抗癌藥用植物，其地下莖和塊根在印度固有醫藥中被廣泛地使用。由於它的多元用途，其需求量持續上升，市場供應不穩定且量亦不足。因為破壞性的採收方式和砍伐森林造成生育地的破壞，使問題更趨嚴重。現在 Curculigo orchioides 在原始生育地已瀕臨絕種，因此其種源的保育是非常重要的。本論文報導由莖頂和地下莖圓片誘導多芽繁殖的成功方法。我們發現地下莖近端的圓片比莖頂和地下莖遠端更適宜用來誘導多芽體的產生；且 6-benzylaminopurine (BAP)和 kinetin 兩者合用時 (各 1 mg/L)，對地下莖近端圓片之再生作用的增強效果比莖頂更好。添加 1 mg/L indole butyric acid (IBA) 之 1/2 強度的 MS 液體培養基可適用於發根。由試管培養所得的小植株經溫室馴化階段後，移植到自然環境中，有 90%的生存率。

關鍵詞：地下莖近端圓片、多芽誘導、*Curculigo orchioides*。

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