
***In vitro* propagation of *Curculago orchioides* from rhizome bud**

Shende, C.B.¹, Undal, V.S.^{1*} and Chaudhari, U.S.²

¹Department of Botany, Sant Gadge Baba Amravati University, Amravati 444-602, India

²Shri Shivaji College of Agril Biotechnology, Shivaji Nagar Amravati, India

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The *Curculigo orchioides* Gaertn. is an endangered, medicinally important perennial herb of the family Hypoxidaceae. The plants are naturally propagated through underground bulbils and seeds. The present report developed an efficient protocol for *in vitro* propagation using different concentration and combination of plant growth regulators (PGRs) through the shoot, callus and root induction and finally after acclimatization transferred to soil. Best multiple shoots were obtained from the rhizome bud culture on MS medium supplemented with 0.25 and 0.50 mg/l BAP and KIN. The combination of BAP and IAA at 1.50 and 0.25 mg/l, respectively were found suitable for the best callus formation, while less response of callus induction was observed in response of the combination of 0.50 mg/l BAP + 1.00 mg/l NAA and 0.50 mg/l KIN + 1.00 mg/l NAA. The effect of combination/concentration of KIN + IAA for callus induction was found negative. The high roots formations with root length were achieved on MS medium fortified with 0.25 and 0.50 mg/l of NAA and IAA respectively. *In vitro* raised plantlets were transferred to pots containing a mixture of sand: soil: manure in the ratio of 1:1:1(w/w) under net house for acclimation with period of two-three weeks. After proper acclimatization, the plants were transferred to field condition and attained normal growth.

Key words: *Curculago orchioides*, medicinal herb, shoot culture, callus culture, micropropagation, plant regeneration

Abbreviation – :NAA - α -naphthalene acetic acid, BAP -6-benzyl- amino-purine, KIN- kinetin, IAA -indole 3-acetic acid, HgCl₂- mercuric chloride, HCl-hydrochloric acid, NaOH-sodium chloride, PGRs-plant growth regulators

Introduction

The *Curculigo orchioides* (also called "Kali musli") is an endangered, medicinal important, stem less perennial herb belonging to the family Hypoxydaceae. Naturally, plants are propagated through underground bulbils and seeds. The plant is medicinally important, tuberous roots of the plant are widely used as tonic for strength, vigour and vitality due to the presence of

* Corresponding author: Undal, V.S.; e-mail: vinodsundal@rediffmail.com

flavanone glycoside-I and other steroidal saponins (Rastogi and Mehrotra, 1993). The tuberous roots are valued in both Indian and Chinese herbal remedies and powdered rhizome applied to cuts is said to stop bleeding and dry up wounds (Anonymous, 1979). The rhizome is also prescribed for the treatment of piles, jaundice, asthma, diarrhoea (Kirtikar and Basu, 1935) and on pimples (Bhamare, 1998). Moreover, the plant is reported to be an anticarcinogenic herb; the extract from its rhizome is hypoglycemic and anticancerous (Dhar *et al.*, 1962). The total produce per year varies from 400 to 500 g per plant and the cost of produce is around Rs 200 per kg of root (Shah, 1978).

Reliable methods of mass propagation of endangered species are highly desirable in order to meet the demand of botanical gardens and plant traders and eventually restore the plants to their natural environment (Giusti *et al.*, 2002). Methods for rapid multiplication of *C. orchoides* are highly advantageous to meet the commercial demand and to conserve the valuable endangered plants (Rout *et al.*, 2000; Ramawat *et al.*, 2004). Plant tissue culture is the only way to increase the number of plant within a short time period. Theoretically, a single cell or piece of plant tissue can produce an infinite number of new plants. The main industrial goal of plant tissue culture is to produce a large number of plants in a month instead of years (Haque *et al.*, 2009). However, the underground parts pose serious problem of contamination when used as an explant and aerial parts like leaf, stem of monocots are difficult materials to regenerate. In spite of this, regeneration from leaf explants has been reported in several monocots like *Curculigo* (Suri *et al.*, 1998a), *Chlorophytum* (Arora *et al.*, 2006), *Curcuma* (Prakash *et al.*, 2004), *Lilium* (Bachetta *et al.*, 2003), and *Agapanthus* (Suzuki *et al.*, 2002). Earlier reports on *C. orchoides* described regeneration from stem-disc explants (Suri *et al.*, 1998a), leaf explants in static (Suri *et al.*, 1998b; Prajapati *et al.*, 2003) and liquid medium (Suri *et al.*, 2000).

The aim of present study was to establish an efficient and appropriate protocol for optimal shoot, callus, root formation and transferred to field for survival from *in vitro* cultured rhizome and axillary buds of *C. orchoides*. The method could make the basis for genetic improvement of *C. orchoides* and serve in the multiplication and conservation of endangered medicinal important plant resource.

Materials and methods

The successful *in vitro* propagation methods proceeded through a sequence of stages each with specific sets of requirement and proper precautions. The stages are -:1) Sterilization and initiation of aseptic culture, 2)

Shoot formation, 3) Callus formation, 4) Root formation, and 5) Hardening of in vitro grown plantlets.

Plant material

Healthy plantlets with rhizome sprouts of *C. orchioides* Gaertn. were collected from natural places of Melghat Tiger Reserve (MTR) Maharashtra, India (Latitude-21° 15'N and 21° 45'N, Longitude-76° 57' E and 77° 30' E, Altitude-varies from 312 to 1178 m above MSL). Melghat forest belongs to central Indian Highlands representing Maikal hill range of Satpudas. The vicinity abundantly rich in biological diversity, rare and endemic flora of North, North East and Western Ghats were investigated. The total rainfall of the forest is ranging from 950 mm to 1400 mm and temperature 4 °C- 46 °C.

Sterilization and culture medium

The rhizome buds were used from the vigorously growing plants and were washed for 10-12 minutes under continuous stream of running tap water to remove soil particles. Explants were surface sterilized with 0.1 % (w/v) of HgCl₂ for 3-5 minutes and then instantly rinsed three times using autoclaved double distilled water under aseptic condition in Laminar chamber. The sterilized explants were then implanted onto the modified Murashige and Skoog's (1962) agar-gelled medium fortified with various concentrations/combinations of growth hormones. The pH of the media were adjusted to 5.7 using 0.1N HCl or 0.1N NaOH before addition of 0.8% agar (w/v) and autoclaved at 121°C for 15-20 minutes. The medium was supplemented as follows:-1) For shoot induction, 0.25, 0.5, 1.0, 1.5 and 2.0 mg/l BAP and KIN alone (Table 1, 2) For callus induction, the combination of increasing concentration of BAP + decreasing concentration of IAA; increasing concentration of KIN + decreasing concentration of IAA; increasing concentration of BAP + decreasing concentration of NAA and increasing concentration of KIN + decreasing concentration of NAA (Table 2 and 3). For root induction, 0.25, 0.5, 1.0, 1.5 and 2.0 mg/l NAA and IAA alone (Table 3). The MS medium without growth regulators was used as control for shoot, callus and root induction. The medium was dispensed into culture bottles with tight cap. The excised rhizome bud explants 0.3-0.6 cm in length were inoculated in these culture bottles for shoot proliferation. The culture bottles were maintained in the culture room with temperature 24 ± 3°C, relative humidity 85-90% and photoperiod of 16 hours light [2000-3000 lux and 8 hours dark]. At 20-30 days interval, subcultures were done. The regenerated

shoots were separated individually from the clump and cultured in separate bottle for optimum growth.

Acclimatization

The rooted cultures were established in sterile mixture of sand: soil: manure in the ratio of 1:1:1(w/w) under greenhouse in the plastic pots (Fig. D). Potted plants were maintained in the net house at 25°C and 75-80% relative humidity in shade upto 4 weeks before transfer to the field. After proper acclimatization, the plants were transferred to field condition and attained normal growth without sign of any morphological variations.

Results

The response of *C. orchioides* Gaertn. rhizome sprouts and bud as explants cultured on different shoot proliferation media over a period of 3-4 weeks were presented in Table 1. The response of different PGRs on proliferations of shoot, callus and rooting were constantly recorded one week after inoculation in order to assay the complete regeneration up to field transfer.

Multiple shoot induction

The rhizome buds cultured on MS medium with different concentration of BAP, KIN were tested for shoot multiplication, and results are presented in Table 1. Without growth hormones, 20% shoot induction observed onto MS medium with shoot length 1.84 ± 0.01 . The BAP alone was efficient in shoot induction, and was high (80 %) observed at 0.25 mg/l, with shoot length 8.60 ± 0.17 (Fig. A), while further increases in the BAP level gradually decreased shoot proliferation. The concentration of BAP at 0.50 mg/l showed highest number of shoots (1.00 ± 0.24) with shoots length (9.58 ± 0.31) out of all concentration used. The effect of KIN alone was maximum, found on 0.50 mg/l level and produced 40 % shoot formation with 0.40 ± 0.24 and 2.80 ± 0.17 number of shoot and shoot length respectively (Table 1). With increasing level of BAP, progressively decreased shoot induction percentage, number of shoot and shoot length. The response of BAP on shoot induction was significant ($P < 0.01$) than KIN.

Callus induction

The response of callus induction was determined for different media (Table 2). Out of twenty-four concentration/ combination media tested, the best

callus induction was observed on MS medium fortified with 1.50 mg/l BAP + 0.25 mg/l IAA (Fig. B). Total six to seven weeks required for the formation of entire, greenish and white friable callus. The combination of 0.50 mg/l BAP + 1.00 mg/l NAA and 0.50 mg/l Kin + 1.00 mg/l NAA were less effective for callus induction. No others treatment were found suitable for callus induction in present protocol (Table 2).

Induction of rooting and acclimatization

The treatment of NAA and IAA alone started with concentration 0.25 mg/l and end with 2.00mg/l. Without supplement of PGR served as control for verification (Table 3). Maximum and efficiently rotting was observed on MS medium supplemented with 0.25 mg/l NAA with number of roots (2.0 ± 0.02) and root length (9.68 ± 0.20) in the current study (Fig. C). Forty percent rooting was accounted using 0.50 mg/l IAA with high root length (3.90 ± 0.23). On the other hand, 20 % rooting were achieved via 1.00 mg/l IAA with less root length (1.04 ± 0.26). With increasing level of NAA and IAA, inhibited number of root and root length (Table 3). The best response on root number and root length accounted on medium supplemented with NAA which found significant ($P < 0.01$) than IAA.

The superior grown hardened plants were transferred to the field after 48 days (Fig. D). Almost 80 % of the regenerated plants survived and showed healthy growth of rhizome and roots, without sign of any morphological variations.

Table1. Effect of MS medium containing different concentration of PGRs on shoot growth from rhizome bud of *C. orchoides*

MS media + growth hormones	Concentration (mg/l)	Shoot induction (%)	Number of shoot/ Explant (Mean \pm SE)	Shoot length (cm) (Mean \pm SE)
BAP	0.0	20	0.20 ± 0.02	1.84 ± 0.01
	0.25	80	0.80 ± 0.01	8.60 ± 0.17
	0.50	60	1.00 ± 0.24	9.58 ± 0.31
	1.00	40	0.40 ± 0.24	2.40 ± 0.14
	1.50	--	--	--
	2.00	--	--	--
KIN	0.0	20	0.20 ± 0.02	1.84 ± 0.01
	0.25	40	0.40 ± 0.14	2.18 ± 0.13
	0.50	40	0.40 ± 0.24	2.80 ± 0.17
	1.00	20	0.20 ± 0.02	0.86 ± 0.06
	1.50	--	--	--
	2.00	--	--	--

Table 2. Effect of MS medium containing different concentration/combination of PGRs on callus formation from axillary bud of *C. orchoides*

Concentration of BAP (mg/l)	Concentration of IAA (mg/l)	Morphological response	Concentration of BAP (mg/l)	Concentration of NAA (mg/l)	Morphological response
0.0	2.00	--	0.0	2.00	--
0.25	1.50	--	0.25	1.50	--
0.50	1.00	--	0.50	1.00	+
1.00	1.50	--	1.00	1.50	--
1.50	0.25	++	1.50	0.25	--
2.00	0.00	--	2.00	0.00	--
Concentration of KIN (mg/l)	Concentration of IAA (mg/l)		Concentration of KIN (mg/l)	Concentration of NAA (mg/l)	
0.0	2.00	--	0.0	2.00	--
0.25	1.50	--	0.25	1.50	--
0.50	40	--	0.50	1.00	+
1.00	1.00	--	1.00	1.50	--
1.50	0.50	--	1.50	0.25	--
2.00	0.25	--	2.00	0.00	--

(+ indicates less than 50 % callus proliferation; ++ indicates more than 50 % callus proliferation and -- indicates no response of callus proliferation)

Table 3. Effect of MS medium containing different concentration of PGRs on root formation and root length from isolated plantlets of *C. orchoides*

MS media + growth hormones	Concentration (mg/l)	root induction (%)	Number of root/explant (Mean \pm SE)	root length (cm) (Mean \pm SE)
NAA	0.0	00	--	--
	0.25	60	2.0 \pm 0.02	9.68 \pm 0.20
	0.50	60	1.00 \pm 0.24	9.36 \pm 0.25
	1.00	40	0.40 \pm 0.20	3.36 \pm 0.29
	1.50	00	--	--
	2.00	00	--	--
IAA	0.0	00	--	--
	0.25	20	0.60 \pm 0.02	2.90 \pm 0.18
	0.50	40	0.60 \pm 0.04	3.90 \pm 0.23
	1.00	20	0.20 \pm 0.04	1.04 \pm 0.26
	1.50	00	--	--
	2.00	00	--	--

Discussion

Rapid *in vitro* multiplication rate, successful acclimatization and establishment of tissue culture-raised propagules in soil, genetic uniformity and stability of the resultant progeny and cost effectiveness are key parameters of a micro-propagation protocol (Mathur and Mathur, 2003; Debnath *et al.*, 2006).

BAP showed a strong effect with respect to multiplication of shoots (Kavyashree, 2009).

The present results for shoot formation with shoot length was best found on MS media supplemented with 0.50 mg/l BAP (Fig. A) and were in accordance to the report of Prajapati *et al.* (2003). The Bhau and Wakhlu, (2003) observed that high concentration of BAP resulted decrease in shoot multiplication rate of mulberry. The Parallel effects of BAP on shoot multiplication were obtained in present in vitro regeneration protocol (Table 1).



Fig. A-Initiation and multiple shoots regeneration on MS+ 0.25 mg/l BAP, B- Callus initiation from axillary bud explant on MS + 1.50 mg/l BAP and 0.25mg/l IAA, C-Root induction on MS + 0.25 mg/l NAA, D- Hardened plant let.

In contrast, a genus of a related family (Liliaceae), *Chlorophytum*, required a very high concentration of BA (22.2 μ M) for shoot regeneration (Purohit *et al.*, 1994). The effect of BAP showed better results as compared to KIN (Table 1). The similar results were published on shoot induction with the earlier report on *Azadirachta indica* (Arora *et al.*, 2010), *Zingiber officinale* (Balachandran and Bhat, 1990), *Houttuynia cordata* (Handique and Bora, 1999), *Sida cordifolia* (Sivanesan and Jeong, 2007), *Morus alba* (Balakrishnan *et al.*, 2009) and *Podophyllum hexandrum* (Chakraborty *et al.*, 2010).

The callus is an undifferentiated mass of tissue which appears on explants within a few weeks of transfer onto growth medium with suitable hormones (Bhojwani and Razdan, 1996). Of tested concentration/combination in this report for callus induction (Table 2), the MS media in combination of 1.50 mg/l

BAP and 0.25 mg/l IAA found superior (Fig. B). It noted that, amount of callus increased with increased levels of BA from 0.44-6.66 μ M (Alice and Dsouza, 1997). However, the combination of 0.50 mg/l BAP + 1.00 mg/l NAA and 0.50 mg/l Kin + 1.00 mg/l NAA in this study were less effective for callus proliferation. The result obtained in current report on callus proliferation was in agreement with Nagesh *et al.* (2010).

The root initiation in current study was observed onto the MS medium with growth hormones (Table 3). On the other hand inductions of few roots were reported on half strength of MS basal medium without any hormones, however with 0.53 μ M NAA, found improved rooting with their length (Wala and Jasrai, 2003). The best rooting response with root length were accounted on MS + 0.25 mg/l NAA and 0.50 mg/l IAA alone in the present protocol (Fig. C). Similar results on root formation were obtained in *Portulaca grandiflora*. Hook (Jain and Bashir, 2010). In contrast, high concentration of NAA (2.00 mg/l) required to produce best rooting and root length were reported in Ginger (Kambaska and Santilata, 2009).

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). The regenerated rooted plantlets of *C. orchioides* were transferred to plastic pots contained sterile mixture of sand, soil and manure under green house with 75-80 % relative humidity (Fig. D). The plants acclimatization was done at $24 \pm 4^\circ\text{C}$ for ten days and transferred to field condition after four weeks. However, in the following 5-8 weeks, extensive increase in plant height was observed with 80 % survival rate.

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