Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(4): 119-123

Received: 25-05-2016
Accepted: 27-06-2016

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An overview on tissue culture studies of *Curculigo orchioides* Gaertn: An endangered multi-potential medicinal herb

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Abstract
Curculigo orchioides Gaertn. (Hypoxidaceae) is popularly known as black musali in India. 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, to prevent bone loss, for antidiabetic activity, antitumor activity and antibacterial activity. The active principles that have been reported are flavones, glycosides, steroids, saponins, triterpenoids. 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 and habitat destruction has lead to the present endangered status of this plant, the need for *in vitro* conservation of this plant is crucial. Therefore the present review is an attempt to focus on *in vitro* Propagation studies of this important medicinal plant.

Keywords: *Curculigo orchioides*, *in vitro* propagation studies, endangered medicinal plant.

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 plant 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 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 (Dhar *et al*.1968) [8]. The juice extracted from the rhizome has also been used as a tonic to overcome impotency (Chopra *et al*. 1956; Chauhan and Dixit 2007) [6], to prevent bone loss (Cao *et al*. 2008) [5] for antidiabetic activity (Madhavan *et al*. 2007) [14], antitumor activity (Singh *et al*. 2008) [23], hepatoprotective activity (Rao *et al*. 1984a; 1984b Venukumar and Latha *et al*. 2002) [33]. Estrogenic activity (Vijayanarayana *et al*., 2007) [34] antibacterial activity (Nagesh and Shanthamma, 2009) [20, 21] and antifungal activity (Jaiswal *et al*., 1984) [11].

The active principles that have been reported are flavones, glycosides, steroids, saponins, triterpenoids (Kubo *et al*. 1983; Misra *et al*. 1984; Misra *et al* 1990; Xu *et al*. 1992; Lakshmi 2004; Wu *et al*., 2005; Dall'Acqua *et al*., 2009; Zuo *et al*., 2010) [12, 16, 15, 37, 13, 36, 9, 38].

The demand of the raw materials and derivatives of the plant for the indigenous drug industries is satisfied mainly from the wild source, depleting the natural population.

Advanced biotechnological methods of culturing plant cells and tissues should provide new means for conserving and rapidly propagating valuable, rare, and endangered medicinal plants. Therefore, herewith we present an overview on *in vitro* propagation protocols developed by previous reporters using different explants on different types of media, on different concentrations and combinations of growth regulators for mass multiplication and conservation of this multipotential medicinal plant: C. *orchioides.*

***In vitro* propagation studies**

The plant is propagated itself in nature through seeds and grows only during rainy season. 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. 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) [2, 1]. Therefore, the need for conservation of this plant is crucial.

Shoot tip culture

Francis *et al*. (2007) [10] developed an efficient protocol for *in vitro* clonal propagation of *Curculigo orchioides* Gaertn. through apical meristem culture. Multiple shoots were induced from apical meristems grown on Murashige and Skoog (MS) basal medium supplemented with 1.5 mg/l6-benzyladenine (BA), 100 mg/l adenine sulfate (Ads) and 3% sucrose. Inclusion of indole-3-butyric acid (IBA) or indole- 3-acetic acid (IAA) in the culture medium improved the formation of multiple shoots. The highest frequency of multiplication was obtained on MS medium supplemented with 1.5 mg/l BA, 100 mg/l Ads, 0.25 mg/l IBA and 3% sucrose. Nagesh (2008a) [18] observed a synergistic effect between 6-benzylaminopurine (BAP) and kinetin (Kn) (each at 1 mg/L) on the maximum number of shoot buds multiplication from shoot tip explants.

Rhizome disc culture

Nagesh (2008b) [19] find that proximal rhizome discs are optimal for high frequency shoot bud formation than shoot tip and distal rhizome disc. Synergistic effect of 6-benzylaminopurine (BAP) and kinetin (Kn) (each at 1 mg/L) on the regeneration of shoot buds from proximal rhizome disc was reported.

De novo shoot bud formation from rhizome disc

Nagesh *et al* (2009) [21] reported the occurrence of strong polarity towards shoot bud induction and the effect of cytokinin(s) on each segment of stem axis, of *Curculigo orchioides* Gaertn. (Hypoxidaceae) have been documented. Maximum number of shoot buds arising *de novo* from the stem discs (cross section) explanted from proximal end on MS medium fortified with BAP and Kn 1 mg/L each. Stem discs from distal end were less efficient in shoot bud induction. A combination of two cytokinins (BAP and Kn) as a synergistic effect on shoot buds induction from each segment of stem axis and also reported strong polarity within the explant.

Leaf culture

Suri *et al* (1999) [30] suggested a method for large-scale multiplication of *C. orchioides* through bulbil formation of leaf explant in shake flask culture. Suri *et al* (1998) [29] developed method for rapid multiplication through direct organogenesis and bulbil formation *in vitro* leaf and underground stem explants produced maximum number of shoots on B5 medium supplemented with 4.4 µmol/l benzylaminopurine.

Augustine and Souza 1997 [3] reported that the leaf explants of *C. orchioides* cultured on a MS medium without cytokinins produced a limited number of plantlets that originated directly from the cut end of the midrib. BA (0.44-5.66 µM /L) was needed to produce plantlets from rhizome explants. A higher concentration of BA (2.22-4.44 µM /L) resulted in nodular callus that when transferred to cytokinin-free medium formed shoots.

Thomas 2007 noticed that direct inoculation of leaf pieces on MS medium supplemented with various concentrations of benzylaminopurine (BAP) (2-8 µM /L) or thidiazuron (TDZ) (2-8 µM /L) alone or in combination with naphthaleneacetic acid (NAA) (0.5 and 1.0 µM /L) produced low shoot induction both in terms of percentage of response and number of shoots per explant. Hence, leaf explants were pretreated with 15, 25 or 50 µM /L TDZ, for 6, 24 or 48 h with the aim of improving shoot regeneration from cultured explants. The pretreatment of explants with 15 µmol/L TDZ for 24 h significantly promoted the formation of adventitious shoots and the maximum response was observed on MS medium supplemented with 6 µM /L TDZ.

Prajapati *et al*., (2003) [24] inoculated leaf explants on different strengths of MS basal media with 1.5% sucrose concentration and reported that 3% sucrose concentration showed moderate response. The explants in MS basal medium gave direct organogenesis, but only with single shoots. And also noticed among the cytokinins 6-benzylaminopurine (BAP) gave better results compared to kinetin.

Shoot regeneration

A method has been developed by Suri *et al*., 2009 for rapid multiplication of *Curculigo orchioides* (Hypoxidaceae) through direct organogenesis and bulbil formation *in vitro*. Leaf and underground stem explants produced maximum number of shoots (four and 10 per explant, respectively) on B5 medium supplemented with 4.4 µM BAP. Higher concentrations of BAP (≥22 µM) in the medium completely inhibited the growth and shoot proliferation from the leaf explants. Stem-disc-derived callus produced numerous bulbils on the medium containing BAP (8.8–35.2 µM) and sucrose (0.12-0.23 M). Maximum number of bulbils (10 per explant, 0.19 g fresh weight per bulbil) were recorded on the medium supplemented with 35.2 µM BAP and 0.18 M sucrose. Isolated bulbils increased in size (maximum growth index 36.4) and produced secondary bulbils on transfer to medium of the same composition. A maximum number of secondary bulbils (six per bulbil) were produced on the medium containing 35.2 µM BAP and 0.23 M sucrose.

Direct Somatic embryogenesis

Regeneration of plant lets through, direct somatic embryogenesis and shoot regeneration from leaf and stem explants have been reported i*n C. orchioides* by Augustine *et al*., 1998; Suri *et al* 1999 [30]. They suggested a method for rapid multiplication through somatic embryogenesis and bulbil formation directly from leaf explants. An extract from *in vitro* cultures of *C. orchioides* grown as bulbils in shake flasks, afforded two new glucosides of substituted benzylbenzoate curculigoside C and curculigoside D together with two known compounds - curculigoside A and curculigoside B. Their structures were elucidated on the basis of spectral evidence, in particular by using 2D NMR methods. Their vasoactive properties were assessed in isolated rat aortic rings.

Callus induction and Somatic embryogenesis

Nageh *et al* (2010) [22], **r**esponses of rhizome discs from proximal to distal end was tested on MS basal medium fortified with different concentrations and combinations of auxins and cytokinins for induction of callus, luxuriant callus was induced

from the proximal end than distal end of the shoot axis. Two types of callus recognized according to color and texture. Type I callus was soft and white, with a smooth, wet-looking surface and was composed mostly of long, tubular or irregular cells which were vacuolated, without evident nuclei. Apparently, these cells were non-embryogenic. Induced on MS medium supplemented with 2,4-D (0.5 -3 mg/l) or NAA (0.5 -3 mg/l) alone or NAA combined with cytokinins.

Medium with 0.5 mg/l BAP or Kn (0.5 mg/l) combined with 2, 4-D (0.5-3 mg/l) rhizome derived callus was type II. They observed was crumbly, yellowish, with an uneven surface and consisted mostly of small aggregates made up of small rounded, starch containing cells, that of type II callus possessed embryogenic competence.

They noticed embryogenic calli (type II), after being transferred to hormone-free MS medium supplemented with 0.5-4.0 mg/l BA alone or in combination with NAA at 0.1-0.5 mg/l, smooth, round structures occurred on the surface of embryogenic callus. The high frequencies occurred on MS medium containing 0.1 mg/l BAP at a concentration of 1mg/l.

Encapsulation of shoot and somatic embryoids

The regeneration of plants through the techniques of plant tissue culture and their subsequent acclimatization and delivery to the field poses many problems to make tissue culture technology a viable alternative proposition. The successful demonstration of encapsulation of tissue culture derived propagules in a nutrient gel has initiated a new line of research on synthetic seeds. Synthetic seeds are basically defined as, "encapsulated somatic embryos which functionally mimic seeds and can develop into seedlings under sterile conditions". In a broader sense, it would also refer to encapsulated buds or any other form of meristems which can develop into plants.

Nagesh *et al* 2009 [21] developed protocol for storability and sodium alginate encapsulated de *novo* from the stem discs (cross section). Storability and shoot development of sodium alginate encapsulated shoot buds of *Curculigo orchioides* were tested on half strength Murashige and Skoog (MS) basal medium fortified with coconut water (10% v/v). The frequency of regeneration from encapsulated shoot buds was affected significantly by concentration of sodium alginate and the duration of exposure to calcium chloride. Shoot buds encapsulated with 2.5 % sodium alginate dissolved in MS basal salts solution recorded significantly higher shoot development than other treatments. A relatively short (5 min) incubation with calcium chloride solution provided uniform encapsulation of shoot buds that gave the highest percentage (68%) of shoot development. Encapsulated shoot buds could be stored at 4°C for 50 days without reduction in viability as oppose to non - encapsulated shoot buds which showed 9.5 % viability after 20 days at 4°C.

Anther culture

Augustin *et al*., 2008 anthers cultured in MS liquid medium supplemented with 0.5 mg/l BAP or 0.5-1.0 mg/l NAA multicellular pollen noticed. The multicellular pollen cultured with 0.2-1.0 mg/l 2, 4-D developed into embryos. These embryos converted into plant let on medium containing 0.5 mg/l BAP.

Root induction

Rooting was achieved and reported by number of researchers by transferring the microshoots to half-strength MS medium containing different concentrations of auxins, Wala *et al*., 2003 [35] reported maximum roots induced on medium containing 0.53 µM NAA, 0.25 mg/L IBA and 2% sucrose. Augustine and D' Souza 2007 reported that the shoots were rooted on media supplemented with either (0.54–5.37 µ*M*) of 1-naphthaleneacetic acid (NAA) (0.57–5.71 µ*M*) of indole-3-acetic acid (IAA), or (0.49–4.90 µ*M*) indole-3-butyric acid (IBA).Nagesh *et al*., 2008a [18] reported that the Optimum root induction was achieved on half-strength MS liquid medium supplemented with 1 mg/L of indole-3-butyric acid.

Micropropagated plantlets were hardened in the greenhouse and successfully established in soil. Sharma *et al*., 2008 [28] reported that the effect of three arbuscular mycorrhizal (AM) fungal inocula on post transplanting performance of *in vitro* raised *C. orchioides* plantlets. The three AM fungal inocula consisted of two monospecific cultures of *Glomus geosporum* and *G. microcarpum* and one crude consortium of AM fungal spores isolated from rhizosphere soil of *C. orchioides* growing in natural habitat. *C. orchioides* plantlets responded significantly differently to all three mycorrhizal treatments. The study suggests use of mixed consortium of AM fungi over monospecific cultures for the sustainable cultivation and conservation of endangered medicinal plant: *C. orchioides.*

Acclimatization

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. Plantlets of *C. orchioides* developed *in vitro* were successfully transferred to soil with a high rate of 90% survivability were recorded by many reporters (Suri *et al*.,1999; 2000; Wala *et al*., 2003, Thomas 2007; Nagesh 2008; Nagesh *et al*., 2008) [30, 31, 35, 32, 18, 19,].

Conclusion

Herbal drugs have been extracted from plant cultivated in fields or from their natural habitats. It has been reported that nearly 95% of plants used traditionally as ingredients in crude drugs are collected from natural habitats. Endemicity, restricted distribution, small population, availability in particular season poor seed setting and germination have caused a decline in wild population and also major task for exploitation /extraction. To overcome these, *in vitro* cultures could be used as alternative method for extraction of drugs and also for conservation. From our present review we suggested that improved protocol may be developed and *in vitro* propagated plants could be used as alternative to field grown plant for extraction of drug throughout season and its conservation.

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