Electronic Journal of Plant Breeding, 7(4): 1056-1061 (December 2016)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2016.00144.7

Research Note

In vitro plantlet regeneration study for *in vitro* conservation in *Curculigo orchioides* - an endangered medicinal plant

P.E. Rajasekharan, S. Ganeshan and Sunitha Bhaskaran
Division of Plant Genetic Resources,
Indian Institute of Horticultural Research, Hessaraghatta Lake P.O.
Bangalore 560 089, India
E-mail: pers@iihr.res.in

(Received: 23 March 2015; Accepted: 20 Oct 2016)

Abstract
In vitro studies on *Curculigo orchioides* Gaertn. (Hypoxidaceae) was undertaken to devise a protocol for *in vitro* conservation of this endangered species. Fresh underground tubers with shoot apices were used to initiate cultures. Later the *in vitro* derived leaves and roots were used as explants on MS medium supplemented with 8.87µM BA, to obtain multiple shoots. Callus phase was avoided to maintain true-to type cultures and direct encourage regeneration. Simultaneous rooting enabled successful establishment *extra vitrum*, with 100% survival rates. *In vitro* conservation was accomplished at 10^0 C and cultures could be maintained for a minimum period of 1 year without an intermediate subculture.

Key words
Endangered, *Extra vitrum*, *In vitro* conservation

Curculigo (Hypoxidaceae) is a small genus comprising of about 12 *species* of herbs with tuberous roots, many of which are ornamental. *Curculigo orchioides* Gaertn is considered to be of high medicinal value and all parts of the plant (tuber, root, leaf, whole plant) have therapeutic properties. *Curculigo orchioides* is a monocot with tuberous root stocks small, geophilous, perennial herbs with long, cylindric, root-stock; leaves basal, sessile, linear or lanceolate, acute, plicate, glabrous, nerved and tips often bearing bulbils; flowers small yellow on very short scape, distichous, hidden in leaf sheath perianth gamophyllous. Rootstock bearing several fleshy lateral roots (rhizomes) (Sivarajan and Balachandran, 1999). It is normally propagated vegetatively through suckers. The species is a stemless perennial herb of medicinal importance and a native of India.

Roots of *C. orchioides* are used as a tonic for strength, vigour and vitality due to presence of flavanone glycoside-I, three steroids, six triterpenoids, three saponins and other metabolites (Xu and Xu, 1991, 1992; Xu *et al*., 1992 (a), (b)). Flavanone glycoside-I possesses powerful uterine stimulant activity. It is usually administered in piles, diarrhea, jaundice and asthma. *C. orchioides* has been used along with other plants in several pharmaceutical formulations in Indian system of medicine as a metabolic enhancer and aphrodisiac (Ramawat *et al*., 1997). It is distributed in India, in the sub-tropical Himalayas from Kumaon eastwards and in the Western Ghats from Konkan southwards (frequently occurs in large scale in hilly areas and rarely in the plains). This species is naturally propagated through seeds and underground bulbils, but over-exploitation associated with poor seed set and germination associated with improper replenishment, has made it an endangered species (FRLHT, 1999).

In order to reverse the trend of their steady decline from the region of origin and multiply them to sustain the supply of raw materials through cultivation, we need to acclimatize and domesticate them under different agro-ecosystems. *In vitro* techniques can offer a rapid means for multiplication of such elite and rare germplasm (Rajasekharan and Ganeshan, 2002) and an alternative method of *ex situ* conservation (Elgelman, 2004).

Plantlet regeneration from *in vitro* root (intercalary expanded portion of root) and in vitro conservation of *C. orchioides* is reported in this publication for the first time. A reproducible protocol for *in vitro* conservation of this endangered medicinal plant is reported.

Plant material: C. orchioides was initially established in IIHR field gene bank, formed the explants for initiating cultures *in vitro* was derived from this plant material.

The tuberous roots (cleared of root hairs) were washed thoroughly with water for 30 minutes and further cleaned with 4% cleansol (liquid detergent) for 30 minutes prior to treatment with 70% ethanol for 100 seconds under aseptic condition. Subsequently the explants were sterilized with 0.1% mercuric chloride for 8 minutes followed by 3 to 4 washes with sterile double distilled water to remove traces of mercury and chlorine.

The sterile explants were trimmed and the outer leaf sheaths were removed (ensuring that the core central shoot apices does not get damaged;

 DOI: 10.5958/0975-928X.2016.00144.7

inoculation was carried out) and inoculated to initiate primary cultures primarily after which, *in vitro* derived leaves and roots were used.

Culture Media and Initiation: Moorashige & Skoog (1962) basal medium was used for shoot and root proliferation. The medium was supplemented with 3% W/V sucrose and gelled with 6.5 % W/V bacteriological grade agar agar, (Hi Media, India). The pH of the medium was adjusted to 5.8 prior to autoclaving. 15 ml of molten medium w dispensed into sterile 25 x 150 cm culture tubes. For each treatment, 10 replicates were used and all the experiments were repeated thrice. The cultures were incubated at 25±2^{o} C with 50 to 55 % RH and a 16 h photoperiod, provided by cool 'Philips' white fluorescent tubes with an intensity of 31.55 $\mu m^{-2}S^{-1}$. Details of Treatments imposed are given in Table 1.Cultures were initially kept at SCC (Standard Culture Conditions) for a contamination free establishment.

Multiplication: Multiple shoots were established on MS media containing 2 mg/l BAP and on WPM (Woody Plant Medium, Lloyd and Macoy 19) containing 4.44µM BAP, maintained at 26 $\pm$ 2^{0}C with 16 hours and 8 hours photoperiod. Number of multiple shoots produced by different explants was recorded separately for (i) the tuberous root (underground tuber) with shoot apices (ii) *in vitro* derived leaf (iii*) in vitro* derived roots. A minimum of 120 cultures were raised in order to study the period of successful conservation under standard culture condition (SCC) and 10^{0}C. The cultures were incubated at 25±2^{0}C with 50 to 55 % RH and a 16 h photoperiod, provided by cool 'Philips' white fluorescent tubes with an intensity of 31.55 $\mu m^{-2}S^{-1}$.

Rooting: Simulating with shoot growth and proliferation, rooting was observed irrespective of the multiplication media used. Therefore no specific rooting media was required to be employed.

Hardening: Rooted vitroplants were removed from culture tubes and washed were transferred into polythene bags clipped containing 'Soilrite' and kept in glasshouse for a month before transferring them into pots containing sand, soil and FYM (Farm Yard Manure) in the ratio 1:1:1.

Aseptic explants of *C. orchioides* (underground tubers) were obtained using 70 % alcohol (for 90 seconds) and 0.1% $HgCl_2$, (for 5 minutes) and initiated on MS Basal medium without growth regulators. Bud break was observed after 45 days of inoculation followed by multiplication. Different media combinations were used and optimum multiplication rate was obtained in HMS+8.87µM BAP. Number of shoots was minimum; tubers and shoot length were maximum when fresh tuber was used as explants. Leaf explants produced maximum no of shoots and roots. Root explants yielded minimum roots, tubers and shoots length. Within 6 months number of shoots was increased to three fold and roots increased to 4 times and tubers 3 times and shoot length 2 times at SCC.

WPM medium with 2 BAP responded with maximum no of shoots, root tubers and shoot length followed by HMS and MS basal at SCC. At 10^{o} C (low temperature) fresh tubers produced maximum shoots, roots and shoot length followed by in vitro derived roots and leaf.

There was no significant difference in the different media used with respect to number of shoots, as they were on par irrespective of the presence or absence of growth regulators (Table 3). Also, irrespective of the explants used, the number of shoots produced was on par. When fresh tubers were used as explants, they produced maximum number of roots and leaves resulting in taller plants (Table 1). Under Standard Culture Conditions, the cultures derived from all explants could be maintained for a period of 6 months without intermittent subcultures and a maximum of 9.11$\pm$0.779 shoots were obtained (Table 2). This high multiplication rate was responsible continue maintenance at SCC and subculture became inevitable.

a)Culture initiation from underground tubers, in vitro derived leaves in vitro derived roots and in vitro plantlet production: With an optimal multiplication rate, an average of 5.86$\pm$0.33 shoots in 6 months was obtained when MS media supplemented with 8.87 was used (Table 3). On the plantlet leaf tip, presence of *in vitro* bud was observed, which in turn developed into plantlets. Roots started developing from the base of shoots on MS media, with or without growth regulators. Among several roots formed, 3 to 4 developed into tubers.

Bulbil formation and direct regeneration was observed from *in vitro* derived *leaves* of plantlets obtained from underground tubers. Direct regeneration was observed from the dorsal and ventral surface of the *in vitro* leaf explants on MS basal media, MS media containing 2 mg/l BAP and WPM containing 4.44µM. A maximum of 3.45 shoots were obtained within 2 months of inoculation. Direct regeneration in nature was observed only from leaf tips. However, under *in vitro* conditions this phenomenon was observed from all parts of the leaf (tips, lamina, leaf blade, leaf end, and cut ends). Rooting was simultaneous with shoot multiplication (2 to 3 roots differentiated into tubers). Buds arose from the

 DOI: 10.5958/0975-928X.2016.00144.7

leaves of *in vitro* derived plantlets, which in turn gave rise to plantlets.

Shoot emergence from *in vitro* roots of plantlets established through underground tubers and from plantlets established through *in vitro* leaf with 8.87µM (with or without agar gelling agent). Shoot emergence was observed from the intercalate expanded portion of the *in vitro* roots (this intercalate/interstitial expansion was observed only in roots of some *in vitro* plantlets, while few other roots developed into tubers and remained as normal roots). The expanded portion produced an optimal multiplication rate of 5.8±0.33 shoots in 6 months of culture.

b) Low temperature storage treatment: In vitro plantlets obtained from the above 3 explants, when subjected to low temperature treatment of 10^0 C temperature and reduced light intensity of 2.97 $\mu M^{-2}S^{-1}$, could be maintained for a minimum period of one year without subculture (Table 5). Multiplication rate and shoot length were reduced when compared to the cultures maintained at SCC (26 + 2^0 C with 16 hour photoperiod). *In vitro* storage using HMS basal proved to be a better storage medium producing multiple shoots at the rate of 4.5±0.269 in comparison to 6.13±0.269 shoots produced on 1/2 strength MS supplemented with 2 mg/l BAP (Table 6). On the leaf tip of conserved plants, presence of *in vitro* leaf buds was observed to develop into plantlets irrespective of the culture media. However, more number of buds was seen on plantlets inoculated in media containing growth regulators.

Vitro plants derived from *in vitro* leaves could also be stored for a minimum period of 1 year without subculture. Reduced growth and multiplication rate was obtained on MS basal medium but more incidence of leaf buds were observed on WPM medium supplemented with 4.44µM.

Plantlets obtained from *in vitro* roots, were relocated to 10^0C chamber with low light intensity (2.97 $\mu M^{-2}S^{-1}$). They could be maintained on MS medium supplemented with 8.87µM BAP. Under such conditions the cultures could be conserved for a period of 1 year without intermittent subculture.

c)Hardening: No mortality was recorded in the hardened plants as they established 100%, irrespective of the explants used for plantlet production, under glasshouse and field conditions.

The results here showed that *C. orchioides* plantlets could be conserved without subculture for 12 months at 10^0 C. It was observed that *C. orchioides* cultures, kept under reduced light and temperature conditions, showed similar rate of multiplication as those kept under SCC, but with significantly reduced shoot length. This may be due to production of endogenous cytokinins. Cultures when shifted from media containing growth regulators to basal media same multiplication rate was maintained supporting the role of endogenous cytokinins in multiplication. Though cultures maintained at SCC and at 10^0 C produced polyphenols, it this did not affect growth or multiplication rate. However, it was observed that when cultures were grown on media containing activated charcoal, multiplication rate got increased, indicating the absorption of polyphenols by charcoal and making the nutrients available to the cultures (George, 1996).

Conservation for one year under 10^0C produced lesser number of shoots when compared to six months storage at SCC thereby, indicating reduced temperature and light reduced the multiplication rate. However there was significant difference among the three explants with respect to number of shoots produced. Fresh tubers as explants produced maximum shoots, followed by *in vitro* root explants and *in vitro* leaf explants. Significant difference were also observed with respect to number of roots, tubers, leaves and shoot length within the three explants wherein fresh tubers produced maximum roots, leaves and shoot length. During storage at 10^0C, the presence and absence of growth regulators appear to play a vital role in determining the multiplication rate. When medium devoid of growth regulators was used, a mean average of 4.5±0.766 shoots was obtained, which increased to 6.13+0.766 when the medium was fortified with growth regulators. Significant difference was also observed with respect to number of roots and number of leaves. However, no significant difference was observed between the media with respect to number of tubers and shoot length as they were on par with and without growth regulators.

At 10^0C, *C. orchioides* could be conserved without subculture for a period of one year. Etiolating of leaves was a rarely observed despite prolonged storage. High incidence of tubers was observed when vitro plants were conserved at 10^0C (11.46±1.47) whereas at SCC, was the mean average tubers obtained when conserved at SCC was 4.6 ±0.52 (Table-5). Possibility of prolonged storage at 10^0C could be attributed to this increased tuber formation. Although root growth showed no effect on survival of culture, it is important to recognize its significance in conservation *in vitro*. Rooted cultures survive longer as they can absorb water and nutrients from the medium more efficiently than shoot culture especially when water and nutrients are nearing exhaustion. A high post conservation survival percentage of vitro plants is essential for establishing *in vitro* active banks. Presence of *in vitro* tubers in *C. orchioides* vitro plants also attributes their longevity under SCC and 10^0C

 DOI: 10.5958/0975-928X.2016.00144.7

storage. These rooted plantlets establish very well under *extra vitrum* conditions with 100% survival rate. Storage systems that minimize growth without sacrificing quality require the manipulation of light, temperature, and medium composition (Wilson *et al.,* 2000). Provision of light during low temperature storage can improved the quality of plantlet during storage. In case of *Hosta* plants 2µmolm^{-2}s^{-1} provision of light during low temperature storage can improve the quality of plantlets during storage. Common factors which need to be adjusted are temperature, light illumination and medium composition (Prurski *et al.,* 2000). In this study, the presence of 2.97 µM^{-2}S^{-1} light at low temperature improved the recovery of vitro plants. *In vitro* cultures of potato choke cherry and Saskatoon cherry are successfully stored under low temperature. Low light significantly improved the quality of potato culture whereas prolonged exposure to the low temperature had reduced the dormancy.

During storage under SCC and 10^{0}C, *C. orchioides* plantlets exhibited presence of leaf buds on the leaf tips and cut ends of intact leaves. Presence of bulbils was also observed at the base of cultures. Both leaf buds and bulbils produced plantlets by direct organogenesis. This reveals the high totipotent nature of *C. orchioides* and can be considered a model plant for *in vitro* studies.

A need to establish conservation strategy for this species has arisen due to its poor seed set and germination and improper replenishment in nature (Gupta and Chadha, 1995). Under these conditions, *C. orchioides* can be propagated by seed, tuber and leaf tips. Under i*n vitro* conditions they exhibit direct organogenesis from tuber, all parts of *in vitro* derived leaf (tip, margin, midrib, cut ends) and even from *in vitro* derived roots (*i.e*. intercalate expanded portion). Regenerating plantlets through somatic embryos and bulbils of leaf (Suri *et al.* 1998, 1999, 2000) has been reported earlier. The aim of this investigation was to develop systems for direct *in vitro* regeneration, using various explants of *Curculigo orchioides* for conservation and optimizing protocols for establishment of *in vitro* active genebank for this endangered species. Regeneration without intervening callus stage can be efficiently used to preserve true to type in the propagation and *in vitro* conservation.

References

Ashmore, S.A. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of Plant genetic resources, IPGRI.

Bajaj, Y.P.S. 1986. *Biotechnology in Agriculture and forestry* Vol.I Trees. Berlin. Germany Springer-Verlag.

Bhat, S.R. and Chandel, K.P.S. 1993. *In vitro* conservation of *Musa* germplasm: Effects of mannitol and temperature on growth and storage. *J. Horticultural Sci*., **68**(6): 841-846.

FRLHT. 1999. The key role of Forestry sector in conserving India's Medicinal Plants: Conceptual and Operational features. FRLHT, Bangalore.

Garg, S.N., Migra, L.N. and Reddy, M.N. 1989. Corchicoside A, an acinol glycoside from *Curculigo orchids*. *Phytochem.*, **28**: 1771-1772.

George, E.F. 1996. Plant propagation by tissue culture. Exegetics, Edinborough U.K.

Gupta, R. and Chadha, K.L. 1995. Medicinal and aromatic plants research in India. *In*: Advances in Horticulture Vol.11. (K.L. Chadha and Gupta R. eds.). Malhotra Publishing House, New Delhi.

Kartha, K.K. 1985. Cryopreservation of Plant cells and organs. Boca Raten, FL, CRC Press, 115-134.

Ramawat, K.G., Jain, S., Suri, S. and Arora, D.K. 1998. Aphrodisiac plants of Aravalli Hills with special reference to safed musli. *In*: Khan. I. (Ed.) Role of Biotechnology in Medicinal aromatic plants Ukaz Publications, Hyderabad India. pp210.

Sivarajan, V.V. and Balachandran, I. 1998. Ayurvedic drugs and their plant sources. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi.

Suri, S.S. Dilip, K.A., Rachana, S. and Kishan, G.R. 1998. Rapid Micropropagation through direct somatic embryogenesis and bulbil formation from leaf explants in *C.orchioides*. *Indian J. Exp. Biol*., **36**: 1130-1135.

Suri, S.S., Sunitha Jain and Kishan, G.R. 1999. Plantlet regeneration and bulbil formation in *in vitro* from leaf and stem explants of *C. orchioides*, an endangered medicinal plant. *Scientia Horticulturae*, **79**: 127-134.

Suri, S.S., Dilip K.A. and Kishan, G.R. 2000. A method of Large-scale multiplication of *Curculigo orchioides* through bulbul formation from leaf explant in shake flask culture. *Indian J. of Exp. Biol*., **38**: 145-148.

Xu, J.P. and X4, R.S. 1991. New Cycloartane sapogenis and its Saporins from *Curculigo orchioides*. *Phytochem.*, **31**: 233-236.

Xu, J.P. and Xy, R.S. 1992. Cycloartane type sapogenis and the glycoside from *C. orchioides*. *Phytochem*., **31**: 2455-2458.

Xy, J.P., Xu, R.S. and Li, X.Y. 1992. Glycosides of acycloartane sapogenins from *C. orchioides*. *Phytochem*., **31**: 233-236.

Xy, J.P., Xy, R.S. and Li, X.Y. 1992. Four new cycloartane saponins from *C. orchoides*. *Planta Med*., **58**: 208-210.

Electronic Journal of Plant Breeding, 7(4): 1056-1061 (December 2016)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2016.00144.7

Table 1. Mean values of the three explants, with regard to growth parameters under SCC

Explants	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
Fresh tuber	5.566	13.7[b]	3.1[b]	5.21[c]	3.53[b]
In vitro derived leaf	6.400	10.83[a]	3.23[b]	4.44[b]	3.36[b]
In vitro derived root	5.8	10.5[a]	2.43[a]	2.77[a]	2.63[a]
Significance 5%	NS	**	**	**	**
SeM	0.3380	0.6082	0.2281	0.1263	0.1217
CD 5%	0.9540	1.7167	0.6438	0.3565	0.3435

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)

Table 2. Mean values of storage period, with regard to growth parameters under SCC

Period	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
1 month	2.73[a]	4.44[a]	1.244[a]	2.31[a]	2.111[a]
6 months	9.11[b]	18.91[b]	4.6[b]	5.96[b]	4.244[b]
Significance 5%	**	**	**	**	**
SeM	0.2759	0.4966	0.1862	0.1031	9.9380
CD 5%	0.7789	1.4017	0.5256	0.2910	0.2804

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)

Table 3. Mean values of different medium, with regard to growth parameters at SCC

Media	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
MS Basal	5.36	8.5[a]	3.33[b]	4.22[b]	3.5[b]
HMS+2mg/l BAP	5.86	11.86[b]	2.06[a]	3.67[a]	3.26[b]
WPM+2mg/l BAP	6.53	14.66[c]	3.36[b]	4.52[b]	2.76[a]
Significance 5%	NS	**	**	**	**
SeM	0.3380	0.6082	0.2281	0.1263	0.1217
CD 5%	0.9540	1.7167	0.6438	0.3565	0.3435

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)

 DOI: 10.5958/0975-928X.2016.00144.7

Table 4. Mean values of the three explants, with regard to growth parameters under 10°C

Explant	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
Fresh tuber	8.15[b]	16.65[b]	11.95[b]	3.54[c]	4.1[c]
In vitro derived leaf	3.65[a]	7.45[a]	6.1[a]	1.9[a]	2.2[a]
In vitro derived root	4.15[a]	8.8[a]	5.0[a]	2.745[b]	2.6[b]
Significance 5%	**	**	**	**	**
SeM	0.3297	0.9628	0.6347	0.1288	0.1274
CD 5%	0.9384	2.740	1.8063	0.3666	0.3627

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)

Table 5. Mean values of storage period, with regard to growth parameters under 10°C

Period	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
1 month	3.366[a]	6.933[a]	3.9[a]	1.97[a]	2.4[a]
1 year	7.266[b]	15.00[b]	11.46[b]	3.48[b]	3.53[b]
Significance 5%	**	**	**	**	**
SeM	0.2692	0.7861	0.5182	0.1051	0.1040
CD 5%	0.7662	2.7400	1.4749	0.2993	0.2962

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)

Table 6. Mean values of different medium, with regard to growth parameters at 10°C

Media	No. of shoots	No. of roots	No. of tubers	Shoot length	No. of leaves
MS Basal	4.5[a]	11.066[a]	7.033	2.806	2.8[a]
HMS+2mg/l BAP	6.133[b]	10.866[b]	8.333	2.65	3.13[b]
Significance 5%	**	**	NS	NS	**
SeM	0.2692	0.7861	0.5182	0.1051	0.1040
CD 5%	0.7662	2.2372	1.4749	0.2993	0.2962

In each column, mean values superscribed by the same letters are not significantly different (p=0.05)