SHORT COMMUNICATION

SHOOT MULTIPLICATION RATE IN DIFFERENT SUBCULTURE CYCLES AND GENETIC FIDELITY IN AN IMPORTANT BUT ENDANGERED MEDICINAL HERB *CURCULIGO ORCHIOIDES* GAERTN.

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Curculigo orchioides **Gaertn. is a perennial herb of monogeneric family- hypoxidacea. *Curculigo orchioides* was multiplied on Murashige and Skoog's medium with half-strength nitrogen and 0.44 µM 6-benzyladenine. The developed shoots demonstrated root induction on same medium. This study reports different subculture cycles and rate of shoot multiplication. Multiple shoots were obtained from leaf explants and propagated up to twelve subculture cycles. Maximum multiplication rate (10.3 shoots/ explant) was obtained from first subculture cycle. However, multiplication in subsequent subcultures declined to 3.85 shoots/explant in 7th subculture cycle. Genetic fidelity of the *in vitro* derived plants was assessed using random amplified polymorphic DNA (RAPD).**

Key words: **6-Benzyladenine, RAPD analysis, subculture**

Curculigo orchioides Gaertn (family-hypoxidaceae) commonly known as kali musli, is an endangered medicinal herb. It appears first with onset of monsoon and last to disappear on completion of monsoon (Francis *et al.* 2007). The leaves, roots and rhizome of *Curculigo* are medicinally useful (Bhamare 1998). The powdered rhizome is an excellent remedy against cuts, bleeding and healing of wounds. The drug is considered as demulcent, diuretic, tonic, aphrodisiac, alternative, anti-pyretic, restorative, anti-inflammatory and hepato-protective activities (Kurma & Mishra 1995). The alcoholic extract has several pharmacological actions such as adaptogenic, anti-inflammatory, anticonvulsant, sedative, androgenic as well as immuno-promotion activities (Xu *et al.* 1992). The rhizome extract shows hypoglycemic, spasmolytic and anticancer activities.

Removal of plants for medicinal and edible tuberous roots as a substitute for safed musli, coupled with extensive denudation of forests floor caused by cattle grazing, poor seed setting and germination are some of the major causes that contribute to the herb being categorized as a threatened plant (Augustine and D'souza 1997). High incidence of viral and bacterial diseases poses yet another constraint in its multiplication and strengthens the need for *in vitro* techniques for multiplication (Dhenuka *et al.* 1999). There are few reports of rapid multiplication via direct and callus mediated plant regeneration and direct embryogenesis from leaf explants. However there is no report on effect of subculture cycles on shoot multiplication. The present study examined impact of subculture cycles on shoot multiplication of an important but endangered medicinal herb *Curculigo orchioides*.

One of the most crucial concerns in *in vitro* propagation is to retain genomic integrity of the micropropagated plants. Variation in both morphology and

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genotype has been reported to occur during *in vitro* regeneration processes (Kaeppler *et al.* 2000). Molecular tools are more reliable than phenotypic observation for evaluating tissue culture induced variations. Many authors have reported that dedifferentiation of plant tissues for plant regeneration leads to genetic modifications (Hashmi *et al.* 1997, Rani *et al.* 2000). Williams *et al.* (1990) developed Random Amplified Polymorphic DNA (RAPD) technology, which has since been widely used for the genetic analysis of biological systems.

Currently, no study has been reported at the molecular level with the genus *Curculigo orchioides.* In order to begin an extensive molecular study, improving DNA isolation methods and optimizing PCR conditions and RAPD analysis is necessary**.** In this study, genetic fidelity of the *in vitro* derived multiple shoots from different subculture cycles was assessed using RAPD.

Young leaves from *in vitro* raised plants of *Curculigo orchioides* were used as an explant. Leaves were surface sterilized with 0.1% of $HgCl_2$ (1 min). Treated leaves were thoroughly washed with sterilized double distilled water (3-4 times) and inoculated aseptically on culture medium. Leaf explants (~1 cm long) were inoculated on MS medium with half-strength nitrogen salts (ammonium nitrate 0.825gm/l, potassium nitrate 0.9 g/l), 2.0 % sucrose (w/v) and 0.8 % agar-agar (w/v). The medium was supplemented with 0.44 µM benzyladenine (BA).

The pH of media was adjusted to 5.8 and autoclaved at 121ºC (20 min). The cultures were incubated at 25ºC under 16 hour photo-period with 55 µ mol $m^{-2}s^{-1}$ photon flux density. It took about 11-12 weeks for one subculture cycle. Shoot multiplication was recorded for each subculture cycles up to 12 subcultures. For each subculture 100 shoots were assessed along with means and standard error (± SE).

Young leaves from each subculture cycles were subjected to DNA extraction and purification (Doyle & Doyle 1987). The modified PCR conditions were optimized (Akhare *et al.* 2008). The amplification products were revealed using agarose gel electrophoresis system where PCR products electrophoresed on agarose (1.2%, w/v). All the reactions were repeated at least twice, and only the consistently reproducible bands were considered.

For shoot organogenesis in *Curculigo*, MS medium with reduced levels of nitrogen (NH_4NO_3 and KNO_3) and 0.44µM BA found suitable**.** Earlier, ornamental plants such as *Begonia, Kalanchoe, Peperomia* have also demonstrated direct shoot development from the leaves. The first visible sign of shoot initiation was observed about 15 days after the inoculation, as a white shiny protuberance developed either from the midrib region or from cut ends of leaf explants. The growing shoots enlarged in size and reached an average length of about 10 cm in 12 weeks. The root induction was observed simultaneously with shoot formation. Many researchers tried different medium and growth regulators for root induction in *C. orchioides* (Wala and Jasrai 2003, Prajapati *et al*. 2003, Francis *et al*. 2007, Thomas *et al.* 2007). The shoots were at first few (1-4) in number. Gradually, it increased to 25 shoots per explant. Shoot cluster having 1-5 shoots, were robust and dark green having 4-8 leaves. The bigger shoot clusters (more than 5 shoots) were comparatively light green with 2-4 leaves. However, no morphological variation was noted among the regenerated plantlets through subcultures.

In this study maximum number of shoot multiples (~10) were obtained from first subculture cycle (Fig. 1). The earlier reports on shoot multiplication of *Curculigo orchioides* are quite varied. Augustine and D'suoza (1997) and Prajapati *et al.* (2003) reported formation of single shoot from a leaf explant using BA as growth regulator, while 5 shoots were obtained using 2,4 D (Prajapati *et al.* 2003). Through indirect organogenesis,

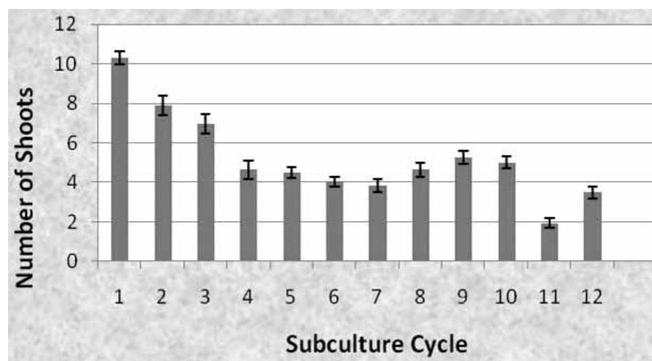

Fig. 1. Effect of subculture cycle on shoot multiplication

4 shoot multiples were obtained using leaf explant (Suri *et al.* 1998). Wala and Jasrai (2003) reported 4-5 shoot multiples from shoot apices using the MS media, while Francis *et al.* (2007) and Sharma *et al.* (2007) reported 6-7 shoots using shoot bud as an explant. The Present study reports about 10 shoots (Fig-2a) in first subculture cycle using MS media supplemented with 0.44µM BA and 50% concentration of nitrogen salts. The results clearly demonstrate that the rate of shoot multiplication decreased gradually from first to seventh subculture cycle. The Maximum number of shoot multiples (~10 shoots /explant) were obtained from first subculture (Fig 2a). The shoot multiplication rate decreased in subsequent subculture cycles (Fig. 1).

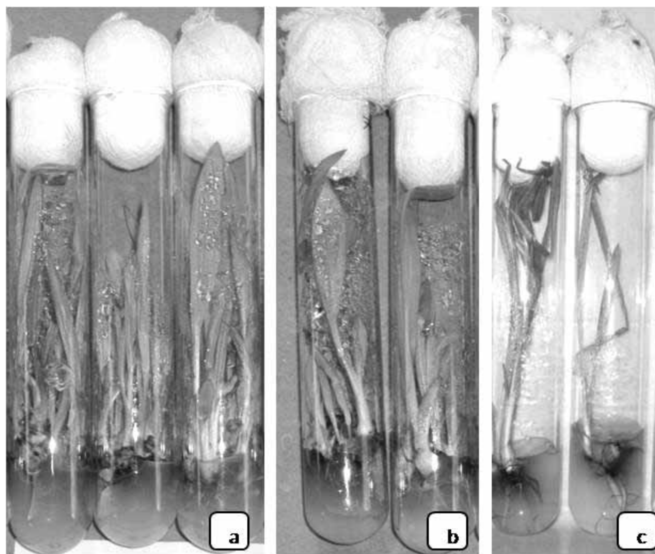

Fig. 2. Multiple Shoots from different subculture cycle (SC), a. 1st SC, b. 7th SC c. 12th SC.

The genetic fidelity of regenerated plants, derived through subculture cycles were subjected to RAPD analysis. Of the 20 primers tested (Table 1), OPE 04 and OPB 04 produced amplification products that were monomorphic across all the micropropagated plants (Fig. 3). Primer OPE 04 produced 5 bands and OPB 04 produced 3 bands common to all plants. No variation was observed among the regenerated plants. This is more important, in light of the fact that each subculture cycle is of 10-12 weeks, thus cultures were more than 70 weeks old. Similar studies in *Vanilla plantifolia* (Reddampalli *et al.* 2007) and *Bambusa balcooa* (Gillis

Table 1. Primers used for RAPD analysis

S. No.	Primer	S. No.	Primer
1.	OPE 01	11.	OPB 01
2.	OPE 02	12.	OPB 02
3.	OPE 03	13.	OPB 03
4.	OPE 04	14.	OPB 04
5.	OPE 05	15.	OPB 05
6.	OPE 06	16.	OPB 06
7.	OPE 07	17.	OPB 07
8.	OPE 08	18.	OPB 08
9.	OPE 09	19.	OPB 09
10.	OPE 10	20.	OPB 10

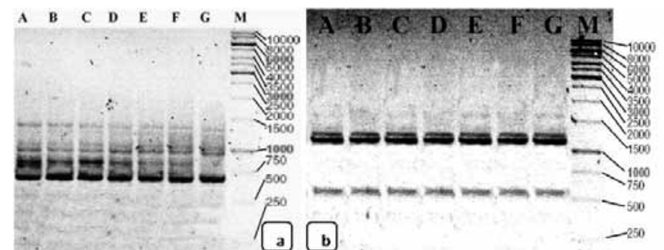

Fig. 3. RAPD profile of *Curculigo orchioides* regenerants with a. primer OPE 04and b. primer OPB 04 from 1st to 7th sub-culture cycle (A-G), M shows 1kb ladder

et al. 2007) reported genetic uniformity among the micropropagated plants.

Earlier uniformity in regenerated plants was noticed in *Crotalaria longipes* (Jayanthi and Mandal 2001) and *Arachis retusa* (Gagliardi *et al.* 2004). Our results indicate that regenerated *Curculigo* plants from different subculture cycles were genetically alike and true-to-type.

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