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Micropropagation of an Endangered Medicinal Plant: *Curculigo orchioides* Gaertn.

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Abstract

Curculigo orchioides Gaertn. is a perennial herb of the family Hypoxidaceae. It is an endangered plant species of medicinal importance. Multiple shoots were obtained from the meristem tip culture on MS medium supplemented with BA (2.21 µM). The shoots were rooted either on half strength of MS basal medium or on the one supplemented with NAA (0.53 µM). *In vitro* plantlets were transferred to pots containing a mixture of vermiculite and soil (1 : 1) for acclimation for a period of two - three weeks. At the end of a three-month period, an average of 125 plants were obtained from a single meristem.

Introduction

Curculigo orchioides Gaertn. (family - Hypoxidaceae), also called "Kali musli" is a monocot with tuberous root stocks. The species is a stemless perennial herb of medicinal importance and a native of India. It is a herb with prominently nerved and plicate leaves. The flowers are bright yellow and distichous. It is normally propagated vegetatively through suckers. This is one of the first plant species that appears in the forest after the first shower of rains and also the last to disappear on completion of monsoon. "Kali musli" is reported to have hypoglycaemic, spasmolytic and anticancer principles. The rhizome is also prescribed for the treatment of piles, jaundice, asthma, diarrhoea (Kirtikar and Basu 1935) and on pimples (Bhamare 1998). This plant species has now become endangered due to depletion in the natural habitat that support vegetation. Among the contributory factors, the following are the major ones: (i) Extensive denudation of the forest floor, caused by cattle grazing and collection of leaf litter (Jasrai and Wala 2000), (ii) removal from

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the wilderness for tuberous roots which are highly priced in the market for its metabolic enhancing principles and aphrodisiac formulations (Ramawat et al. 1998; Subramonium and Gayathri 2002), (iii) poor seed setting and germination (Suri et al. 1999), (iv) high incidence of viral and bacterial diseases affecting rhizomes (Dhenuka et al. 1999), (v) use of the rhizome as an edible flour by many tribal people (Bhattacharjee 1998) and (vi) use of the plant as a substi-tute for safed musli (Bhattacharjee 1998).

Augustine and D'souza (1998) have documented "Kali musli" to be an endangered species. The Department of Biotechnology, Ministry of Science and Technology, New Delhi has also included it in the list of endangered plants (Anonymous 2000). The gradual decline in the population of this species demands launching of conservation efforts so as to ensure continuous and ample supply by establishing a balanced cycle of harvest and renewal (Odum 1971). Such conservation efforts would ensure continuous and ample supply of this valuable material which is in great demand by the pharmaceutical industry. Only a small percentage of medicinal plants, used in the industry are cultivatred. Most of them are collected from the wild, very often in a destructive and unsustainable manner. Keeping the above facts in mind, namely the gradual decline of this endangered species, the present study was undertaken to develop a suitable protocol for its rapid multiplication.

Materials and Methods

The tubers were collected from fields, located in the Jambughoda Wild Life Sanctuary (lat. 22_21´ N to 20_30´ N and long. 73_35´ E to 73_45´ E) in Panchmahal and Vadodara districts of Gujarat state, India. The materials were washed thoroughly under running tap water (1 h) and teepol. There was a serious problem of contamination with almost 70% of the explants, being affected even after a prolonged surface sterilization treatment. Keeping this in view, a long (15 - 18 h) pre-treatment of a more stringent nature was followed.

Shoot apices were excised and stored in a solution containing chloramphenicol (0.1 %), bavistin (0.1 %), polyvinylpyrrolidone (0.1%), activated charcoal (0.1%) and citric acid (0.1%). In the laboratory, the excised apices were kept for pre-treatment (15 - 18 hr) on gyrotary shaker (150 rpm). This pre-treatment solution consisted of chloramphenicol (0.5%) and bavistin (0.25%). The treated apices were washed thoroughly (three - four times) with distilled water. These explants were then surface sterilized with $HgCl_2$ (0.1%; w/v). After repeated rinsing (five times) with distilled water, two - three leaves were removed and meristem tips (10 - 15 mm size) were inoculated

(Fig. 1) on MS medium supplemented with different concentrations of BA (0.44 - 5.3 µM). The pH of all the media was adjusted to 5.8 ± 0.1 prior to gelling with 0.8% (w/v) agar-agar (Glaxo, bacteriological grade), dispensed (10 ml) into culture tubes (25 ∇ 150 mm) and sterilized by autoclaving (121_C for 15 min). The cultures were maintained in the culture room under a regime of 16 h photoperiod (intensity - 40 µEcm^{-2}/min/sec) at 25_C. All experiments were conducted at least three times with 15 replicates each.

The developed shoots were excised, subjected to vertical cuts (two to three) and then transferred to MS with BA (2.21 µM) for multiple shoot induction. The multiple shoots were isolated and transferred (after three weeks) to MS liquid medium containing different concentrations of NAA (0.53 - 5.3 µM) for root induction. After two weeks, the rooted shoots were transferred to the plastic net-pots (3 cm diam.) in protrays containing a mixture of soil and vermiculite (1 : 1). Prior to their transfer, the plants were treated (20 min) with bavistin (0.01%). The plants were irrigated with one-fourth strength of MS (5 - 6 ml/pot) and also sprayed with 0.01% bavistin to control the fungal growth. A high humidity condition was maintained by regular spray of distilled water at an hourly interval. Following hardening, the plantlets were transplanted to earthen pots containing a mixture of garden soil and compost (1 : 1).

Results and Discussion

To our knowledge there are no published reports on multiplication of *Curculigo* from shoot tips. The plant generation from meristems is considered to be one of the most promising ways for multiplying a selected variety true to its type. Such individuals are genetically similar showing the same agronomic characteristics. *C. orchioides* in its natural conditions is propagated through suckers, which are highly prone to viral infections (Dhenuka et al. 1999). Meristem culture provides a powerful tool to eliminate virus, leading to production of healthy and vigorously growing planting material (Natayanaswamy 1997).

Various concentrations of BA (Table 1) were helpful in inducing the growth of meristem tip. However, the best and rapid growth was observed on MS medium with lowest concentrations of BA (0.44 µM). In contrast, a genus of a related family (Liliaceae), *Chlorophytum,* required a very high concentration of BA (22.2 µM) for shoot regeneration (Purohit et al. 1994).

Vertical cuts on *in vitro* shoots caused the formation of multiple shoots on MS medium containing different concentrations of BA (Table 2). However, the maximum number of multiple shoots were obtained (Fig. 2) with a low level of

BA (2.21 µM). Further increase in the concentration of BA had no effect on the number of multiple shoots. In contrast, in *Crinum* (Amaryllidaceae), the enhanced level of BA had stimulating effect on the total number of regenerated plantlets (Slabbert et al. 1993). Similar results were reported in some members of the Liliaceae and Amaryllidaceae. These results confirmed that some plant species have enough levels of endogenous hormones and does not require high levels of exogenous growth regulators for plant regeneration (Hussey 1982).

Table 1. Effect of various concentrations of BA on shoot growth from meristem-tip.

Concentration (µM)	Response (%) (Mean ± S.E.)
0.44	100.0 ± 0.00
1.10	70.4 ± 3.34
2.21	45.6 ± 2.90
5.3	-

- indicates no response.

Table 2. Effect of various concentrations of BA on multiple shoot formation from *in vitro* shoots of *Curculigo*.

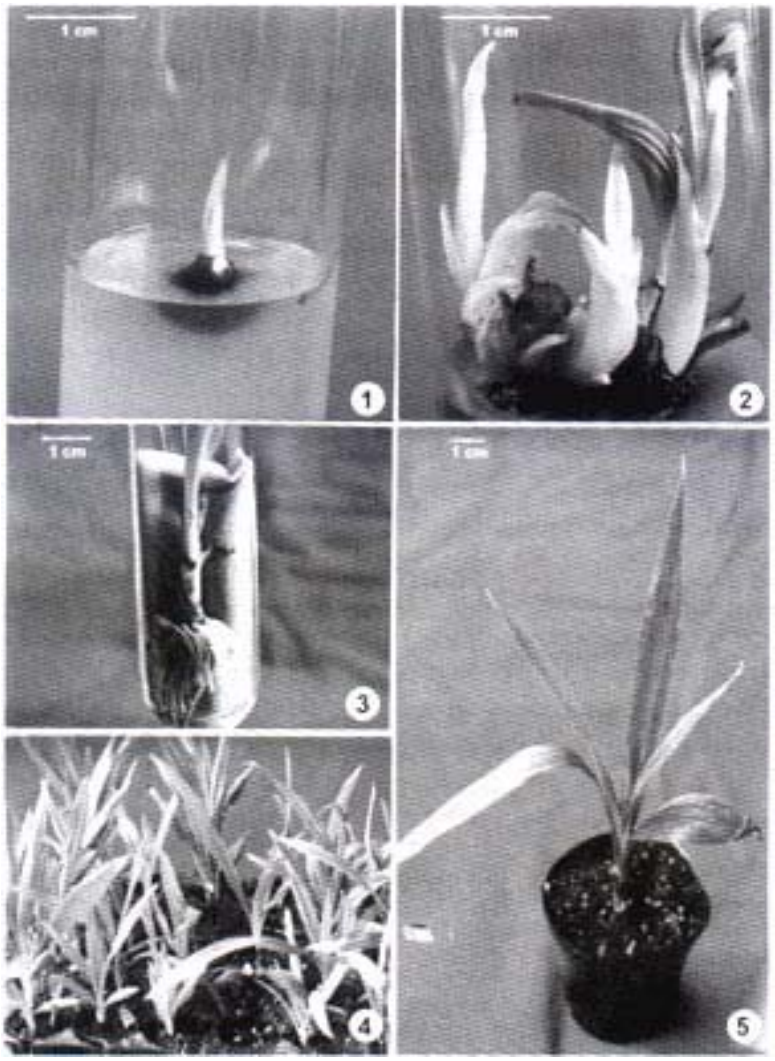
Concentration (µM)	Response (%) (Mean ± S.E.)	Number of shoots/explant
0.0	20.2 ± 2.40	2
1.10	20.2 ± 2.40	4
2.21	100.0 ± 0.00	4 - 5
5.3	-	-

- indicates no response.

Multiple shoots were separated and transferred to liquid MS (half strength), containing different concentrations of NAA for root induction. Transfer to agar based semi-solid medium was not suitable for root induction. However, according to Nadgauda et al. (1978), transfer of *in vitro* multiplied shoots from solid- to liquid medium with filter paper platform was necessary to ensure a healthy root system in turmeric. Emergence of roots occurred within a period of 15 - 20 days. Further incubation of one week led to a very vigorous root growth (Fig. 3). The maximum root growth was recorded on MS with 0.53 µM of NAA as a supplement (Table 3).

Few roots (two - four) were also noted from shoots which were incubated on half strength of MS basal medium without any hormones. Yang (1977) reported

asparagus rooting on a medium containing NAA. Good rooting response was obtained for *Chlorophytum* in auxin-free liquid medium and also with auxin up to 9.8 µM (Purohit et al. 1994).



Figs. 1 - 5: 1. Meristem tip inoculated on MS containing BA. 2. Formation of multiple shoots. 3. Extensive root growth on half strength of MS. 4. Acclimatization of regenerated plantlets. 5. Hardened plant ready for transplantation (Horizontal bar in the figures indicate increase/decrease in magnification of 1 cm).

The rooted plantlets were transferred to a mixture of soil and vermiculite (1 : 1) in the protrays for further development and hardening (Fig. 4). However, almost all the regenerated plantlets showed fungal infection and

eventual wilting. Therefore, in subsequent exercises a sterilized mixture of soil and vermiculite was used with good results. Use of autoclaved mixture of soil and vermiculite (1 : 1) was found mandatory for the acclimation of *Alpinia* (Rolf and Ricardo 1995). During hardening, the plantlets were irrigated with one-fourth strength of MS basal medium (without sugar and vitamins) for one week. This helped the plantlets to recover the shock resulting from a change of environment. Earlier, Kar and Sen (1985) reported maintenance of plantlets in

Table 3. Effect of various concentrations of NAA on root induction from *in vitro* raised shoots of *Curculigo*.

Concentration (μM)	Response (%) (Mean ± S.E.)	Number of roots/shoot
0.0	50.7 ± 3.07	2-4
0.53	100.0 ± 0.00	12-15
2.65	80.4 ± 3.4	> 15
5.3	10.2 ± 2.05	12-15

half strength of MS medium, prior to their transfer to the soil. To check fungal growth, plants were sprayed with a 0.1% bavistin once a week. Humidity was maintained by covering it with rigid plastic cover and frequently spraying of water. Similar process of maintaining humidity was practiced for hardening of banana (Jasrai et al. 2000) and *Alpinia* (Rolf and Ricardo 1995). The hardened plants were transferred to the field after 45 days of good growth (Fig. 5). Almost 96 % of the regenerated plants survived and showed a vigorous growth of rhizome and roots without any morphological variations.

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