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Induction of embryos and plantlets from anthers of *Curculigo orchioides* Gaertn.—An endangered medicinal herb

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Curculigo orchioides Gaertn. is an endangered medicinal herb belonging to Amaryllidaceae. Anthers cultured in MS liquid medium supplemented with 0.5 mg/L BAP or 0.5-1.0 mg/L NAA formed multicellular pollen. However, they did not develop further into embryos. The multicellular pollen cultured with 0.2-1.0 mg/L 2,4-D developed into embryos when transferred to a hormone free medium. Continued culture of embryos on the medium free of growth substances resulted only in root formation. The embryos were, however, converted into plants when they were transferred to a medium with BAP (0.5 mg/L). The matured plants were successfully transferred to the soil.

Keywords: Amaryllidaceae, anther culture, *Curculigo*, embryogenesis

Introduction

Curculigo orchioides Gaertn. is a small geophilous perennial medicinal herb belonging to the family Amaryllydaceae. The extract of the plant is hypoglycaemic and has proven anticarcinogenic activity against Saracoma 180 in mice[1,2]. It has been shown to have antioxidant activity in carbon tetrachloride induced hepatopathy in rats[3]. The plant is also used as a reputed rejuvenative drug. The rhizome is prescribed as remedy in case of piles, jaundice, asthma, diarrhea and gonorrhea. Due to its various medicinal properties, the plant has been overexploited[4] and is, therefore, been included in the list of threatened plants[5].

There are reports of *in vitro* propagation of *Curculigo* using rhizome, apical meristem and leaf explants[6-12]. However, there are no reports of anther culture of *Curculigo*, although induction of embryos from anthers and converting them into plants from species belonging to the related families Liliaceae and Iridaceae has been obtained. Plantlets have been produced from anthers of *Asparagus officinalis*[13-15], the Asiatic hybrid lily[16], *Lilium longiflorum*[17,18], *Gladiolus*[19], *Narcissus tazetta*[20] and *Scilla indica*[21]. Although embryos could be obtained through anther culture in *Amaryllis,* they gave rise to only roots and did not develop into plantlets[22].

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Curculigo propagates itself in nature by underground bulbils and by seeds. The bulbils are produced only during the rainy season and are limited in number. Further, the seed set and germination is poor. As a result, the restoration of the plant under natural circumstances is slow and difficult. Hence, methods for large scale *in vitro* propagation are needed to meet the commercial demand and to conserve the valuable plant. Tissue culture techniques using leaf and stem explants have been reported which, however, yield only a small number of propagules[6-8]. Regeneration of the plant from microspores opens up possibilities for the production of propagules in large numbers. The present paper reports, for the first time, induction of embryos by anther culture of *Curculigo* of the family Amaryllidaceae and the successful conversion of the embryos into plants.

Materials and Methods

Curculigo orchioides plants growing on the campus of St Aloysius College, Mangalore were used for the experiments. *Curculigo* flowers once a year just before and during the monsoon season (May-September). The inflorescence is a scape, which remains underground till it emerges out with the onset of rains. The inflorescences with buds in various stages of development were collected by clearing the soil around the rhizome to expose the scapes.

The entire inflorescence was surface sterilized first with 0.1% Bavistin (Carbendazim), a broad based

fungicide, for 5 min, followed by rinsing with sterile distilled water. Further sterilization was done using a solution of 0.1% mercuric chloride and 0.1% sodium lauryl sulphate for 3 min, and finally repeated rinsing with sterile distilled water. The size of the buds was measured using an electronic caliper. The buds were then sorted out on the basis of size and correlated to the stage of development of the pollen. The buds of size 1.05 mm having anthers with uninucleate microspores were opened under the laminar airflow bench. One anther was used to confirm the stage of development of the microspore. The other 5 anthers from the same flower were inoculated into liquid MS[23] medium supplemented with 20 g/L sucrose and varying concentrations of BAP, NAA or 2,4-D singly in Petriplates 5 cm in diameter. Culture of isolated microspores as an alternative to anther culture was also tested. For isolation of microspores, anthers having uninucleate microspores were placed in a sterile centrifuge tube containing 10 mL of sterile distilled water. They were crushed gently by pressing them against the sides of the tube with a sterilized glass rod. The anther debris was removed by filtering the suspension through a sterilized 325 μm sieve. The microspore suspension was centrifuged at 800 rpm for 5 min and the supernatant containing the fine debris was discarded. The pellet of microspores was resuspended in fresh sterile distilled water and centrifuged again. The resulting pellet was now suspended in a liquid MS medium without any growth regulators. Aliquots of this suspension of microspores were pipetted onto either solid or liquid MS medium supplemented with 20 g/L sucrose, with varying concentrations of BAP, NAA and 2,4-D.

The anthers in which multicellular pollen was formed were transferred to MS media with same level of BAP, NAA or 2 4-D as the initiation media. After a culture period of 2 wk, the anthers were transferred to a medium free of growth substances. The anthers wherein the multicellular pollen had developed into embryos were either cultured further on the medium without growth substances, or transferred to MS medium with varying concentrations of BAP, for the conversion of embryos to plants. The cultures were maintained at a 16 h photoperiod with a photon-flux density of 10-15 $\mu E\ m^{-2}\ s^{-1}$ provided by cool daylight fluorescent tube lights. Each experiment was repeated 3 times with 10 replications. Statistical analysis was done using the GraphPad Instat Statistics Version 2.

Results

Induction of Multicellular Pollen

Isolated microspores cultured either in liquid or on solid medium with any of the growth substances tested did not show any divisions. When entire anthers were cultured on media without any growth regulators, no divisions of the microspores were seen. On the other hand, when the anthers were cultured with low amounts (0.1-0.5 mg/L) of BAP, the uninucleate microspore started dividing on the 5th d of incubation (Fig. 1A). It first became multinucleate (Fig. 1B) and then turned multicellular; 5.45% of the anthers had multicellular pollen with 0.1-0.5 mg/L BAP within 2 wk in culture (Table 1). With higher levels of BAP, multicellular pollen was not formed in any of the anthers. In anthers, cultured with either low amounts of NAA (0.1-0.4 mg/L) or high amounts of NAA (> 1.0 mg/L), multicellular pollen was not induced. Multicellular pollen was formed only with 0.5 mg/L NAA in 4.62% of the anthers and with 1.0 mg/L NAA in 1.98% of the anthers. The highest percentage of anthers (23.2%) developed multicellular pollen with 0.5 mg/L 2,4-D. With higher and lower concentrations of 2,4-D, the percentage of anthers with multicellular pollen was lower than that obtained with 0.5 mg/L 2,4-D. With very high levels (> 5 mg/L) of 2,4-D, no multicellular pollen was formed in the anthers (Table 1).

Development of Embryos

Multicellular pollen induced with BA and NAA did not develop further when the anthers continued to be cultured in the initiating medium. There was no further development of the multicellular pollen also when these anthers were later transferred to a medium free of growth substances. Multicellular pollen formed anthers cultured with 2,4-D produced meristematic clumps within 2 wk of culture when transferred to a MS medium without growth regulators. The meristematic clumps could be easily identified by presence of cells with dense cytoplasm and dark stained nuclei. These clumps formed pro-embryo like structures, which soon developed into globular embryoids in 3-4 wk (Fig. 1C). The globular embryoids developed into elongated embryos after 4 wk in culture and emerged out of the pollen wall (Fig. 1D). The growing embryos ruptured the anther wall. Several embryos were seen protruding out of the dehisced anthers after 6 wk in culture (Fig. 1E).

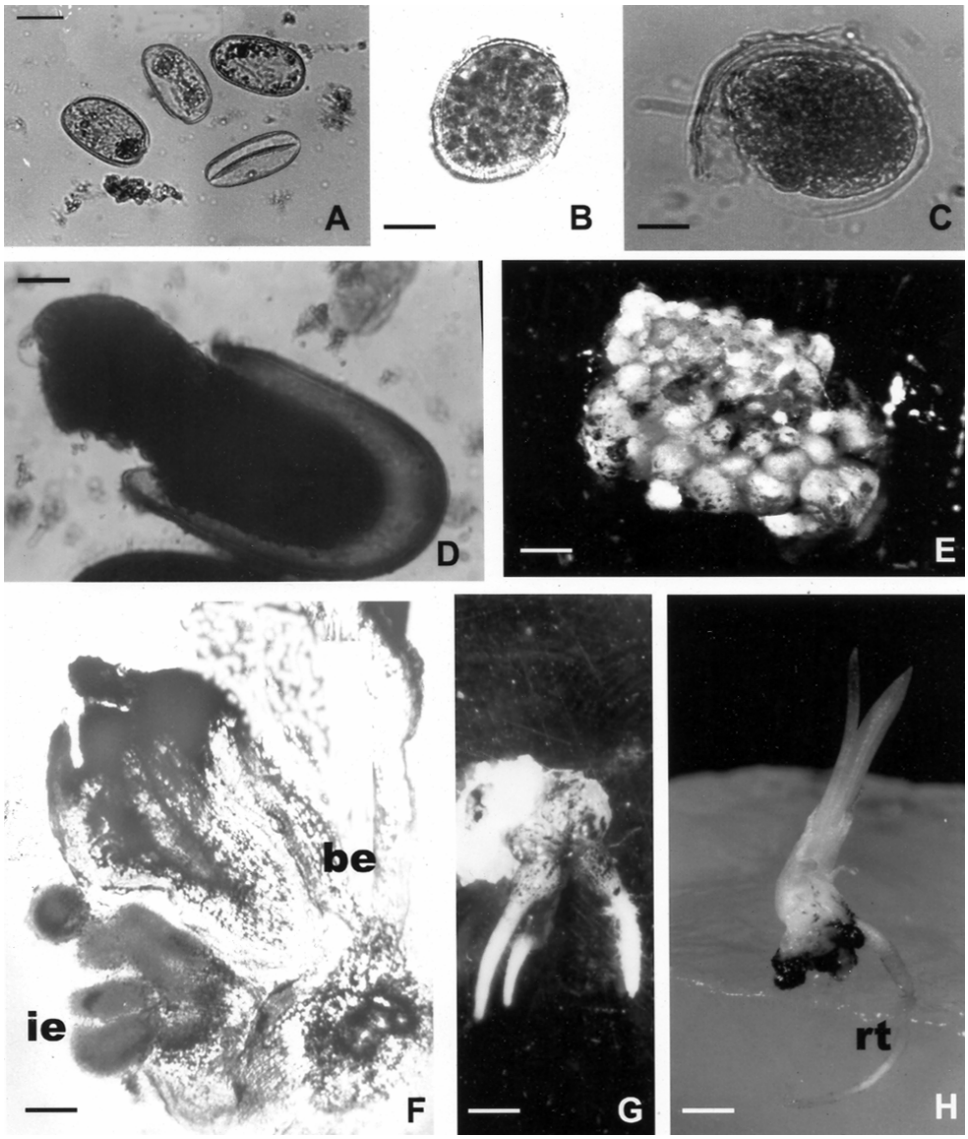

Fig. 1—Stages in the formation of embryos and plantlets from anthers of *C. orchioides*: A. Dividing microspores (bar = 1.8 µm), B. Multinucleate microspore (bar = 2.8 µm), C. Globular embryo formed from the multicellular pollen (bar = 3 µm), D. Elongated embryo after 4 wk in culture emerging out of the pollen wall (bar = 1.9 µm), E. Several embryos protruding out of a dehisced anther after 6 wk in culture (bar =18.2 mm), F. Section of a bipolar embryo (be; incipient embryos) showing definite shoot and root poles (bar = 9.1 µm), H. Embryos giving rise to only roots when cultured on medium free of growth regulators (bar = 5.6 mm), and I. Embryos germinated to form complete plants on MS medium with 0.5 mg/L BAP (rt = root; bar = 6 mm).

Conversion of Embryos into Plants

Sections of the embryos show definite shoot and root poles (Fig. 1F). If they were left in the medium free of growth substances, they did not develop into plants but gave rise only to roots (Fig. 1G). Transfer of embryos to MS medium with low levels (0.1-0.3 mg/L) BAP also resulted in embryos developing only roots (Table 2). When the embryos were transferred to MS medium with 0.4-0.5 mg/L BAP, both shoot and root were formed giving rise to complete plantlets (Fig. 1H). However, with higher levels of BAP, neither root nor shoot initials developed into roots or shoots. Matured plants were successfully transferred to the soil.

Discussion

In most plant species, induction of embryogenesis from microspores at the uninucleate stage is the most efficient way to induce androgenesis, either from cultured anthers or from isolated microspores[24,25]. In

Table 1—Percentage of anthers of *C. orchioides* with multicellular pollen induced with various concentrations of BAP, NAA and 2,4-D

Growth substance	Conc. (mg/L)	Anthers with multicellular pollen (%)[1]
BAP	0	0
	0.5	5.45 ± 1.3ac
	1.0	0
NAA	0-0.4	0
	0.5	4.62 ± 1.1a
	1.0	1.98 ± 0.7b
	2.0	0
2,4-D	0	0
	0.2	6.99 ± 0.9c
	0.5	23.28 ± 3.4d
	1.0	0.87 ± 0.2e
	5.0	0

[1]Values are mean ± SD of three independent experiments each with 10 replicatesValues within the column followed by different letters differ significantly from one another (P = 0.05)

Table 2—Conversion response of embryos of *C. orchioides* on MS medium with various amounts of BAP

BAP (mg/L)	Nature of response
0	Only root pole develops into root
0.1-0.3	Only root pole develops into root
0.4-0.5	Both root and shoot poles develop to form complete plants
> 0.5	Neither root nor shoot pole develops

Each experiment was repeated thrice with 10 replicates in each

C*urculigo,* anthers with microspores at the uninucleate stage were found to be suitable for the induction of embryos. Isolated microspores have proved to be a promising system for increased androgenesis in several plants[26-29]. They also offer advantages, such as, direct monitoring of microspore development and avoidance of plants from somatic tissues of the anther[30]. However, in *Curculigo*, isolated microspores did not develop into embryos with any of the growth regulators tested. Similar results have been reported in barley where no embryos were obtained from isolated pollen, although appreciable embryogenesis was seen when entire anthers were cultured[31]. The effect of auxin in anther culture of monocots has been studied widely and, in general, monocots appear to be auxin dependant to induce mitosis of pollen leading to embryogenesis[32-34]. In rice, 2,4-D is required for induction of androgenesis from anthers[35]. In the case of *Curculigo*, although multicellular pollen was formed with BAP and NAA, only multicellular pollen induced with 2,4-D developed further into embryos.

Authors have been able to induce, for the first time, microspore derived embryos and plantlets from anthers of *C. orchioides*, a member of Amaryllidaceae, and the plants were successfully trasferred to the soil.

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References

1 Bishit B S & Nayar S L, Pharmacognostic study of the rhizome of *Curculigo orchioides* Gaertn, *J Sci Ind Res*, 19C (1960) 25-27.

2 Dhar M L, Dhar M M, Dhawan B N, Mehrotra B N & Ray C, Screening of Indian Plants for Biological activity. Part 1, *Indian J Exp Biol*, 6 (1968) 232-247.

3 Venukumar M R & Latha M S, Antioxidant activity of *Curculigo orchioides* in carbon tetrachloride induced hepatopathy in rats, *Indian J Clin Biochem*, 17 (2002) 80-87.

4 Wala B B & Jasrai Y T, Micropropagation of an endangered medicinal plant: *Curculigo orchioides* Gaertn., *Plant Tissue Cult*, 13 (2003) 13-19.

5 Ansari A A, Threatened medicinal plants of Madhaulia forest of Gorakhpur, *J Econ Taxon Bot*, 17 (1993) 24.

6 Augustine A C & D'Souza L, Regeneration of an anticarcinogenic herb, *Curculigo orchioides* (Gaertn.), *In Vitro Cell Dev Biol (Plant)*, 33 (1997) 111-113.

7 Suri S S, Arora D K, Sharma R & Ramawat K G, Rapid micropropagation through direct somatic embryogenesis and bulbil formation from leaf explants in *Curculigo orchiodes*, *Indian J Exp Biol*, 36 (1998) 1130-1131.

8 Dhenuka S, Balakrishna P & Anand A, Indirect organogenesis from the leaf explants of medicinally important plant *Curculigo orchioides* Gaertn., *J Plant Biochem Biotechnol*, 8 (1999) 113-115.

9 Suri S S, Jain S & Ramawat K G, Plantlet regeneration and bulbil formation *in vitro* from leaf and stem explants of *Curculigo orchioides*, an endangered medicinal plant, *Sci Hortic*, 79 (1999) 127-134.

10 Prajapati H A, Mehta S R, Patel D H & Subramanian R B, Direct *in vitro* regeneration of *Curculigo orchioides* Gaertn., an endangered anticarcinogenic herb, *Curr Sci*, 84 (2003) 747-749.

11 Prajapati H A, Mehta S R & Subramanian R B, *In vitro* regeneration in *Curculigo orchioides* Gaertn., an endangered medicinal herb, *Phytomorphology*, 54 (2004) 85-95.

12 Francis S V, Senapati S K & Rout G R, Rapid clonal propagation of *Curculigo orchioides* Gaertn., an endangered medicinal plant, *In Vitro Cell Dev Biol (Plant),* 43 (2007) 140-143.

13 Pelletier G, Raquin C & Simon G, La culture *in vitro* d'anthere d'Asperge (*Asparagus officinalis*), *CR Acad Sci Ser D*, 274 (1972) 848-851.

14 Hondelmann W & Wilberg B, Breeding all male varieties of Aspargaus by utilization of anther culture and tissue culture, *Z Pflanzenz*, 69 (1973) 19-24.

15 Feng X R & Wolyn D J, Development of haploid Asparagus embryos from liquid cultures of anther derived calli enhanced by ancymidol, *Plant Cell Rep*, 12 (1993) 281-288.

16 Han D S, Niimi Y & Nakano M, Regeneration of haploid plants from anther cultures of Asiatic hybrid lily 'Connecticut King', *Plant Cell Tissue Organ Cult*, 47 (1997) 153-158.

17 Sharp W R, Raskin R S & Sommer H E, Haploidy in Lilium, *Phytomorphology*, 21 (1971) 334-336.

18 Arzate-Fernandez A M, Nakazakit T, Yamagata H & Tanisaka T, Production of doubled-haploid plants from *Lilium longiflorum* Thunb. anther culture, *Plant Sci,* 123 (1997) 179-187.

19 Bajaj Y P S, Sidhu M M S & Gill A P S, Some factors affecting the *in vitro* propagation of Gladiolus, *Sci Hortic*, 17 (1982) 32-37.

20 Chen L, Zhu X, Gu L & Wu J, Efficient callus induction and plant regeneration from anther of Chinese narcissus (*Narcissus tazetta* L. var. *chinensis* Roem), *Plant Cell Rep*, 24 (2005) 401-407.

21 Chakravarty B & Sen S, Regeneration through somatic embryogenesis from anther explants of *Scilla indica* (Roxb.) Baker, *Plant Cell Tissue Organ Cult*, 19 (1989) 71-75.

22 Bapat V A & Narayanswamy S, Growth and organogenesis in explanted tissues of Amaryllis in culture, *Bull Torrey Bot Club,* 103 (1976) 53-56.

23 Murashige T & Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Physiol Plant,* 15 (1962) 473-497.

24 Touraev A, Pfosser M & Heberle-Bors E, The microspore: A haploid multipurpose cell, *Adv Bot Res,* 35 (2001) 53-109.

25 Seguí-Simarro J M & Nuez F, Meiotic metaphase I to telophase II is the most responsive stage of microspore development for induction of androgenesis in tomato (*Solanum lycopersicum*), *Acta Physiol Plant,* 27 (2005) 675-685.

26 Lichter R, Efficient yield of haploid plants from isolated microspores of different Brassicaceae species, *Plant Breed*, 103 (1989) 119-123.

27 Hu T C, Ziauddin A, Simion E & Kasha K J, Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media 1. Effects of pretreatment, isolation methods, and hormones, *In Vitro Cell Dev Biol (Plant),* 31 (1995) 79-83.

28 Shariatpanahi M E, Bal U, Heberle-Bors E & Touraev A, Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis, *Physiol Plant,* 127 (2006) 519-534.

29 Testillano P, Georgiev S, Mogensen H L, Coronado M J, Dumas C *et al*, Spontaneous chromosome doubling results from nuclear fusion during *in vitro* maize induced microspore embryogenesis, *Chromosoma,* 112 (2004) 342-349.

30 Mordhorst A P & Lorz H, Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores are influenced by the amount and composition of nitrogen sources in culture media, *J Plant Physiol*,142 (1993) 485-492.

31 Sarah L, Robert O & James M D, Barley anther culture: Pretreatment with mannitol stimulates production of microspore-derived embryos, *Plant Cell Tissue Organ Cult,* 20 (1990) 233-240.

32 Niizeki H & Oona K, Induction of haploid rice plants from anther culture, *Proc Jpn Acad,* 44 (1968) 554-557.

33 Ball S T, Zhou H P & Konzak C F, Influence of 2,4-D, IAA and duration of callus induction in anther culture of spring wheat, *Plant Sci,* 90 (1993) 195-200.

34 Bhaskaran S & Smith R H, Regeneration in cereal tissue culture: A review, *Crop Sci*, 30 (1990)1328-1136.

35 Chen J X, Sun Z X & Song Z S, Rice tissue culture and somaclonal variation, in *Tissue culture of field crops,* edited by C J Yan (*Shangai Press, Shangai*) 1991, 117-128.