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Direct Somatic Embryogenesis of *Curculigo orchioides* Gaertn., an Endangered Medicinal Herb

T. Dennis Thomas*, Alphonsa Jacob

Post Graduate and Research Department of Botany, St. Thomas College, Pala, Arunapuram-686574, Kottayam (Dt), Kerala, India

Abstract

***In vitro* multiplication of *Curculigo orchioides* was achieved by direct somatic embryogenesis in young leaf segments. Immature leaf segments of about 0.5 cm in length were cultured on MS medium supplemented with different concentrations of BAP (2-10 μM) or Kin (2-10 μM). Optimum response in terms of per cent cultures responding (89%) and the number of embryos per explant (16) were observed on MS medium supplemented with 8 μM BAP. The emergence of several somatic embryos on the adaxial side of the leaf segments was observed one month after the culture. Germinated somatic embryos were grown up to about 1.5 cm length before transferring to maturation medium. For maturation, the individual embryos were isolated and transferred to MS medium supplemented with BAP (5 μM) and NAA (0.5 μM). The plantlets emerged from the embryos were transferred to soil containing 1 peat: 1 sand with 90% success. The embryos were formed directly on the leaf segments without any callus phase. Direct regeneration of somatic embryos is important for the conservation of this endangered species, as rare somaclonal variants are likely to arise than from indirect regeneration.**

Key words: Endangered species, leaf segment culture, medicinal plant, direct somatic embryogenesis

Introduction

Curculigo orchioides Gaertn., (Family Hypoxidaceae) is an endangered medicinal plant. The rhizome extract of this plant consists of a number of useful compounds like flavonone glycoside I, three kinds each of steroids and saponins and six types of triterpenoids (Garg et al. 1989; Xu and Xu 1992; Xu et al. 1992; Tandon and Shukla 1995). The anticarcinogenic activity of this plant against sarcoma 180 in mouse is also reported (Dhar et al. 1968). This subterranean herb is naturally available only during rainy season and due to excessive exploitation the species has become endangered. *C. orchioides* propagates naturally through seeds but poor seed set and germination are the limiting factors for natural propagation (Gupta and Chadha 1995). The plants can also be propagated through underground rhizomes. But poor growth and insect attack always limit this method of conventional propagation. Plant tissue culture offers many attractive techniques for mass multiplication of plants (Bhojwani and Razdan 1996). *In vitro* micropropagation using different explants was reported in *C. orchioides* (Suri et al. 1999; Prajapati et al. 2003). However somatic embryogenesis has not been reported in this plant.

Plants are distinctive in their ability to produce somatic embryos (Gray 1989). Somatic embryos and zygotic embryos are structurally and physiologically similar, but the former lacks protective seed coats and nutritive accessory tissues (Gray and Purohit 1991a). A quiescent resting phase found in seeds is also absent in somatic embryos (Gray and Purohit 1991b). Standardizing protocols for somatic embryogenesis is important in the study of basic mechanisms of embryogenesis, morphogenesis and senescence. Somatic embryogenesis and synthetic seeds could be employed for the large scale *in vitro* micropagation of this plant. Thus, the present study was carried out with the objective to induce somatic embryogenesis in *C. orchioides* from immature leaf segments.

* Corresponding author, E-mail: den_thuruthiyil@yahoo.com
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Materials and Methods

Plant Material

The leaves of *C. orchioides*, used for the experiments, were collected from mature plants growing in the botanical garden of the college. The leaves were collected 10, 20, 30 and 40 days after emergence and were cut into small segments, each measuring about 0.5 cm in length. The surface sterilized leaf segments were cultured on MS (Murashige and Skoog 1962) medium supplemented with various concentrations of BAP (2-10 μM) and Kin (2-10 μM). The leaf segments were placed on media with its abaxial side touching the media.

In another experiment, the leaf segments were cultured on MS medium containing 8 μM BAP with increasing concentrations of sucrose, fructose or glucose to determine the influence of sugar source and concentration on embryogenesis.

Embryo maturation and transplantation

For somatic embryo maturation, individual embryos measuring about 1.5 cm in length were isolated and subcultured on half strength MS medium supplemented with BAP (5 μM) and NAA (0.5 μM). Well developed plantlets derived from embryos were then transferred to *ex vitro* acclimatization conditions in 1 peat: 1 sand mixture in plastic pots, and maintained under 16 h light/8 h dark at 25±2℃ with intermittent misting. Relative humidity was decreased gradually from 95% at the beginning to 70% over a period of six weeks of acclimatization. Survival rate was recorded. The acclimatized plants were transferred to the greenhouse conditions in earthen pots. After three months, plants were transferred to field and further studies are in progress to evaluate the field performance of the *in vitro* regenerated plants.

Culture conditions

All the media, unless otherwise mentioned, contained 30 g/L sucrose and gelled with 0.8% agar and the pH was adjusted to 5.8 after adding the growth regulators. The media were steam sterilized in an autoclave under 1.5 kg/cm^2 and 121℃ for 15 min. All the cultures were grown at 25±2℃ under 16 h photoperiod supplied by two fluorescent tubes. Twenty four cultures were raised for each treatment and all experiments were repeated three times. Analysis of variance and Duncan's multiple range test was used for comparison among treatment means.

Results and Discussion

On MS medium supplemented with various concentrations of BAP (2-10 μM) or Kin (2-10 μM), cultured leaf segments produced swellings on its adaxial surface near the cut end, which later transformed into somatic embryos. The growth of the embryos was slow and occasionally got detached from the parent leaf segments and continued their growth. There was no callusing at any stage of the somatic embryo development. The maximum response in terms of per cent cultures responding and the number of embryos per explant was observed on MS medium supplemented with 8 μM BAP (Table 1). On this medium, 89% cultures responded with an average 16 embryos per explant. The leaf age also plays a major role in somatic embryogenesis. The maximun response was observed when 20 day-old explants were used for culture experiments (Table 2). Hence, all further experiments were conducted with 20-day-old explants. Low frequency of embryogenesis and embryogenic induction related to the age of the explant suggest that the intrinsic physiological stage of the explant plays a decisive role in inducing embryogenesis. Such observations were reported in somatic embryogenesis from zygotic embryo cultures of several genera (Mathur et al. 2000; Gogate and Nadgauda 2003).

Among the different carbohydrate sources (sucrose, fructose

Table 1. Effect of different growth regulators on somatic embryogenesis from leaf segments in *C. orchioides*.

Cytokinin conc. (μM)	% cultures responding	Number of somatic embryos per explant
BAP		
0	0	0
2	61	3±0.3a
4	78	6±0.5b
6	80	9±0.7c
8	89	16±1.4e
10	71	11±0.6d
Kin		
0	0	0
2	45	3±0.4a
4	61	6±0.6b
6	68	8±0.7c
8	76	6±0.2b
10	59	5±0.6b

Twenty days old embryos were cultured on MS medium supplemented with 300 mM sucrose for 90 days. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

Table 2. Effect of explant (leaf) age on somatic embryogenesis.

Explant age (d)	% cultures responding	Number of somatic embryos per explant
10	45	6±0.3a
20	89	16±1.2c
30	65	9±0.6b
40	58	7±0.7a

Explants were cultured on MS medium supplemented with 8 μM BAP for 90 days. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

and glucose) and concentrations (1-4%) used, the maximum number of embryos per explant was observed when leaf segments were transferred to MS medium supplemented with 8 μM BAP and 2% sucrose. On this medium, the highest number of 19 embryos per explant was produced in 90 days. However, the maximum per cent (89%) of the cultures responded when MS medium was supplemented with 8 μM BAP and 3% sucrose. The use of fructose was not effective, with very few embryos formed as compared to other sugar concentrations (Figure 1 and 2). The importance of different sugars and their concentrations on somatic embryogenesis is well documented in different systems (Jehan et al. 1994; Samoylov et al. 1998; Nakagawa et al. 2001; Anthony et al. 2004). The carbohydrates probably influence the embryogenic pathway physiologically, rather than as an energy supply, since their effects usually occur at low concentrations (Kochba et al. 1978). Sugars at high concentrations (400 mM and above) usually have a negative role in somatic embryo induction. This is generally attributed to the strong osmotic stress regulated by sugars, which might change metabolic regulation (Nakagawa et al. 2001).

The embryos emerged as small outgrowths on the adaxial surface of the leaf segments. The visible sign of these outgrowths were observed 15 days after culture. Several fully developed white globular embryos were observed 45 days after culture (Figure 3a and 3b). The cultured leaf segments turned dark as the embryos develop. According to some workers, it is very difficult to observe different stages of embryogenesis in monocots. Moreover, the heart shaped stage is absent in monocots as they have only one cotyledon (Sivakumar et al. 2003). Somatic embryo development was non-synchronized, with embryos in different developmental stages were observed in the same culture (Figure 4a). The embryos measuring about 1.5 cm were isolated individually and transferred to MS medium supplemented with BAP (5 μM) and NAA (0.5 μM) for maturation (Figure 4b). All the embryos developed into plantlets in the maturation medium six weeks after subculture (Figure

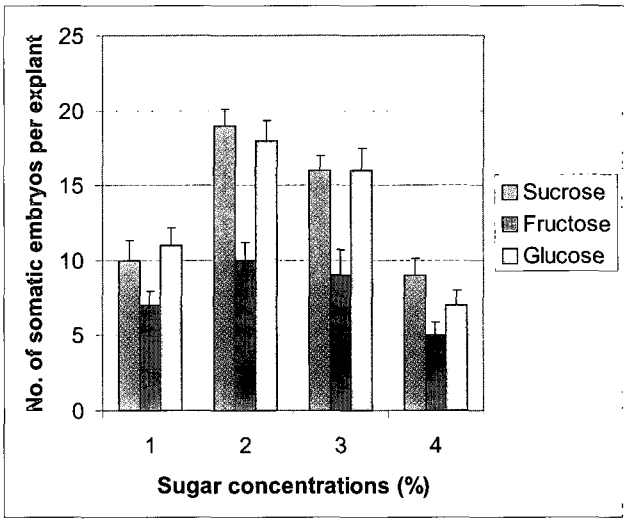

Figure 1. Influence of different concentrations of sucrose, fructose and glucose in conjunction with 8 μM BAP on number of somatic embryos per explant during embryogenesis in *C. orchioides.* Results expressed as means of three replicates ± SD.

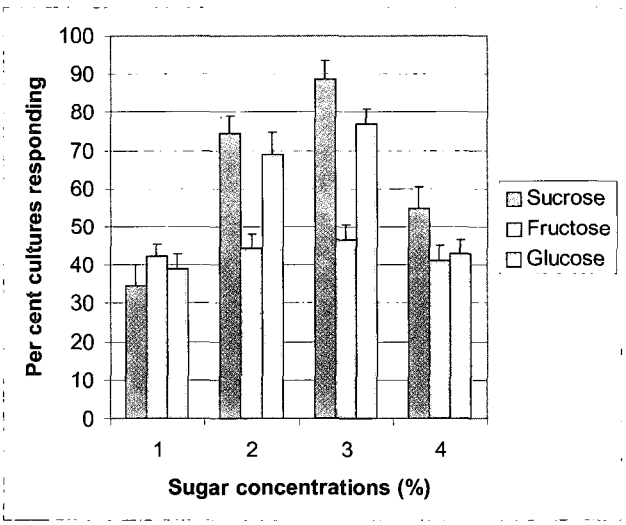

Figure 2. The influence of different concentrations of sucrose, fructose and glucose in conjunction with 8 μM BAP on per cent cultures responding during embryogenesis in *C. orchioides.* Results expressed as means of three replicates ± SD.

4c). The plantlets were eventually transferred to 1 peat: 1 sand mixture in plastic pots with 90 % success (Figure 4d). The embryos were formed on the leaf surface without any callus phase. Similar results were observed in bottle palm (Sarasan et al. 2002). The direct regeneration of embryos in the present investigation is less likely to generate somaclonal variants than indirect regeneration from callus tissue (Merkle 1999). To the best of our knowledge this is the first report on the somatic embryogenesis in this taxon.

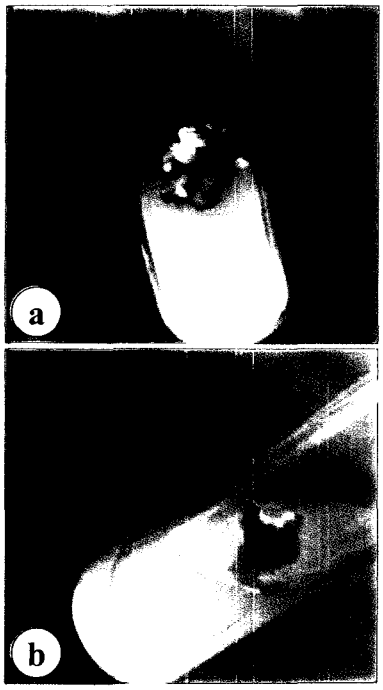

Figure 3. Different stages of direct embryogenesis in *C. orchioides* from leaf pieces.
A. Initiation of globular embryos from the explant on MS medium supplemented with 8 μM BAP 45 days after culture.
B. Somatic embryos emerged from leaf explants on MS medium supplemented with 8 μM BAP.

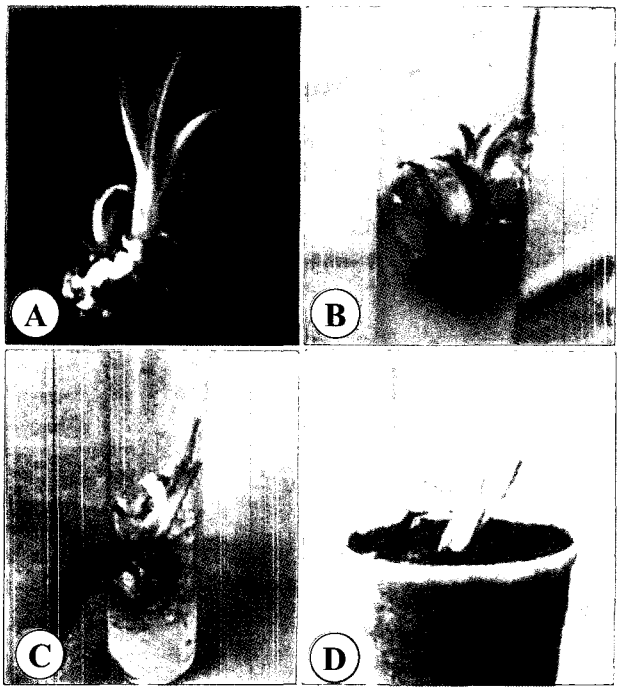

Figure 4. Somatic embryo maturation and transplantation to soil.
A. A group of germinating embryos at different stages of development on MS medium supplemented BAP (8 μM) 90 days after culture.
B. Isolated embryos on MS medium supplemented with BAP (5 μM) and NAA (0.5 μM) for embryo maturation at the time of culture.
C. Rooting of the embryos on MS medium supplemented with BAP (5 μM) and NAA (0.5 μM) 60 days after culture.
D. A transplanted plant two weeks after transfer. New leaves have started emerging.

In conclusion, we have been able to achieve direct somatic embryogenesis from leaf segments of *C. orchioides*. The protocol described here could not only be used for the propagation of this plant but also for the genetic transformation procedure. This technique could also provide ample evidence for the better understanding of the embryogenesis events in *C. orchioides*.

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