The Bioscan 7(1) : 39-41, 2012
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

HISTOLOGICAL EVIDANCES FOR DE NOVO SHOOT FORMATION IN AN ENDANGERED MEDICINAL HERB- CURCULIGO ORCHIOIDES GAERTN

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KEY WORDS
Organogenesis
Histological
Curculigo

Received on :
12.09.2011

Accepted on :
27.12.2011

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ABSTRACT
In vitro derived leaf explants of *Curculigo orchioides* Gaertn. were cultured on MS media containing half-strength nitrogen salts and 0.44 μM BA. The histological events leading to de novo shoot formation were evaluated. The studies clearly demonstrated initiation of the shoot buds from the phloem parenchyma cells on 9th day of culture initiation. The present work focuses on the sequence of events leading to the shoot organogenesis of Kali musli.

INTRODUCTION

Curculigo orchioides Gaertn (family-hypoxidaceae) commonly known as Kali musli, is the first to appear with onset of monsoon and last to disappear on completion of monsoon (Francis *et al.,* 2007). In india it is distributed in sub-tropical Himalayas from Kumaon eastward and in the western ghats from Gujarat-Maharastra southwards. 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 and 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).

Phytochemical investigations of the rhizome revealed the presence of curculigoside, orcinol glycosides (Li *et al.,* 2003), 2,6,- dimethoxyl benzoic acid (Chen and Mei, 1999), curculigoside A-C (Chen and Mei, 1999), curculigol , curculigo saponins A-M (Xu *et al.,* 1992), 2, 3, 4, 7-tetramethoxyxanthone 1,3,7- trimethoxylanthine, daucosterole (Li *et al.,* 2003).

Removal of plants for medicinal and edible tuberous roots as a substitute for safed musli, coupled with extensive denudation of forest floor caused by cattle grazing (Jasrai and Wala, 2000), 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). Hence the methods for large scale *in vitro* propagation are needed to meet the commercial demand and to conserve this valuable plant. Micropropagation offers the possibility of large scale multiplication of this endangered plant.

Our studies on *Curculigo orchioides* showed that shoot regeneration occurs via directly after a short culture period of 3 weeks (Wala and Jasrai, 2003). These cultures have been able to produce viable plants by subsequent rooting. However, the occurrence of direct organogenesis has to be clearly demonstrated by anatomical evidences.

The present work focuses on the sequence of events, specially at the anatomical level, leading to the process of direct organogenesis of *Curculigo orchioides*. This is the first study to report histological evidences for shoot formation in this important and endangered species.

MATERIALS AND METHODS

Young leaves from *in vitro* raised shoots 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 (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 0.9gm/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 medium was adjusted to 5.8 and autoclaved at 121ºC (20 min). The cultures were

incubated at 25ºC under 16h photo-period with 55 μ mol $m^{-2}s^{-1}$ photon flux density.

Explant samples were collected on 5th day to 25th days of culture and fixed in FAA (formalin: acetic acid: ethanol). The fixed samples were dehydrated in a graded series of ethanol/ tertiary butanol, followed by infiltration and embedding in paraffin wax (Merck, melting point 58-60ºC). Serial sections (10 μm thick) were cut with a rotary microtome (Zeus 150R). The sections were affixed to slide, dewaxed and stained with safranin (Johansen, 1940). The stained sections were studied under microscope with attached camera (Lawrence and Mayo, India). Micro-photographs were taken and histological data of different stages of organogenesis were recorded.

RESULTS AND DISCUSSION

Middle lamina of the leaf explants (Fig. 1a) gave significant morphogenetic response on MS medium supplemented with half-strength nitrogen salts and 0.44 μM BA. This protocol resulted in 100 % shoot regeneration response from inoculated explants (Fig. 1b). Earlier reports also demonstrate direct shoot organogesis from leaf explant using BA as growth regulator (Augustine and D'suoza, 1997; Prajapati *et al.,* 2003; Wala and Jasrai, 2003).

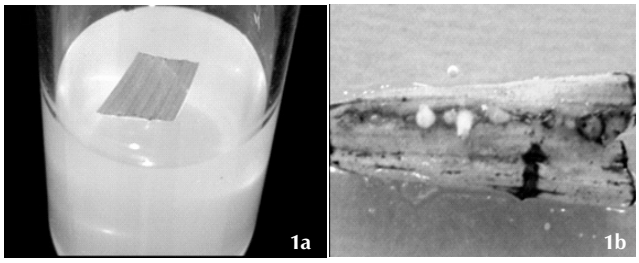

Figure 1: *De novo* shoot formation in Kali musli a. Leaf explants, b. formation of shoots

Plant tissue culture system coupled with histology offer the possibility of identifying the point determination of cell differentiation leading to morphogenesis (Fukuda and Komamine, 1985). Thin sections of the leaf explants revealed origin of new structures from the cell initials associated to vascular bundles. Vasculature of this plant displays xylem to the adaxial side and phloem to the abaxial side with sclerenchymatous cells towards upper and lower epidermis respectively. Histological examination of shoot formation process showed pro-meristemoids being formed in the sub-epidermal region in the midrib and veins. These structures developed subsequently into shoot primordia.

On MS medium with half-strength N and low level of BA, the cultured explants exhibited cell cluster in the phloem parenchyma associated with vasculature in veins and midrib on 9th day of the culture. The predominance of cell divisions in response to BA has been demonstrated earlier (Saravitz *et al.,* 1993; Villalobos *et al.,* 1985; Von Arnold and Gronroos, 1986). The capacity of cell to respond to specific signals, probably of a hormonal nature, could lead parenchymatous cell to dedifferentiate; subsequently forming a meristemoid and finally an organ (Thorpe, 1980).

Judging from the cell division pattern and arrangement for the pro-meristemoid indicate likely origin from a single cell in Kali musli (Fig. 2a). Within next 2-3 days, the parenchymatous cells exhibited periclinal divisions. In *Passiflora edulis* (Gloria *et al.*, 1999) and *Fraser fir* (Saravitz *et al.*, 1993) similar divisions were observed in the mesophyll cells close to the vasculature.

The continuous divisions in these cells lead to the formation of meristematic zone (Saravitz *et al.,* 1993) consisting of small cells with a dense cytoplasm. On day 15th of culture, with further cell divisions the vascular system seemed disorganized (Fig. 2b). Eventually, epidermal layers ruptured as the divisions progressed. The first leaf primordia with meristemetic dome was observed on 22nd day (Fig. 2c), confirming that these structures originated from the cell located as sub-epidermal layers. The inception of new leaf primordial is indicated by the densely stained and well arranged cells.

Within next two days next leaf primordia was observed (Fig. 2d) with cells arranged into longitudinal files covered by periclinally devided elongated cells. A thick and continuous layer of elongated cells suppose to develop the epidermal cells of shoots.

The data clearly demonstrate that the shoots of *Curculigo orchioides* developed from the meristemoids, as a result of mitotic divisions in phloem parenchyma during first week of culture. Similarly in *Begonia erythrophyla* (Burritt and Leung, 1996) and conifers (Villalobos *et al.*, 1985; Von Arnold and Gronroos, 1986; Saravitz *et al.,* 1993) formation of shoots were reported directly from paranchymatous cells of epidermal origin.

The histological studies with *Curculigo orchioides* clearly showed that plant regeneration occurred via *de novo* bud differentiation in around 3 weeks of culture. These cultures

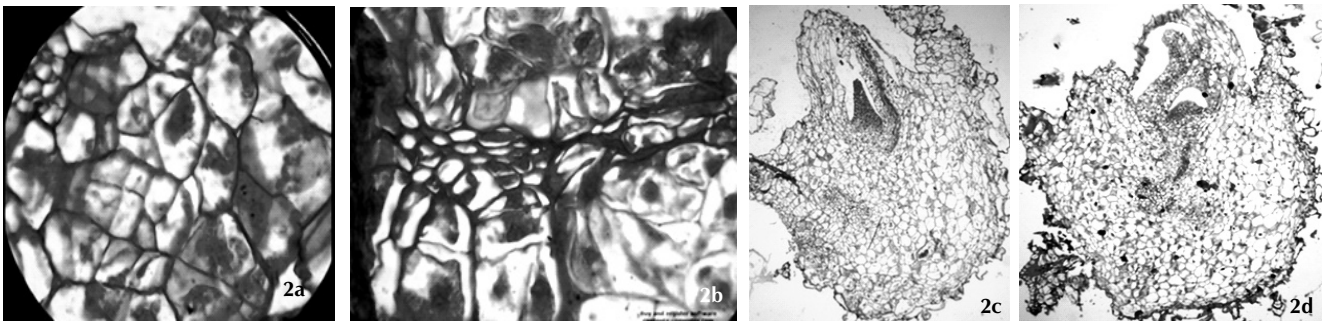

Figure 2: T.S. of leaf explants of Kali musli on MS medium after a. 9th day, b.15th day, c. 22nd day and d. 24th day of culture. (a. and b. 100x , c. and d. 40x)

were further processed for induction of root and hardening of generated plantlets (Wala and Jasrai, 2003).

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