Histological Analysis of the Callogenesis and Organogenesis from Root Segments of *Curcuma zedoaria* Roscoe

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ABSTRACT

Callus was induced from root segments taken from in vitro *grown plants of* Curcuma zedoaria *Roscoe. The explants were cultured on agar-solidified Murashige and Skoog medium supplemented with 13.4mM of* **a***-naphthaleneacetic acid and 2.2mM of 6-benzylaminopurine at 25ºC in the dark. Histological analysis revealed that callus was formed from the hypertrophied cortical parenchyma cells of the explant. Some of these cells underwent division while the surrounding cells accumulated starch. Callus was capable of shoot bud regeneration after 70 days when it was transfered to liquid medium of the same composition. After 30 days in liquid medium, buds developed from nodular structures. The adventitious shoots developed extensive root systems when they were placed on agar-solidified Murashige and Skoog medium without growth regulators at 25º C in the light. The establishment of these plantlets in soil was about 95%.*

Key words: *Adventitious bud, anatomy, morphogenesis, Zingiberaceae*

INTRODUCTION

Zedoary (*Curcuma zedoaria* Roscoe), a vegetatively propagated species of the Zingiberaceae family, is an aromatic herbaceous plant with a rhizome. As a common house-hold remedy, it has been used extensively as an expectorant, demulcent, diuretic, rubefacient, cholagogue and for treatment of gastritis (Miyake, 1986). Pharmaceutical companies have translated the commercially valuable therapeutic properties of the rhizome into products that medical doctors prescribe to effectively treat gastritis and problems with digestion (Yasuda *et al.,* 1988). In addition to the well-known effect of zedoary as a stomachic, this specie has antiinflammatory potency related to its antioxidant effect (Yoshioka *et al*., 1998) and

has demonstrated to be hepatoprotective (Matsuda *et al*., 1998) and cytotoxic against human ovarian cancer cells (Syu *et al*., 1998).

It is important to note that, as is the case with ginger (Babu *et al,*. 1992), the absence of seed set in *Curcuma zedoaria* renders conventional breeding methods ineffective. *In vitro* culture represents an alternative method for the creation and exploitation of the resultant variation. Thus, this technique offers significant potential for crop improvement.

While there are no reports for *C. zedoaria*, morphogenesis *in vitro* has been reported for some members of the Zingiberaceae family (Roy & Pal, 1991; Babu *et al,*. 1992; Balachandran *et al*., 1990; Kackar *et al.,* 1993; Reghunath & Priyadarshan, 1993; Dogra *et al.,* 1994; Hung, 1995; Illg &

Faria, 1995) as well as for the *Curcuma* genus (Dekkers *et al.,* 1991; Barthakur & Bordoloi, 1992).

To date, no detailed histological analysis of the complete process of *de novo* shoot bud formation in Zingiberaceae has been undertaken. The aims of the work were to study the organogenesis in *C. zedoaria* root segment, and to describe the histological changes leading to the induction of meristematic tissue which produces meristemoids and, finally, shoot primordia.

MATERIALS AND METHODS

Root segment explants of 1cm length and 1mm diameter were excised from *in vitro* growing *Curcuma zedoaria* plants obtained from rhizome cuttings cultured on 0.75% (w/v) agar-solidified (Sigma) Murashige & Skoog (1962) (MS) basal medium. The cultures were incubated at 25°C under light with a photoperiod of 16h and an irradiance of 30 μmolm^{-2}s^{-1} provided by "Philips" cool white fluorescent tubes. The explants were placed on the same medium, supplemented with 3.0% (w/v) sucrose,13.4μM of α-naphthaleneacetic acid (NAA) and 2.2μM of 6-benzylaminopurine (BA). The pH was adjusted to 5.8 prior to autoclaving. Cultures were incubated in total darkness. After 70 days calluses excised from explants were transfered to MS liquid medium of the same composition and kept under similar conditions.

During the culture, samples were periodically taken and fixed in FAA 50 (Sass, 1951) or Karnovsky solution (Karnovsky, 1965). After fixation in formalin, glacial acetic acid and 50% ethyl alcohol (FAA 50), samples were dehydrated through an ethanol series, embedded in paraffin, sectioned at 8 μm (Sass, 1951) and stained in fuchsin/astra blue combination (Roeser, 1972).

Samples fixed in Karnovsky solution were dehydrated through an ethanol series, and infiltrated with glycol methacrylate resin (Reichert-Jung, Germany). Sections were cut (5 μm) and stained with toluidine blue O (Sakai, 1973). All samples were observed with a Nikon (Labophot) microscope. Starch was identified using iodide zinc chloride in non-fixed, free-hand sectioned samples (Strasburger, 1913) and with polarized light in fixed sectioned samples.

Rooting of regenerated shoots was performed in 0.75% (w/v) agar-solidified (Sigma) MS medium, containing 3.0% (w/v) sucrose under the conditions previously described. Acclimatization was performed under greenhouse conditions, in plastic pots consisting of 6x10x15 cm units containing autoclaved sand, covered with a rigid plastic cover that provided an environment with high relative humidity. Plants were sprayed regularly with water (Illg & Faria, 1995).

RESULTS AND DISCUSSION

The anatomy of *Curcuma* root explants followed the pattern described by Tomlinson (1969) for the Zingiberaceae family. After 3 days of culture, the first explant alterations could be detected when several cortical parenchyma cells were hypertrophied (Figure 1 arrow).

Within 20 days of culture, the histological analysis showed that a large number of lateral roots were produced from the explant pericycle. After 30 days of culture, some of the outer cortical parenchyma cells exhibited starch accumulation and the hypertrophy process intensified resulting in rupture of epidermis. Thus, the callus could be seen on the explant as well as on the surface of its lateral roots.
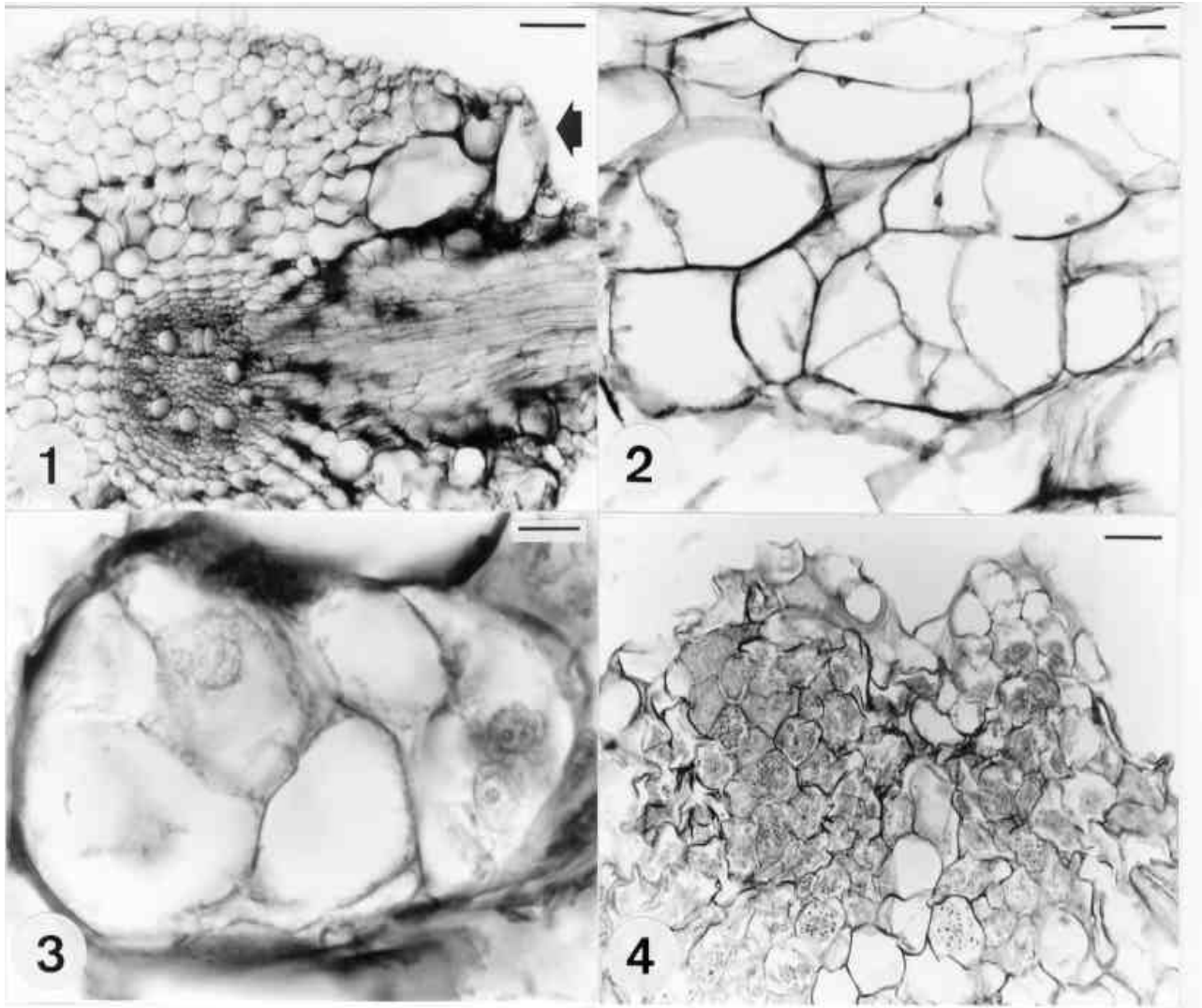
Figure 1-4 - Transverse section of *C. zedoaria* radicular explant cultured in MS solid medium supplemented with 13.4µM NAA and 2.2µM BA in the dark. Figure1- Explant hypertrophied parenchyma cell (arrow) after 3 days in culture. Bar=100µm. Figures 2, 3: Hypertrophied parenchyma cell showing division after 60 days of culture. Bar=50µm and 10µm, respectively. Figure 4: The compact sector of the *C. zedoaria* callus showing starch accumulation after 70 days of culture. Bar=100µm.

Within 60 days of culture, the callus exhibited loosely hypertrophied cells arranged around the vascular cylinder. In some areas the hypertrophied cells divided several times giving rise to various daughter cells included in the original cell wall (Figures 2, 3). Those derived cells were dedifferentiated and also underwent division. Therefore, the callus in *Curcuma zedoaria* arose from hypertrophied parenchyma cell division. A similar process was reported by Gautheret (1957) in *Amorphophallus rivieri*. Figueiredo *et al.* (1989) detected that only the cortical tissue was transformed into callus in *Datura insignis*. They also observed that the meristematic centers were always formed close to the starch accumulating cells.

Either on one end or on both ends of the explant, several calluses could be observed on a single root after 70 days of culture. In some instances, one or more calluses could also be detected on a lateral root. A similar process was described in *Iris* by Laublin *et al.* (1991). At the moment of callus transference in MS liquid medium, its structure was not homogeneous. The inner cells were

elongated, vacuolated and loosely arranged, giving the callus a friable aspect. However, similar to those reported by Laublin *et al*. (1991), the outer cells were isodiametric, cytoplasmically dense and closely arranged. These compact sectors of the callus revealed conspicuous levels of starch accumulation (Figure 4).

Soon after being transfered to the MS liquid medium, some of the outer cells of the calluses underwent divisions (Figures 5, 6) leading to the formation of meristematic areas similar to those described by Saravitz *et al*. (1993) as meristemoid areas (Figure 7). These meristemoids were constituted of densely cytoplasmic isodiametric cells with prominent, enlarged nuclei and nucleoli (Figure 7). According to Thorpe (1980), the capacity of a cell to respond to specific signals, probably of a hormonal nature, could lead a parenchyma cell to dedifferentiate; subsequently forming a meristemoid and finally an organ. There is strong evidence that the combination NAA + BA enhances shoot regeneration from *Alstroemeria* (Gonzales-Benito & Alderson, 1992) and *Dianthus chinensis* (Jethwani & Kothari, 1996) organogenic calluses. The effect of combining these two growth regulators was also described for micropropagation experiments in *Curcuma amada* (Barthakur & Bordoloi, 1992) and other members of the Zingiberaceae family (Babu *et al.,* 1992; Reghunath & Priyadarshan, 1993; Dogra *et al.,* 1994; Hung, 1995; Illg & Faria, 1995).

The involvement of starch accumulating cells in the shoot-forming process, as described by Sharma *et al*. (1993), could also be observed in this study.

According to these authors, the degraded by-products of starch, along with free sugars from the medium were utilized as an energy source for meristemoids and bud differentiation.

After 30 days in MS liquid medium, the meristemoids (Figure 7) elongated vertically and acquired well defined shoot-bud primordial shape (Figures 8, 9). The structure of the shoot apex was normal (Figure 10). The top of the axis was dome-shaped and two distinct zones were identified: tunica and corpus. Leaf primordia arose gradually around the shoot apex. The area joining the shoot buds to the callus was of significant width and their vascular tissue could be distinctly seen as originating from the callus tissue (Figure 10). The frequency of shoot primordia formation on the surface area of each callus was 2.3cm^{-2}(mean of 10 samples).

In order to induce rooting, shoots of 5.0cm developed from the callus were placed on agar-solidified MS medium without plant growth regulators, under standard light conditions. An extensive root system developed within 40 days in 100% of the plantlets. The establishment of the rooted plantlets in sand was 95% when a high level of humidity was maintained for the first 20 days after transfer to soil.

According to Babu *et al*. (1992), when already differentiated tissues are used for plant regeneration, there may be an increased rate of somaclonal variation through an intermediary callus phase. This source of variation could be exploited for crop improvement specially since other conventional methods have shown to be ineffective (Silvarolla, 1992) as for *C. zedoaria*.
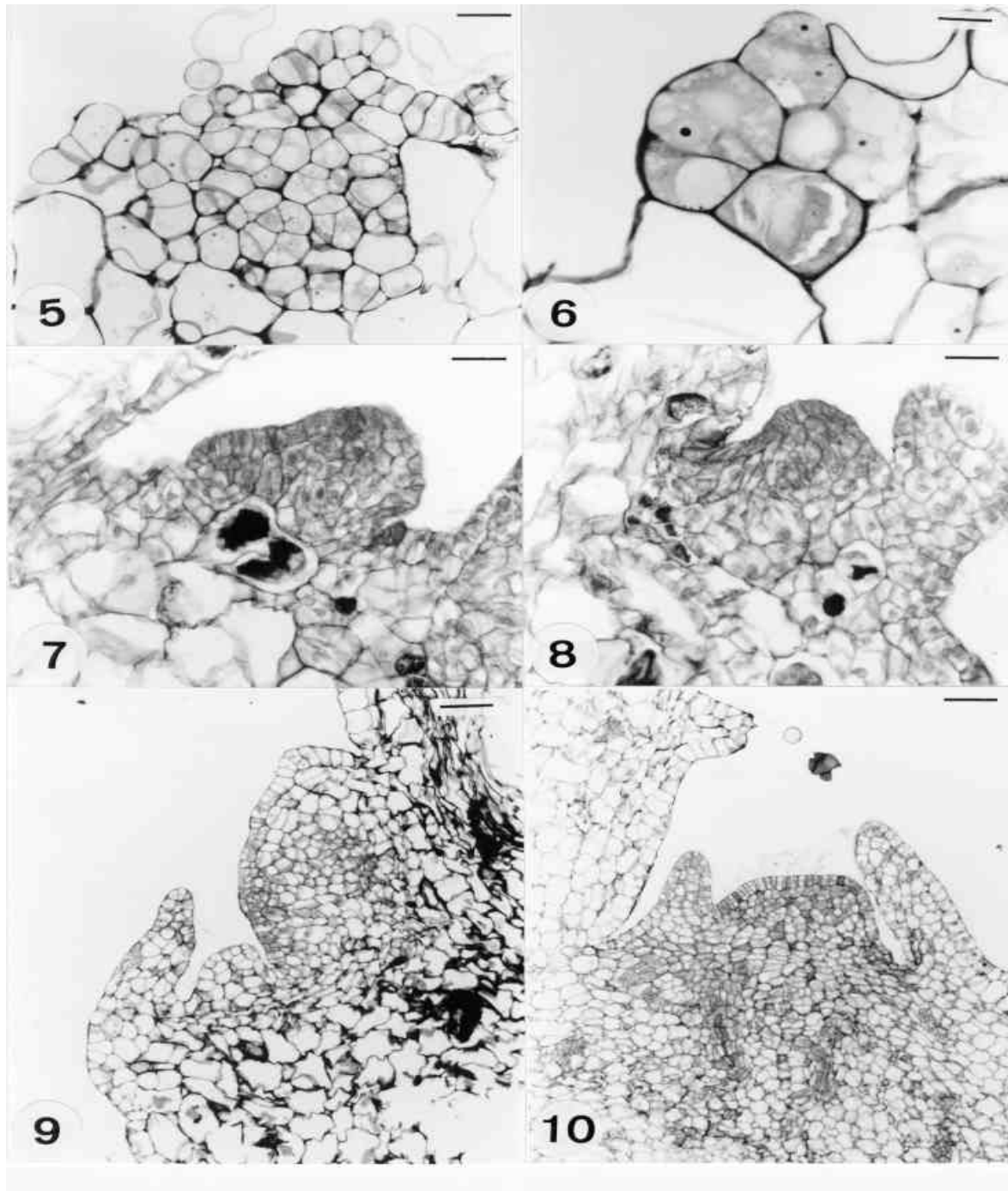
Figure 5 – 10 - Longitudinal axial sections of *Curcuma zedoaria* Roscoe organogenic calluses cultured in MS liquid medium supplemented with 13.4μM NAA and 2.2μM BA in the dark. Figures 5 and 6- meristematic area after 10 days in liquid medium. Bar =50μm and 26μm, respectively. Figure 7- meristemoid constituted of isodiametric densely citoplasmic cells after 20 days in liquid medium. Bar = 19μm. Figures 8and 9- shoot-bud primordia after 30 days in liquid medium. Bar =37μm and 100μm, respectively. Figure 10- shoot apex and its vascular connection with the callus tissue. Bar = 100μm.

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RESUMO

Calo de *Curcuma zedoaria* Roscoe foram induzidos a partir de segmentos de raízes de plantas cultivadas *in vitro*. Os explantes foram inoculados em meio de Murashige & Skoog solidificado com ágar e suplementado com 13,4μM de ácido α-naftaleno acético e 2,2μM de 6-benzilaminopurina e mantidos no escuro a 25°C. As análises histológicas realizadas revelaram que os callus eram formados a partir de células hipertrofiadas do parênquima cortical do explante. Algumas destas células entravam em divisão, enquanto as células vizinhas a estas acumulavam amido. Após 70 dias, calos transferidos para meio de Murashige & Skoog líquido de mesma composição, eram capazes de regenerar plantas. Após 30 dias em meio líquido, gemas se desenvolveram de estruturas nodulares. Estas gemas adventícias formaram um abundante sistema radicular quando transferidas para meio de Murashige & Skoog solidificado com ágar, sem regulador de crescimento e mantidas a 25°C na luz. A taxa de sobrevivência das plantas foi de 95%.

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