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Histological Analyses of the Development of a Friable Embryogenic Callus of African Marigold (*Tagetes erecta* L.)

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Abstract

Histological analysis was carried out during the sequence leading to the formation of embryogenic callus and somatic embryos from cotyledon explants of African marigold (*Tagetes erecta*). Tissues at various developmental stages were excised and observed by light, fluorescent and scanning electron microscopy. Meristematic activity started on cotyledon procambium cells leading to formation of nodular structures. Friable callus was composed by narrow strands of meristematic cells interspersed with large intercellular space, and embedded in a mucilaginous substance. Somatic embryos showed abnormal shape with poorly developed cotyledons and no shoot apex. The origin of the somatic embryos is discussed.

1. Introduction

African marigold (*Tagetes erecta* L.) is an important ornamental plant. Different kinds of thiophenes have been characterized on this species. Thiophenes are secondary metabolites with nematicidal properties which accumulate in roots of many Asteraceous plant species (Bohlmann *et al.*, 1973).

Studies on *in vitro* tissue and cell culture are mostly concerned with analyzing the influence of medium factors on callus or/and regeneration, but few works are concerned with studying the developmental process, investigating the cells that originate the callus and the regenerants. These kinds of studies are very important when working with direct genetic transformation, because the best stage and cell type for transformation can be predicted.

Plant regeneration from tissue culture can be obtained by embryogenesis or organogenesis. In plants of the *Asteraceae*, the majority of the regeneration reports are of organogenic type and, so far, only a few species have been regenerated by embryogenesis in this family (May and Trigiano, 1991). In a previous work we reported the induction of a friable embryogenic callus from cotyledon explants of African marigold, and development of somatic embryos from this callus (Bespalhok and Hattori, 1998). In that paper, histological aspects of embryogenesis were briefly mentioned, showing the embryogenic nature of the callus, but without description of cellular events that lead to callus and embryo formation. Such information is important to understand the process of somatic embryogenesis and might help to improve this system.

Here we analyze the development of embryogenic callus and somatic embryos of African marigold by light, fluorescence and scanning electron microscopy and discuss the possible origin of the friable callus and somatic embryos.

2. Material and Methods

2.1 Plant materials and culture methods

The basic medium (BM) was composed of MS (Murashige and Skoog, 1962) salts and organic compounds, 30 gl^{-1} sucrose and 8 gl^{-1} agar (Ina, type BA-30). The pH was adjusted to 5.6-5.8 before adding the gelling agent and media were autoclaved for 20 min (120C). Petri dishes (9×1.5 cm) with 25 ml of medium and sealed with Parafilm were used.

Embryogenic calli were induced as previously reported (Bespalhok and Hattori, 1998). Achenes of an African marigold line (Matsunaga Seed Co., Japan) were sterilized in ethanol (70%, 1 min) followed by treatment with sodium hypochlorite (0.2% in 0.1 M KH_2PO_4, 20 min) and then rinsed 3 times in sterile distilled water. Achenes were germinated on MS medium under continuous light (20 μmol $m^{-2}s^{-1}$). From one week old seedlings, cotyledons were explanted and pre-treated in a filter-sterilized solution of sodium citrate (65 mM) for 1 min to avoid browning. First, the cotyledons were cultured in BM supplemented with 2.0 mgl^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.2 mgl^{-1} kinetin. After 5 weeks, the explants were transferred to the second step

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medium which was composed of BM supplemented with 0.02 mgl^{-1} thidiazuron (TDZ). After 4 weeks, somatic embryos and compact embryogenic callus developed on TDZ supplemented medium. The compact embryogenic callus was maintained by subcultures every 2 weeks in BM supplemented with 2.0 mgl^{-1} 2,4-D. For somatic embryo development, friable embryogenic callus (that developed on 2.0 mgl^{-1} 2,4-D supplemented medium) was transferred to the medium with 3.0 mgl^{-1} (±) cis,trans-abscissic acid (ABA, Sigma) and 60 gl^{-1} sucrose. ABA was filter sterilized and added aseptically to autoclaved medium. All experiments were held in dark condition at 23°C.

2.2 Histology and light microscopy

Histological sectioning was performed according to Mendoza *et al.* (1993) with some modifications. Samples were fixed in FAA (formalin, acetic acid, 70% ethanol 5: 5: 90), dehydrated in a grade acetone series (30, 50, 70, 90, 95, 100, 100%) at 30 min intervals, embedded in resin which contained glycol methacrylate (as main component), and sectioned to 6 μm on a rotary microtome. Sections were stained with toluidine blue (0.05%, 2 min) and observed under a light microscope. Some histological samples were stained with Periodic acid-Schiff's (PAS) stain to observe carbohydrates.

2.3 Fluorescence microscopy

For fluorescence microscopy, sections were stained with decolorized aniline blue (0.1% in aqueous 0.1 M K_3PO_4, 1 h), dried and mounted in eukitt. Observations were made with an Olympus fluorescence microscope.

2.4 Scanning electron microscopy (SEM)

For scanning electron microscopy observations, samples were dipped in liquid nitrogen and viewed under a Hitachi S-2300 scanning electron microscope with cryo-stage.

3. Results

3.1 Histological observations

Prior to the culture, cotyledon explants of African marigold consisted of uniform epidermal and mesophyll cells, interspersed with vascular tissue (**Fig. 1-a**). After 5 d culture, proliferation was observed in the vascular parenchyma and in mesophyll cells around the vascular system (**Fig. 1-b**). This localized proliferation resulted in the formation of multiple nodular structures after 15 days of culture (**Fig. 1-c**). Nodular structures were characterized by having meristematic, isodiametric cells in the outer layers and more differentiated, vacuolated cells in the inner layers. No vascular elements were observed in the nodular tissue. In contrast to proliferation observed in the vascular parenchyma and mesophyll cells, the parenchyma and epidermal cells started to degenerate. A similar tissue specificity response to auxin has been shown in carrot (Guzzo *et al.*, 1994) indicating that totipotency is not intrinsic to all plant cells.

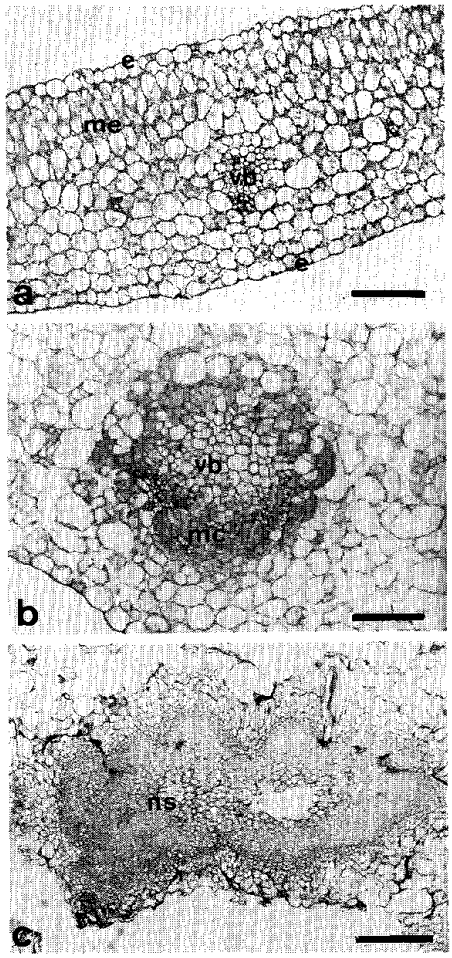

Fig. 1 Histological sections of cotyledon explants of African marigold on 2,4-D (2.0 mgl^{-1}) and kinetin (0.2 mgl^{-1}) supplemented medium: (a) cotyledon explant prior to culture (bar=100 μm); (b) mitotic activity around vascular bundles after 5 days of culture (bar=100 μm); (c) meristematic nodule developed after 15 days of culture (bar=200 μm). Abbreviations: e-epidermis; mc-meristematic cells; me-mesophyll cells; ns-nodular structure; vb-vascular bundles.

Compact embryogenic callus developed when explants with nodular structures were transferred to TDZ supplemented medium. After 4 week culture, the compact callus was composed of a broad meristematic zone with somatic embryos on its surface (**Fig. 2-a**). Globular to torpedo stages of embryo development were observed. These embryos seemed to be growing by secondary embryogenesis, probably from the epidermis of other embryos (**Fig. 2-a**).

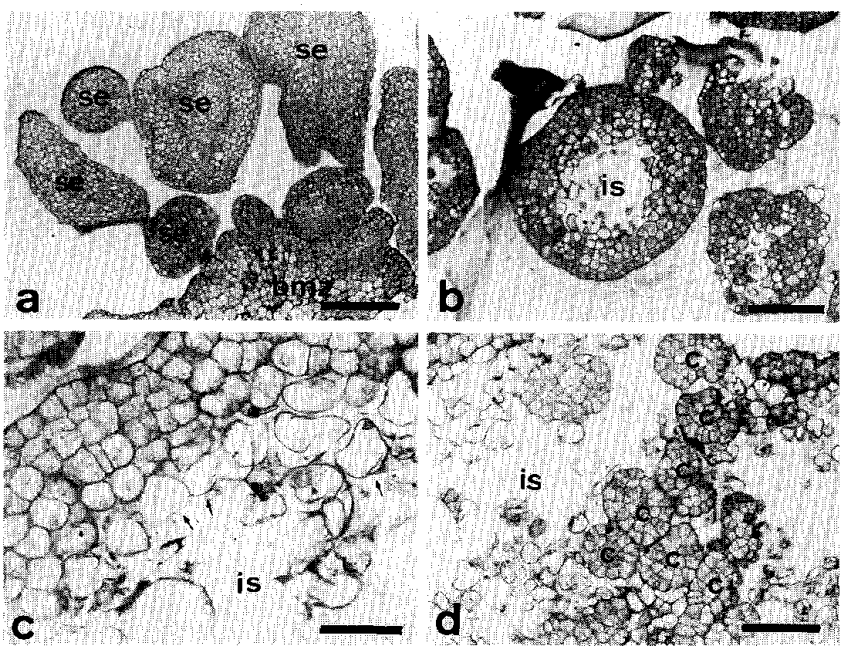

Fig. 2 Histological cross sections of African marigold embryogenic callus: (a) compact embryogenic callus showing somatic embryos on its surface (bar=200μm); (b-d) friable embryogenic callus (b) showing a circular shape narrow strand meristem (bar=200μm) (c) detail of the narrow strand meristem showing separating cells (arrow) (bar=50μm), and (d) cluster of embryogenic cells (bar=100μm). Abbreviations: c-cluster; is-intercellular space; se -somatic embryo.

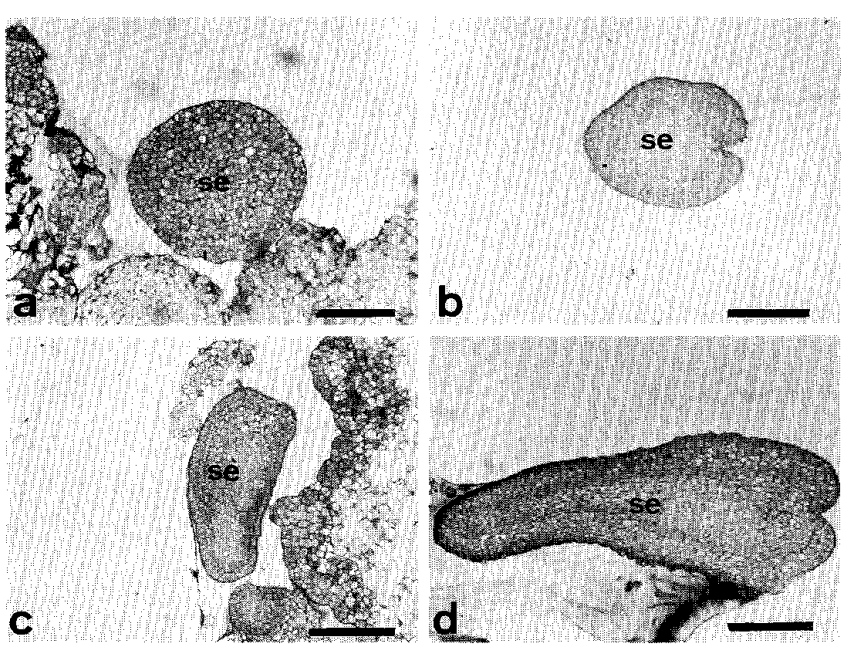

Fig. 3 Histological cross sections of African marigold somatic embryo formation. (a) A globular embryo (bar=100μm); (b) a heart shape embryo (bar=200μm); (c) a torpedo embryo (bar=200μm) and (d) a mature embryo showing reduced cotyledon formation and no shoot apex (bar=200μm). Abbreviations: se-somatic embryo.

On maintenance medium (2.0mgl^{-1} 2,4-D) the embryogenic callus gradually changed from compact to friable. Friable embryogenic callus was poorly organized and composed of two different types of cells ; 1) small isodiametric cells with densely stained cytoplasm and nucleous, which are characteristic of meristematic cells and 2) large, elongated, vacuolated cells. The isodiametric cells were grouped together as narrow strands (**Fig. 2-b, c**) or small clusters (**Fig. 2-d**). The narrow strands of embryogenic cells were

mainly structured in a circular shape. Large intercellular spaces were observed between and inside the narrow strands (**Fig. 2-b**). These strands were composed of 2-5 layers of embryogenic cells and division was only towards the inner side where separating cells could be seen while a smooth surface developed in the outside layer (**Fig. 2-c**). On these calli no embryo development or vascular elements were observed, confirming their dedifferentiated state. Small clusters of embryogenic cells were observed mainly in the inner side of the embryogenic narrow strands (**Fig. 2-d**). Friable embryogenic callus observed in the cultures are very similar to type II callus reported in some cereals (Fransz and Schez, 1991).

A mucilaginous substance was observed around the friable embryogenic callus. When stained with toluidine blue, it got a purple-reddish color indicating methacromasia. In the friable callus, the mucilaginous substance completed the intercellular spaces between the cells aggregates. The mucilaginous substance stained positive with PAS, showing that it must consist of a polysaccharide component (data not shown).

Upon transfer to medium with ABA, the friable embryogenic callus started differentiation of the somatic embryos. After a week of culture, globular (**Fig. 3-a**) and heart shape embryos (**Fig. 3-b**) were observed and after 10 days the torpedo-cotyledon stage embryos (**Fig. 3-c**) could be seen. The mature embryo (**Fig. 3-d**) had a well developed root pole but the apical meristem was absent, and cotyledon formation was reduced. No connection was observed between embryogenic callus and somatic embryos.

3.2 Fluorescence microscopic observations

With aniline blue staining under fluorescent light, callose could be seen as weakly fluorescent spots around some cells of the friable callus (**Fig. 4-a, b**) while other cells showed a cell plate without a surrounding callosic wall. The cells walled with callose were observed either at the narrow meristematic strands (**Fig. 4-a**) or at the cluster of embryogenic cells (**Fig. 4-b**), and in both cases only single cells were stained.

3.3 Scanning electron microscopic observations

SEM images of the friable embryogenic callus showed 2 different types of cells: non embryogenic cells (**Fig. 5-a**) and embryogenic ones (**Fig. 5-b**). The embryogenic cells were small, isodiametric and structured in small clusters (**Fig. 5-b**). The mucilaginous substance was also observed by SEM between the embryogenic cells (**Fig. 5-c**). The non embryogenic cells were bigger than the embryogenic ones and were individualized (**Fig. 5-a**).

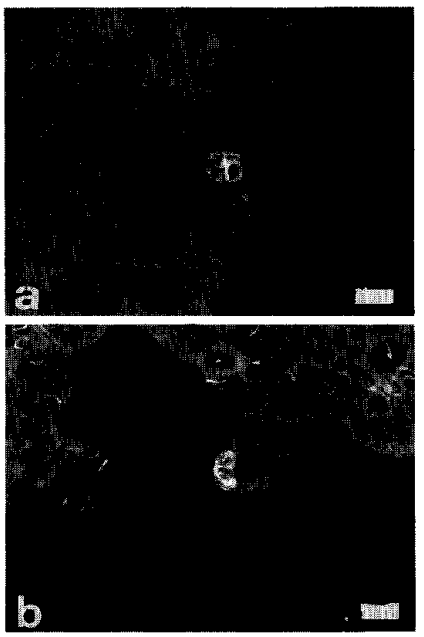

Fig. 4 Fluorescence image showing callosic wall in embryogenic cells (a) in a cluster and (b) in narrow strand meristem (bar=200μm).

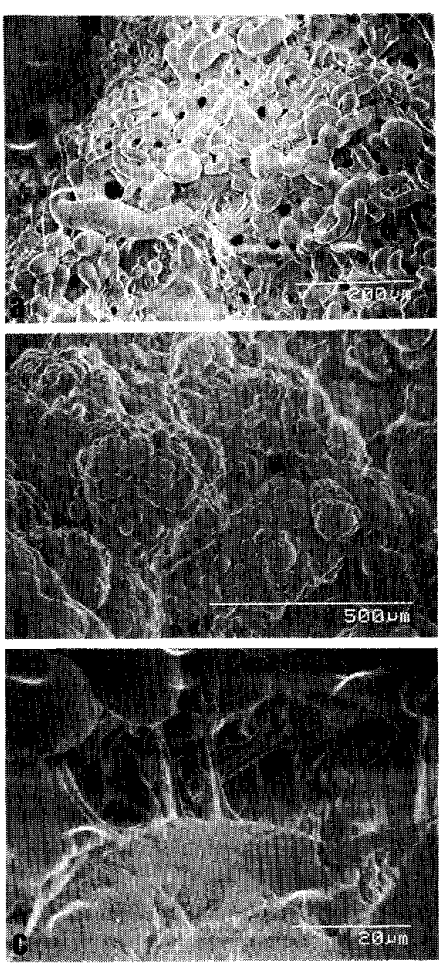

Fig. 5 Scanning electron micrograps of African marigold somatic embryogenesis. (a) Friable, non embryogenic cells; (b) embryogenic cells and (c) detail of mucilaginous substance around embryogenic cells.

SEM images of somatic embryos showed different stages of embryogenesis: globular (**Fig. 6-a**) and

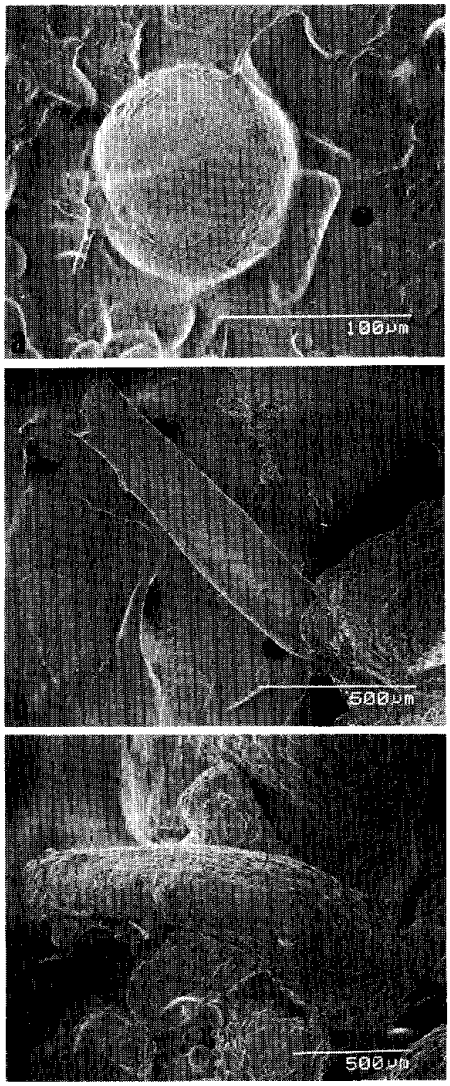

Fig. 6 Scanning electron micrographs of African marigold somatic embryos. (a) Globular embryo, (b) torpedo embryo developing on TDZ and (c) ABA supplemented medium.

torpedo (**Fig. 6-b, c**). Somatic embryos developing on TDZ supplemented medium had a smooth epidermis (**Fig. 6-b**) while the ones developing on ABA supplemented medium had a wrinkled epidermis (**Fig. 6-c**).

Discussion

Our light microscopic observations in the development of a friable embryogenic callus of African marigold suggest that the friable embryogenic callus originates from the nodular structures, after various subcultures in medium with 2,4-D. Probably, as the nodules grew, the more external meristematic layers led to the formation of the narrow meristematic strands, and the more vacuolated cells inside the nodule degenerated with time forming the large intercellular spaces. The circular shape showed by some narrow strands reinforces this hypothesis. Further development of the narrow strands led to the formation of small cluster of cells, which resulted from the breaking of the narrow strands.

Callus of African marigold has been very difficult to culture. Long time callus formation has been prevented because callus turned dark brown upon subculture probably due to phenol accumulation (Ketel, 1986). Phenol accumulation is related to differentiated tissue. The friable embryogenic callus analyzed in our work could be maintained for more than 2 years probably because of its dedifferentiated nature.

The nodular structures originated from meristematic activity of vascular parenchyma cells and mesophyll cells around the vascular system. The precise localization of the origin of competent cells is important when using genetic manipulation. These kinds of studies are still limited, but in some cases information is available. For instance, in cassava cotyledon culture (Konan *et al.*, 1994), *Brassica campestris* cotyledon culture (Hachey *et al.*, 1991), and sugarcane leaf culture (Sumardi *et al.*, 1988), as in our case, mitotic activity started in the vascular parenchyma. In mungbean (Mendoza *et al.*, 1993) and Camellia japonica (Barciela and Vieitez, 1993) cotyledon culture, meristematic activity originated from epidermal and subepidermal cells.

Fluorescence microscopy showed that some cells on the friable callus were surrounded by a callosic wall. Callose deposition can obstruct connections between contiguous cells, such as the plasmodesmata or the sieve pores in vascular tissue (Kauss, 1996) thereby contributing to physical isolation of these cells. In *Cichorium* (Dubois *et al.*, 1990, Dubois *et al.*, 1991) and *Camellia japonica* (Pedroso and Pais, 1995) callosic wall was observed in single cells from which embryogenic cells developed. The observation of a callosic wall around embryogenic cells reinforces the hypothesis that physiological isolation of competent cells from surrounding tissue is a prerequisite for organized development (Pedroso and Pais, 1995; Williams and Maheswaran, 1986). Therefore, callosic deposition can be used as an early marker for embryogenic competent cells.

The ontogeny and origin of somatic embryos have been frequently discussed. Generally, a single cell origin has been suggested (Jones and Rost, 1989), but in some cases somatic embryos seem to originate from groups of embryogenic cells (Verdeil and Buffard-Morrel, 1995). Our observations of single cells walled with callose in the friable callus provide evidence for a single cell origin of indirect somatic embryogenesis in African marigold.

A mucilaginous substance was present between embryogenic tissues and cells, and was composed a polysaccharide substance. A similar mucilaginous substance has been found in type II callus of maize

(Fransz and Schez, 1991) and friable embryogenic callus of *Coffea canephora* (Nakamura *et al.*, 1994). One of the possible roles of the mucilaginous substance could be in the diffusion of nutrients via intercellular space (Fransz and Schez, 1991).

The somatic embryos had poorly developed cotyledons and no shoot apex. This has prevented the conversion of somatic embryos to plantlets. Abnormal or incomplete formation of somatic embryos are a common phenomena in tissue culture and in some cases could be related to deficient auxin polar transport, as has been shown in carrot (Schiavone and Cooke, 1987). Probably, a high concentration of 2,4-D left in the tissue must have prevented the formation of a internal auxin gradient, which is necessary for normal polar auxin transport.

Experiments are now being conducted to obtain conversion of somatic embryos to whole plants.

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