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Morphological and anatomical observations of abnormal somatic embryos from anther cultures of *Citrus reticulata*

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Abstract

A morphological and anatomical study of regenerants obtained from mandarin anther culture was carried out. Beside morphologically normal somatic embryos, abnormal structures were originated in the course of somatic embryogenesis. Anatomical anomalies can be found at several growth stages, causing the formation of slender stems, stubby structures, non-functional leaves. When too long, some structures are subject to shedding, with the formation of various abscission zones. Most of them are subject to degeneration, although many are capable of further, localized, morphogenesis. A thorough knowledge of morphology and anatomy of normal and abnormal regenerants could make possible to select and subculture the lines considered most suitable for conversion into plantlets.

Additional key words: mandarin, *in vitro* culture, anatomy, abnormal structures.

Introduction

The application of biotechnologies is encouraged in *Citrus* species where the complicated reproductive system has been an obstacle to genetic improvement. *In vitro* culture of ovules, from ovaries and immature fruits, was one the first tools used to obtain virus-free nucellar plants from polyembryonic *Citrus* cultivars (Bitters *et al*. 1970). Moreover, tissue explants and embryogenic cells have, generally, a high capacity to regenerate plants and can be used in protoplast fusion and genetic transformation (Vardi and Galun 1989, Vardi and Spiegel-Roy 1992, Tavano *et al*. 2009). Somatic embryos, embryogenic callus and cell culture from *in vitro* ovule culture have also been used to develop the cryopreservation method for germplasm conservation (Sakai *et al*. 1991, Engelmann *et al*. 1994, Pérez *et al*. 1997).

Although in the genus *Citrus* somatic embryogenesis was reported a long time ago (Maheshwari and Ranga Swamy 1958), a number of difficulties have been encountered in establishing reliable protocols. In the abundant literature on *Citrus* somatic embryogenesis, it appears that results vary greatly depending on the genotypes (Mendes-da-Gloria *et al*. 2001, Tomaz *et al*. 2001, Niedz *et al*. 2002, Ramirez *et al*. 2003).

Anther culture is the most common method for haploid production, and it is also suitable for somatic embryogenesis in a number of fruit trees, including *Citrus* species (Germanà *et al*. 1994, Germanà 2003a). Some problems have been reported concerning the multiplication step of embryogenic calli, among which are the lack of synchrony in embryo development and the risk of morphological abnormalities. Morphological alterations occurring in the course of somatic embryo development, such as embryo fusion, lack of suitable apical meristem or loss of bipolarity (Alemanno *et al.* 1996), have been held responsible for poor yield of vital embryos in several species as different as *Pisum sativum* (Griga 2002), *Arachis hypogea* (Chengalrayan *et al*. 1997), *Quercus robur* (Zegzouti *et al*. 2001), *Acacia mangium* (Xie and Hong 2001), *Carica papaya* (Fernando *et al*. 2001) and *Gossypium hirsutum* (Hussain *et al.* 2009).

The production on a wide scale of normal somatic embryos is not always possible for most *Citrus* species, as the development and germination of normal bipolar embryos from embryogenic callus after the globular

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phase is sometimes problematic. Beyond this stage, embryo development often shows varying degrees of abnormality (Button *et al*. 1974, Niedz *et al*. 2002). In *Citrus sinensis*, for example, somatic embryos displayed abnormal morphology (non-responsive or teratoma type embryo) and the effects of various semipermeable membranes were studied to normalize embryo development (Niedz *et al*. 2002). Ricci *et al*. (2002) reported on embryo development from calli in a number of *Citrus* varieties, where different sources and concentrations of sugars, gibberellic acid (GA_3) and activated charcoal were applied. Sometimes defects lead to poor survival of embryos and mandarin has proven to be particularly vulnerable in this respect compared to other *Citrus* genotypes (Tomaz *et al*. 2001, Ricci *et al*. 2002, Germanà 2003b).

The present study has been carried out with the aim of describing the anatomical aspects of imperfect somatic embryos, produced at the rate of about 30 % of the total number, during the proliferation of long-term embryogenic callus obtained through mandarin anther culture.

Materials and methods

Embryogenic callus and somatic embryos were obtained from anther culture of *Citrus reticulata* Blanco, cultivar Mandarino Tardivo di Ciaculli N.L. 19 (MTC N.L. 19) in accordance with the method described by Germanà (2003b). After 2 - 3 months of culture, anthers start to produce calli. Most calli are not morphogenic, but some of them appear to be highly embryogenic. Embryogenic calli are friable and white and differentiate into clumps of embryoids (Fig. 1*A*). About 70 % of embryos obtained are well-structured and show normal developmental patterns: globular, heart, torpedo and cotyledonary stages. Secondary embryoids developed as well.

For histological analysis, samples showing an abnormal development were collected from culture, photographed with a camera (*Olympus DP10*) applied to a stereomicroscope (*Wild M 3Z*, Heerbrugg, Switzeland), and fixed in 2 % formaldehyde and 2 % glutaraldehyde, buffered with 0.05 M phosphate buffer at pH 7.2. The specimens were then dehydrated in methyl cellosolve followed by absolute ethanol and embedded in *Technovit*

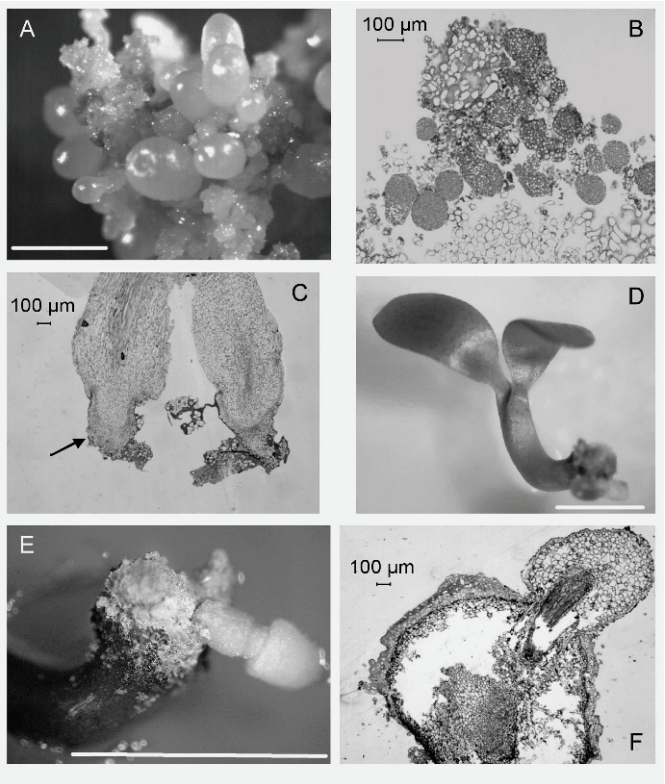

Fig. 1. *A* - Embryogenic callus from mandarin anther culture; *B* - A large number of somatic embryos are initiated in a peripheral zone of competent callus; a loose connection seems to exist between callus cells and embryos; *C* - Lower half of somatic embryos (see Fig. 4*E*). The bottom sections that developed inside the callus are clearly visible because of a marked girth restriction (*arrow*); *D* - Well developed somatic embryo. At the bottom a callus can be noticed, and the beginning of adventitious root growth; *E* - Root structure originated from callus; *F* - Longitudinal section of the root end shown in Fig. 1*E,* the embryo at the bottom end is swollen, but most of its abundant parenchyma has collapsed, and the vascular connection with the growing root has been interrupted (*bars* = 5 mm in *A, D, E).*

7100 (*Heraeus Kulzer & Co.*, Wehrheim, Germany) in accordance with the manufacturer's instructions. Semi-thin (3 - 4 μm) sections were obtained using a glass knife (*Reichert-Jung 2040* autocut microtome, Depew, NY, USA); the sections, after drying at room temperature, were stained with the *Periodic Acid Schiff* reaction and counterstained with *Amido Black 10B* (Ruzin 1999). The sections were mounted in Canada balsam with a cover slip and examined under a light microscope (*Polyvar*, *Reichert-Jung*). The photographs were taken using an optical microscope *Leica DC 100* (Wetzlar, Germany) equipped with an image analysis software (*Leica IM50* and *QWin*).

Results

Somatic embryos usually develop on peripheral callus layers, and the callus from which they are produced is highly friable; this is due in part to degeneration of the neighbouring cells, and in part to a modification of living callus towards an aerenchyma-like organization (Fig. 1*B*). Somatic embryos can also develop more internally in the callus mass, but only in spots in which there are large gaps where the surrounding callus is not able to exert pressure on the competent cells. This effect can also be noticed in more developed embryos, whose difference in thickness is related to their position in the callus mass (Fig. 1*C*).

Some embryos start to germinate, without developing a real root system (Fig. 1*D*); they rather produce callus from which small root structures originate (Fig. 1*E*). These roots, however, do not seem to have many chances of survival or of performing adequately their functions, as the swollen base of the plantlet appears almost empty due to a collapse of the abundant and large celled parenchyma. Vascular connection with the stem central cylinder is therefore shown to be insufficient (Fig. 1*F*). Even in healthy embryos a tendency exists to develop adventitious roots immediately above the root tip (Fig. 2*A*).

Other irregular structures can be produced during somatic embryogenesis. Quite common are cylinder shaped structures, which are usually photosynthetic when young (Fig. 2*B*). They may develop as such, sometimes vertically, more often horizontally on the substrate, or carrying some cotyledonary leaves (Fig. 2*C*), or more complex structures (Fig. 2*D*); the presence of cotyle-

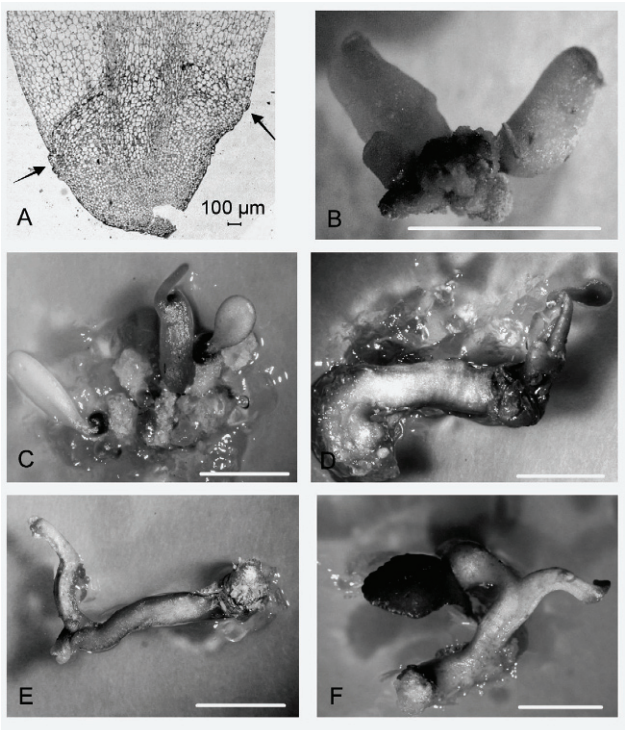

Fig. 2. *A* - Longitudinal section of the root tip of a healthy embryo: immediately behind the apex two adventitious primordia (*arrows*) are visible and close to emergence, vascular connection to the root central cylinder being under way; *B* - Two imperfect somatic embryos, each about 4 mm long, have originated from the callus, one of which (*left*) has lost its photosynthetic capacity and has turned brown; *C* - This callus has produced, among other structures, an irregular somatic embryo which, besides having only one developing cotyledonary leaf, is abnormally swollen; *D* - This structure, after losing photosynthetic capacity, has developed callus at both ends; the callus that was closer to the substrate surface differentiated an imperfect somatic embryo; *E, F* - Complex and hard to classify structures (*bars* = 5 mm in *B, C, D, E, F*).

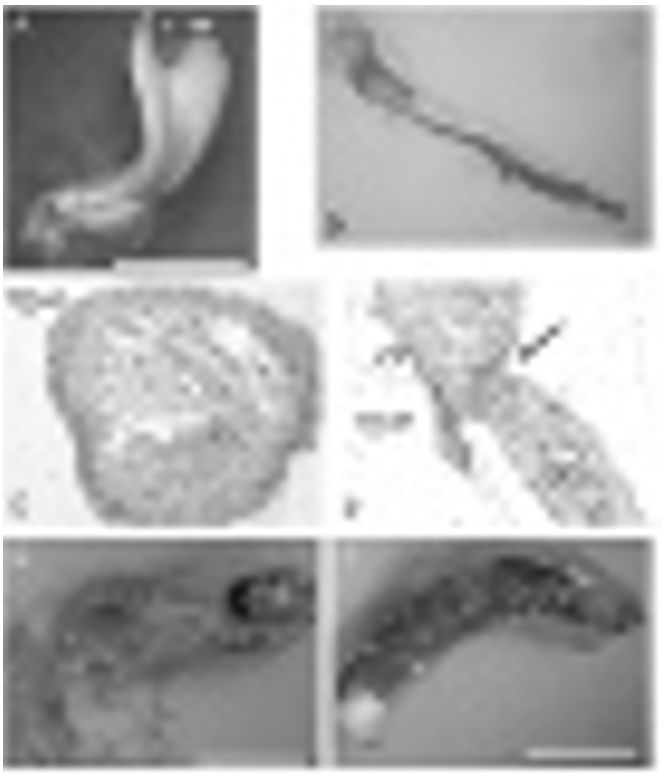

Fig. 3. *A* - Somatic embryo in which leaf growth has been privileged; the largest cotyledonary leaf is no longer green; *B* - Slender stem originated directly from the callus; *C* - Transverse section of a slender stem. Only half of the central cylinder is differentiated; the parenchyma of the other half is very weak and collapsing. A large schizogenous cavity is also opening in the cortex of the differentiated half; *D* - The formation of an abscission zone in the structure of Fig. 3*B* is under way by creating a layer of dead cells (*arrow*): the border cells are typically enriched with polyphenols to better seal the eventual fracture surface; *E, F* - Stubby structures at different degrees of ageing and internal degeneration. In *F*, the surface is still green, decoloration starting to appear at both ends, one of which also shows signs of tissue collapse (*bars* = 5 mm in *A, B, E, F*).

donary leaves indicates their embryogenic origin, and there are many intermediary stages between the cylinder shape and the possible development into a plantlet (Fig. 2*E*,*F*). Other structures found in culture are irregularly developed cotyledons (Fig. 3*A*).

The abnormal shapes formed by the embryogenic callus, other than somatic embryos, show distinct features, described below: *1*) Slender stems – these structures may reach 15 mm in length, and show rudimentary nodes (Fig. 3*B*). They can be considered stems, both for the node/internode sequence, and for the tendency to differentiate a central cylinder, even if the internal parenchyma always collapses before differentiation is completed, thus creating a large cavity. Alternatively, collapse may occur at a later stage, when the central cylinder is only half formed (Fig. 3*C*). Another feature often observed in these structures is the formation of abscission zones, usually at nodes (Fig. 3*D*). *2*) Cylindrical and stubby structures - these structures seem to derive from the stems of the developing embryos (Fig. 2*C*); after an initial regular upright growth and onset of photosynthetic activity, they eventually lie down on the substrate, where, after a further enlargement, a number of different morphogenetic activities take place within them (Fig. 2*D*): callogenesis, organogenesis, secondary somatic embryogenesis. In the course of the process they gradually lose their green colour and develop a hollow core (Fig. 3*E*,*F*, 4*A*,*B*). These structures can also develop abscission zones. In Fig. 2*B* it is possible to observe two cylinder-shaped structures, one of which is green, the other one without chlorophyll (albin); the latter shows an advanced stage of abscission layer formation (Fig. 4*C*). This cylindrical structure undergoes ageing (Fig. 3*E*,*F*). New structures can arise from the peripheral tissues of this degenerating structure although these are never regularly differentiated and are destined to degeneration or to further production of new meristems, tissues and organs. *3*) Leaf structures – leaves have often been recognized among structures developed directly from the callus (Fig. 2*C*, 4*D*) or by other organs. Most commonly they are cotyledonary leaves that have been modified in different ways. Some appear very thin and elongated, often they lose their photosynthetic capacity (Fig. 4*E*) and abscission zones are sometimes developed (Fig. 4*F*, 5*A*). The abscission zone is formed after the concurrence of two mechanisms: size increase (elongation) of competent cells, and separation due to both lysigenous and schizogenous processes. Other leaves

develop with a relatively closer resemblance to normal leaves (Fig. 3*A*, 5*B*), or assume a more fleshy aspect (Fig. 5*C*). In some explants the leaf tissue becomes organogenetic (Fig. 5*D*), and more foliar tissue can be generated (Fig. 5*E*). Leaf-like structures may assume quite odd shapes, such as the spoon-like leaves observed in some explants (Fig. 2*F*, 5*F*).

Discussion

Calli obtained through *Citrus* anther culture, regularly subcultured on fresh medium, have maintained their morphogenic ability for over nine years and have shown themselves to be highly embryogenic. However, the developmental process is accompanied by a high occurrence of imperfect structures which greatly reduce its efficiency.

Such abnormal embryogenic structures display a wide spectrum of histological events which eventually determine loss of vitality and cell degeneration, or regression towards less or poorly differentiated formations. One of the early determinations may be the location of initials, with callus pressure able to influence developing embryos in their earliest stages; this pressure effect has also been noticed in olive somatic embryogenesis (Benelli *et al*. 2001).

Abnormalities in somatic embryo development are described in many other systems, probably caused by inadequate culture conditions; in fact, the response to the different culture conditions are genotype-dependent. In *Theobroma cacao*, different degrees of morphological alterations, such as embryo fusion, formation of more than two cotyledons, or lack of proper apical meristem formation have been described (Alemanno *et al*. 1996). Lack of conversion or low conversion rate of the somatic embryos can be related mainly to abnormalities in the shoot apical meristem or to abnormal formation of the protoderm which can lead to arrest of development of the somatic embryo. In Rangpur lime and Cleopatra mandarin, cell proliferation in the shoot apical region, a precocious elongation of embryo axis, and discontinuity or de-differentation of the protoderm were observed; the ensuing stop of embryo development in embryogenic cultures, which did not continue their normal development, was ascribed to such events (Tomaz *et al.* 2001).

In citrus cv. Mapo tangelo, the albinism was

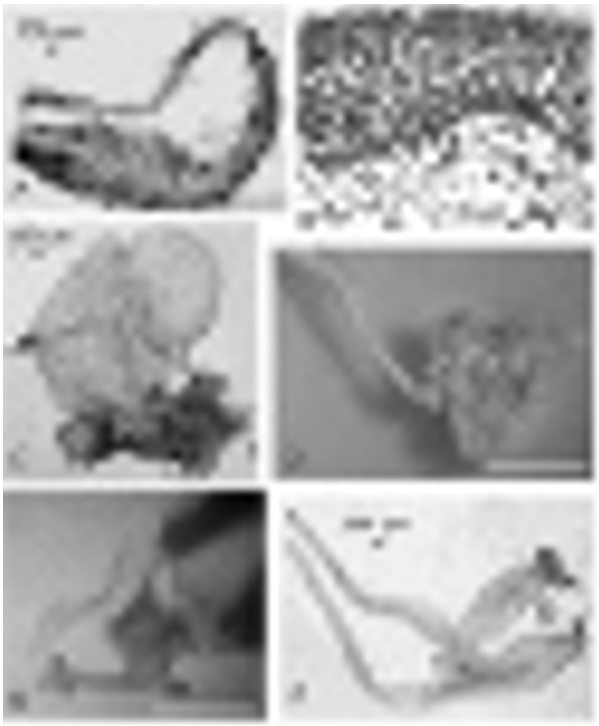

Fig. 4. *A* - Longitudinal section of the distal end (farthest from the callus) of the structure pictured in Fig. 3*E*. The cavity is formed by the collapse of an internal parenchyma, cytologically distinct from the outer parenchyma; *B* - Point of passage between the two parenchymas mentioned for Fig. 4*A*. The difference is striking, involving mainly cell size and shape, wall thickness, intercellular spaces, starch inclusions; *C* - Within the non-photosynthetic structure in Fig. 2*B* an abscission zone is being developed, roughly at half length (*arrow*); *D* - Leaf structure, directly inserted on the callus; *E* - Slender embryo with string-like, non photosynthetic leaves; *F* - Low magnification longitudinal section of two somatic embryos shown in Fig. 4*E*, one of which with two developed leaves (*bars* = 5 mm in *D, E*).

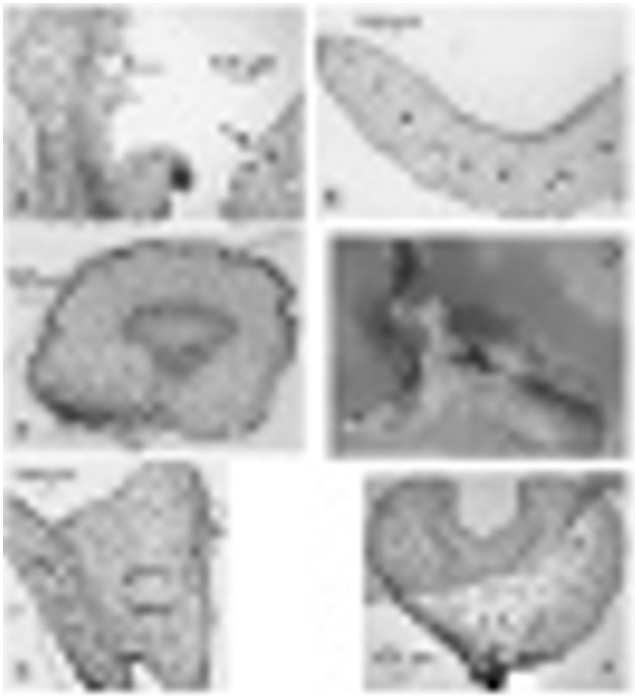

Fig. 5. *A* - Abscission zones (*arrows*) in cotyledonary leaves of sample of Fig. 4*E* ; *B* - Transverse section of the larger leaf of Fig. 3*A*; *C* - Transverse section of the smaller leaf of Fig. 3*A*; *D* - Fleshy somatic embryo, with abundant secondary callogenesis and organogenesis; *E* - This picture combines the transverse section of a leaf of sample in Figure 5*D*, and the longitudinal/tangential section of leaf structure arising perpendicularly from the older leaf, as can be evinced by the orientation of leaf veins; *F* - Transverse section of a spoon-shaped leaf of sample pictured in Figure 2*F* (*bar* = 5 mm in *D*).

observed, with well-structured chlorophyll-deficient embryos. The albino embryos germinated but did not produce plantlets, although several media were tested (Germanà and Reforgiato Recupero 1997). Sometimes teratoma formations were observed. It seems that the frequency of albino plants might depend on the age of anthers (Huang 1986). According to our results, a role in lack of conversion could be exerted by a reduced capacity of the root tip to develop into a taproot, with the production of adventitious roots which can be insufficient or too late.

In our research *Citrus reticulata* demonstrated of about 30 % morphological abnormality; this figure is hard to compare with other results mentioned above, as the rate of abnormal embryos as a rule is not reported in the literature. In peanut (Chengalrayan *et al*. 1997), a protocol was set up to overcome the problem and achieve a 86 - 92 % rate of healthy embryos.

What causes the various imperfect structures is difficult to establish by anatomical investigation alone. It can be hypothesized that growing embryos are affected in their anatomy and histology by a number of negative conditions present in the environment they live in, such as type and concentration of carbon source or plant growth regulators added during culture. With respect to sucrose, for example, previous studies report that this sugar has both a nutritive and an osmotic effect on embryogenesis in several species (Parrot *et al.* 1992), although the specific role of high contents of sucrose in enhancing somatic embryogenesis is not evident.

Many structures are clearly imperfect from the beginning of their growth, like slender stems and individual leaves; others seem to indicate that deviations from normal ontogenesis are determined at later stages.

According to this interpretation of the events, the improvement of the somatic embryogenesis technique should involve a deeper investigation regarding both the physical and chemical conditions in the vessels and of the timing of explant transfer. This study showed the anatomical structures underlying given morphological deviations from the normal embryogenic process, in the belief that histological characterization and evaluation of abnormal morphologies, at all stages of embryo development, is important in estimating embryo quality.

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