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Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae)

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Abstract

Somatic embryos of myrtle (*Myrtus communis* L.) were induced from mature zygotic embryos cultured in MS medium supplemented with several concentrations of 2,4-D (2.26 μM – 18.98 μM) or Picloram (2.07 μM – 16.5 μM) combined with 0.087 M or 0.23 M sucrose. For all the concentrations of 2,4-D or Picloram tested, 0.087 M sucrose proved to be more effective than 0.23 M. The best frequencies of induction were obtained in a medium containing 2.26 μM 2,4-D in which 97.3% of the explants produced somatic embryos. Although most embryos were produced from the adaxial side of the cotyledons, some of them differentiated from the hypocotyl. Secondary somatic embryos were often seen arising from the periphery of the former somatic embryos. Somatic embryo development was not synchronous but practically all the embryos germinated well after being transferred to media containing GA_3 (0.29, 0.58 and 1.44 μM) alone. When benzyladenine was combined with gibberellic acid, germinating somatic embryos produced adventitious shoot buds which contributed to an increase in plantlet regeneration. Histological observations suggested that somatic embryos arise from the upper surface of the cotyledons probably from peripheral cells. Polyphenol-rich cells were usually seen in association with meristematic-like cells from which somatic embryos originate or with earlier steps of somatic embryo differentiation. Regenerated plants were phenotypically normal, showing a diploid ($2n = 22$) set of chromosomes.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – benzyladenine; GA – gibberellic acid; MS – Murashige and Skoog; Picloram – amino-3 ,5,6-trichloropicolinic acid; SE – standard error

Introduction

Myrtle (*Myrtus communis* L.) is a small evergreen tree (up to 4 m) from Asia Minor belonging to the Myrtaceae family (Phillips and Rix, 1989). It is the only species of the genus in Europe but other species such as *Myrtus ugni* and *Myrtus luma* are native to South America (Moussel, 1965). Myrtle trees grow spontaneously in Mediterranean regions, especially in Southern European countries (Greece, Italy and Spain) and in Portugal where it is also grown as an ornamental tree (Chinery, 1984). It occurs in the Azores islands where it is an endangered species (Queirós and Ormonde, 1984). The leaves are strongly aromatic when crushed, and together with flowers and fruits, produce an oil that is used in the cosmetic industry (Polunini, 1984). Infusions of those organs have been employed in folk medicine against disorders of the respiratory and genito-urinary system (Queirós and Ormonde, 1984). The edible fruits are very astringent and a substitute of pepper is obtained from them (Queirós and Ormonde, 1984).

The plant can be vegetatively propagated by rooting of softwood cuttings but the yields are low. *In vitro* propagation techniques could be a useful approach both for the establishment of protocols for rapid multiplication and for studies of genetic manipulation. Protocols for micropropagation of myrtle through axillary shoot development (Khosh-Khui et al., 1984; Nobre, 1994) and somatic embryogenesis (Parra and Amo-Marco, 1999) have already been developed. Although the Myrtaceae family includes more than 3000 species

(Paiva, 1997), some of them belonging to very important economic genera (e.g., *Callistemon, Eucalyptus, Eugenia, Feijoa, Myrciaria, Pimenta, Psidium*), somatic embryogenesis has only been reported in a few species such as *Eucalyptus citriodora* (Muralidharan et al., 1989), *E. grandis* (Watt et al., 1991), *E. gunnii* (Termignoni et al., 1996), *Eugenia jambos* and *E. malaccensis* (Litz, 1984a), *Feijoa sellowiana* (Cruz et al., 1990) and *Myrciaria cauliflora* (Litz, 1984b). Recently, Parra and Amo-Marco (1999) reported the induction of somatic embryogenesis from immature seeds (2-month-old), as well as hypocotyls and cotyledons of *Myrtus communis*. In this study we present the first results of somatic embryogenesis and plant regeneration from mature zygotic embryos of myrtle.

Materials and methods

Fruits of *Myrtus communis* were collected from plants growing at the Botanical Garden of the University of Coimbra, during October and November. Before seed removal, the fruits were washed with running tap water and further sterilized by a 15 min immersion in 30% commercial bleach (Domestos containing 5% of active chlorine). Zygotic embryos were dissected from seeds and sterilized in a 7.5% calcium hypochlorite solution followed by three washes in sterile double distilled water. Whole intact embryos were isolated and cultured in test tubes containing MS (Murashige and Skoog, 1962) basal medium supplemented with 2,4-D (2.26 – 18.97 µM) or Picloram at the same concentrations and 0.087 M or 0.23 M sucrose. All media had the pH adjusted to 5.6 – 5.8 and were solidified by adding 0.8% agar (bacto agar, Difco) before autoclaving at 121 °C for 20 min. Approximately 35–40 embryos were used per treatment and the cultures were maintained in the dark at 25±1 °C. The experiments were repeated three times and the results were scored after 8 weeks of culture. Most of the experiments were performed with mature zygotic embryos. However, a few assays were carried out with globular to torpedo zygotic embryos.

For somatic embryo germination cotyledonary somatic embryos were transferred to a solidified MS basal medium containing 0.058 M sucrose or to the same basal medium plus GA_3 (0.29 or 1.44 µM) or combinations of GA_3 (0.29, 0.58 or 1.44 µM) and BA (0.89 µM). Younger somatic embryos (globular to torpedo stages) with the mother tissue attached were first inoculated on to MS basal medium for a 3 – 4 week period and then the cotyledonary embryos isolated and cultured on the above listed germination media. Results of somatic embryo germination were taken after 4 weeks of culture at 25±1 °C under a 14-h daily illumination regime of 15 – 20 µmol m^{-2} s^{-1} photosynthetically active radiation provided by cool white fluorescent lamps. Plantlets were potted when a size of about 3 cm was reached. Chromosome counts of regenerated plants were taken from aceto-orcein squashes of ethanol-acetic acid (3:1) fixed root tips pre-treated with colchicine (0.05%) for 3.5 h.

For histological studies samples of embryogenic cotyledons were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.0 for 1.5 h and post-fixed in 1% osmium tetroxide prepared in the same buffer for 1 h at room temperature. Dehydration was accomplished in an ethanol series (20, 40, 60, 80, 95 and 100%) and the samples embedded in Spurr's resin (Spurr, 1969). Sections (1 – 3 µm) were made with glass knives on a LKB Ultratome III and stained with 0.2% toluidine blue.

When statistical analysis was performed the data expressed in percentage were first subjected to arcsine transformation and the means corrected for the bias before new conversion of the means and standard error (SE) back into percentages (Zar, 1984). Statistical analysis was performed by submitting transformed data to analysis of variance and the significantly different means were selected by using the Tukey test (p=0.05).

Results

Somatic embryogenesis induction

Somatic embryos were only obtained when cotyledonary-staged zygotic embryos were used. Globular to torpedo-staged zygotic embryos were unable to undergo somatic embryogenesis. Therefore, only the results obtained with cotyledonary-staged zygotic embryos are presented in the following sections. All the media tested were able to support somatic embryogenesis induction over the entire range of concentrations of 2,4-D or Picloram used (Figures 1 and 2). However, the highest frequencies of induction were obtained with 2.26 µM 2,4-D and 4.2 µM Picloram, respectively 97.3 and 95.3%. In media lacking growth regulators the zygotic embryos did not form callus nor produced somatic embryos, but germinated well. When 2,4-D was the auxin present in the induction medium somatic embryo production significantly decreased with in-

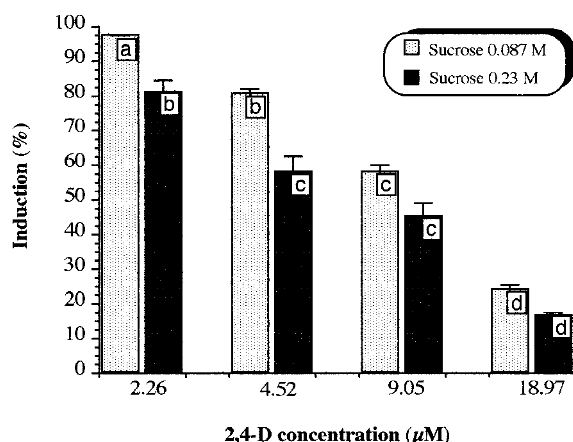

Figure 1. Effect of different concentrations of 2,4-D (combined with 0.087 M or 0.23 M sucrose) on the percentage of zygotic embryos producing somatic embryos in *Myrtus communis.* About 110 zygotic embryos were used in each treatment and the data were collected 8 weeks after the beginning of the experiment. Each value is the mean ± SE of three replicates (35–40 embryos per replicate) and the values followed by the same letter are not significantly different at $p = 0.05$.

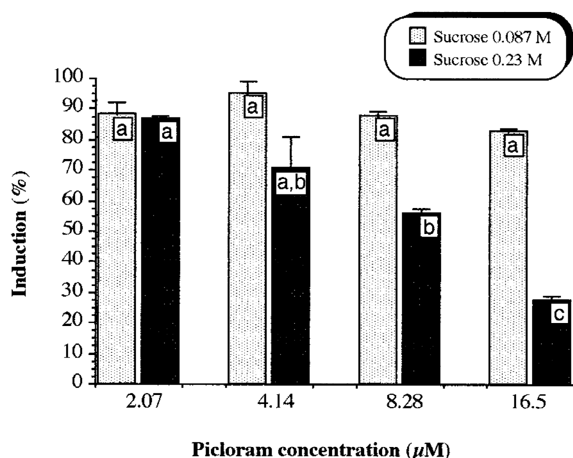

Figure 2. As Figure 1 but for Picloram.

creasing concentrations of this growth regulator (Figure 1); whereas on medium containing the highest Picloram concentration used (16.5 μM), induction of somatic embryogenesis was still very effective, especially in combination with 0.087 M sucrose (Figure 2). The results of Figures 1 and 2 show also that 0.087 M sucrose is more effective than 0.23 M, especially when lower concentrations of 2,4-D (2.26 and 4.52 μM) and higher concentrations of Picloram (8.28 and 16.5 μM) were used.

Globular somatic embryos were first seen after about 3–4 weeks of culture at the surface of callus which developed mainly from the cotyledons (Figure 3A and 3B). In some cases, somatic embryos differentiated from the upper surface of cotyledons almost without callus proliferation and, in a few explants (less than 5%), somatic embryos were seen differentiating directly from the hypocotyl (Figure 3C) or from the shoot apical region. On the same explant somatic embryo ontogeny was not synchronized and embryos at different morphological stages of development could be seen (Figure 3D), resembling their zygotic counterparts. At the end of 8 weeks of culture, the calluses from which somatic embryos had developed were dark, had stopped growth and had lost their embryogenic potential.

Although the number of somatic embryos per embryogenic explant was not counted we could observe that this number was very variable. In some cases only a few embryos (one or two) were obtained whereas in others more than one hundred embryos were produced. Most of the embryos were morphologically normal (Figure 3E). However, a few abnormalities could be seen, mostly represented by embryos showing an abnormal number (1 or 3) of cotyledons (Figures 3D and 3F).

Somatic embryo germination

Myrtle somatic embryos did not germinate on the induction media. To achieve somatic embryo germination (Figure 4A) they had to be removed from the induction medium and transferred to media lacking growth regulators or containing GA_3 alone or combinations of GA_3 and BA. The embryos germinated either attached to the mother tissue or isolated. The first signals of somatic embryo germination, namely the greening of the cotyledons (Figure 4B) and radicle elongation (Figure 4C), were seen after about 1 week on the germination media. One week later the first pair of true leaves could be seen (Figure 4D). Practically all the somatic embryos developed into plantlets on the germination media, even those showing an abnormal number of cotyledons (Figure 4E). When the somatic embryos were produced on media containing the highest concentrations of 2,4-D (18.97 μM) or Picloram (16.5 μM) most of them did not reach the cotyledon stage; these embryos were first transferred to MS basal medium containing 0.058 M sucrose for 3 – 4 weeks to achieve maturation and then placed on the germination media. The total number of plantlets produced by each embryogenic explant was not determined. This number is conditioned by the number

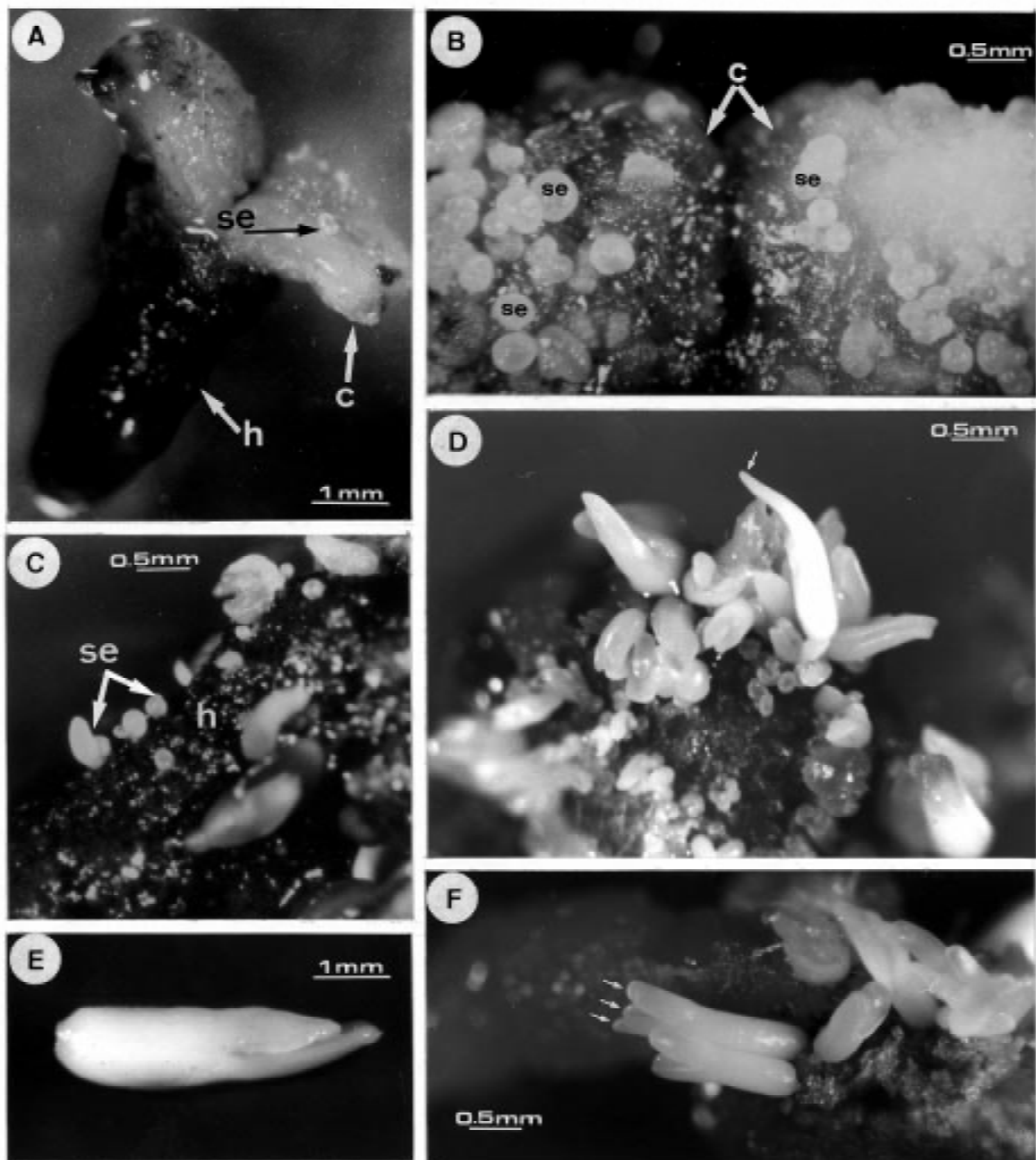

Figure 3. Morphological aspects of somatic embryogenesis in *M. communis*. (*A*) Zygotic embryo after 4 weeks of culture on medium containing 0.087 M sucrose and 4.52 μM 2,4-D. Very young somatic embryos (se) can be seen in the upper face of one cotyledon (c) whereas the hypocotyl (h) appears necrotic. (*B*) Several somatic embryos (se) differentiating on the upper face of the cotyledons (c). (*C*) Somatic embryos (se) developing directly from the hypocotyl (h). (*D*) Numerous somatic embryos in different stages of development. The arrow points to an embryo showing only one cotyledon. (*E*) Cotyledonary somatic embryo. (*F*) Several somatic embryos, one of them (arrows) showing three cotyledons.

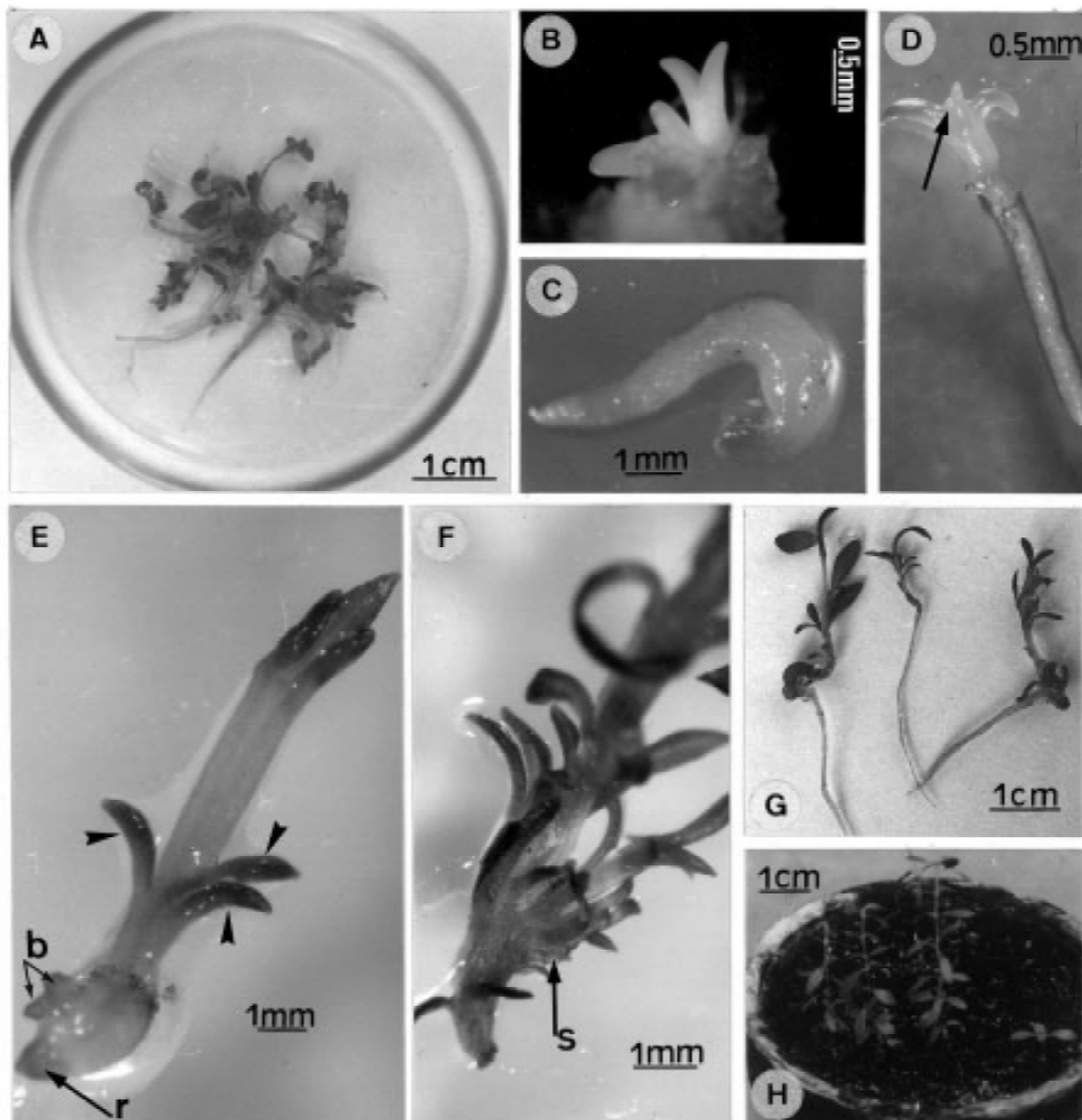

Figure 4. Somatic embryo germination. (*A*) Plantlets of *M. communis* obtained by germination of somatic embryos. (*B* and *C*) Early stages of somatic embryo germination, namely the greenish of cotyledons (*B*) and root elongation (*C*). (*D*) Young plantlet of myrtle showing the first pair of leaves (arrow) after 2 weeks on the germination medium. (*E* – *G*) Adventitious shoot bud development from germinating somatic embryos. Note the early stages of bud (b) differentiation in Figure *E* and the inhibition of root (r) growth. Arrows in Figure *E* show the three cotyledons existing in the somatic embryo. s – adventitious shoots. (*G*) Plantlets of *M. communis* ready to be transferred to soil after 5 weeks on the germination media. (*H*) Plants of *M. communis.*

of adventitious buds forming on the germinating embryos. This kind of morphogenesis was found after 2 weeks of somatic embryo culture on media containing BA (0.89 μM). In these circumstances, root growth was inhibited and buds differentiated at the base of the germinating somatic embryos (Figure 4E). The number of adventitious buds per somatic embryo was variable (from 1 to up to 5). These buds kept on growing and gave rise directly to several shoots at the base of the growing plantlets (Figure 4F). In contrast, when the somatic embryos germinated on media lacking BA the embryos developed normally producing well formed plantlets (Figure 4G). From 37 plantlets that were transferred to soil, 34 (>90%) survived and grew well afterwards (Figure 4H).

Cytological and histological studies

Cytological analysis of root apical meristems of 15 regenerated plantlets, 3 weeks after germination, showed that all had the diploid ($2n$=22) number of chromosomes (Figure 5A). These studies were hampered by the small length of the chromosomes and by the poor affinity of the chromosomes to the stain.

Morphological and histological (Figure 5B) observations showed that somatic embryos arose mainly at the upper face of the cotyledons and revealed the early stages of somatic embryo differentiation (Figure 5C). Somatic embryos seem to arise from cells formed at the periphery of a meristematic zone of cells (Figure 5D) developing below the upper epidermis. In some sections, secondary somatic embryos have been shown to arise directly from the periphery of former somatic embryos (Figure 5E) independently of the induction media used. A very common feature related with the process of somatic embryo ontogeny was the development of a layer of several cells thick separating the somatic embryos from the supporting meristematic layer from which they seem to be originated (Figure 5F). Cells of the delimiting layer accumulated a dense dark material in the vacuole (Figure 5G).

Discussion

Based on the present results, the main factors conditioning somatic embryogenesis in myrtle are culture media composition and the developmental stage of the explant at the time of culture. It was found that an auxin (2,4-D or Picloram) is required for somatic embryogenesis induction. The role of auxins, mainly 2,4-D on somatic embryogenesis induction is well established for many species (Dunstan et al., 1995), including several members of the myrtaceae family (Canhoto et al., 1998a). Among other myrtaceous plants in which somatic embryogenesis has been induced, only in *Eucalyptus globulus* was it found that somatic embryogenesis can occur on media lacking an auxin (Trindade, 1996). However, the embryos thus formed did not develop beyond the globular stage of development. The inhibitory effect of high sucrose levels is not consistent with results obtained in other myrtaceous such as *Feijoa sellowiana* (Canhoto and Cruz, 1994), *Eucalyptus citriodora* (Muralhidaran et al., 1989) and *Myrciaria cauliflora* (Litz, 1984b) and needs further attention. The role of the explant in somatic embryogenesis induction of woody plants is also well documented, the embryonary tissues being the most responsive (Thorpe et al., 1991; Dunstan et al., 1995). In our experiments, only mature cotyledonary zygotic embryos were able to undergo somatic embryogenesis. The results here presented are consistent with those achieved in feijoa (pineapple guava) where somatic embryos differentiate mostly from mature zygotic embryos (Canhoto and Cruz, 1996) but are in contrast with the results recently obtained by Parra and Amo-Marco (1999) in *Myrtus communis*. These authors showed that only 2-month-old immature seeds were able to undergo somatic embryogenesis. Younger seeds or seeds with hard coats (3–4 months after anthesis) did not produce somatic embryos. These differences could be explained by the fact that we used isolated embryos instead of whole seeds. In *Eugenia species* (Litz, 1984a) and *Myrciaria cauliflora* (Litz, 1984b) somatic embryos were only obtained from embryos taken from fruitlets at particular stages of development (0.8 – 1.6 cm length in *Eugenia* and 0.7 – 0.9 in *Myrciaria cauliflora*).

Comparison with other embryogenic systems (Michaux-Ferriére and Carron, 1989; Cruz et al., 1990; Watt et al., 1991; Jha et al., 1992) indicate that number of abnormal embryos produced in myrtle is surprisingly low. Even those embryos showing some type of morphological abnormality were able to germinate. This seems to indicate that the culture conditions are well adjusted for somatic embryo formation and development.

As well as in other myrtaceous (Muralidharan and Mascarenhas, 1995; Canhoto et al., 1996), myrtle somatic embryos also showed the ability to produce secondary embryos or shoot buds thereby increasing the rate of plant regeneration. Morphological obser-

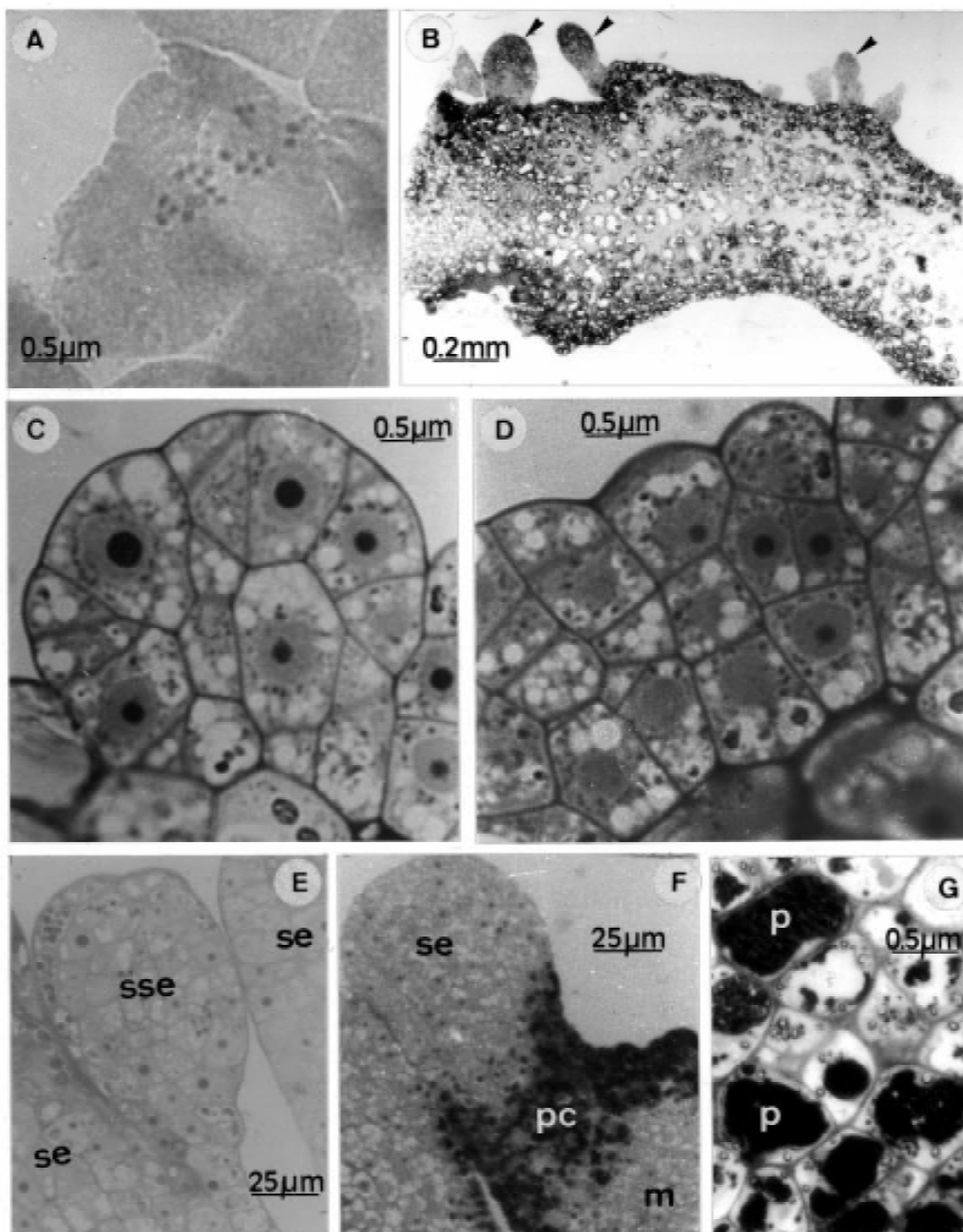

Figure 5. Chromosome number and histological analysis of somatic embryo differentiation. (*A*) Root tip cell of a regenerated plant showing the diploid ($2n = 22$) chromosome number. (*B*) Several somatic embryos (arrows) developing at the upper surface of a cotyledon. (*C*) Section of a somatic embryo at an early stage of differentiation. (*D*) Layer of meristematic cells from which the somatic embryos are produced. (*E*) Secondary somatic embryo (sse) arising from a former somatic embryo (se). (*F*) Transverse section of an embryogenic cotyledon showing a somatic embryo (se) isolated from the meristematic layer (in) of cells tissue by polyphenol-rich (pc) cells. (*G*) Detail of the polyphenol-rich cells. The polyphenols (p) seem to accumulate in the vacuoles.

vations showed that somatic embryos arose mainly from the upper face of the cotyledons. Similar observations were made on feijoa (Canhoto and Cruz, 1996). In this species we were able to show that somatic embryos differentiated directly from epidermal cells or indirectly from groups of meristematic cells near the adaxial surface arising as the result of successive divisions of cells that, under normal conditions, would form the palisade parenchyma (Canhoto and Cruz, 1996). Other authors were also able to show a relation between somatic embryos formation and the presence of a particular type of cell or tissue (Barciela and Vieitez, 1993; Stamp, 1997). In myrtle we could not relate somatic embryo formation with any particular kind of cotyledonary cells but the data seem to indicate that a meristematic zone built up by divisions of epidermal or subepidermal cells is probably the origin of somatic embryos. As well as the primary somatic embryos, secondary embryos of myrtle also seem to originate from peripheral cells. A particular feature of somatic embryo differentiation in myrtle is the association between the developing somatic embryos and phenolic-rich cells. In some cases, these phenolic-rich cells completely delimited the embryos from the supporting tissues. Similar observations were made in our laboratory in other woody species such as feijoa (Canhoto and Cruz, 1996), tamarillo (data not published) and bay laurel (Canhoto et al., 1998b). Other authors have also pointed out this association (Radojevic, 1979). We suggest that phenols can constitute some kind of barrier that isolates embryos from the supporting tissues. Several authors (see Halperin, 1995) have pointed out the relevance of isolation for somatic embryo development. Moreover, phenols can interfere with endogenous auxin levels by affecting the activity of IAA oxidase (Delalonde et al., 1996) and thus conditioning somatic embryo induction.

Future studies in the embryogenic system of myrtle will be focused on the role of polyphenols during somatic embryogenesis induction and in the analysis of protein patterns during the process of somatic embryo differentiation.

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