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Aberrant Citrus Plants Obtained by Somatic Embryogenesis of Nucelli Cultured in Vitro

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Abstract. **Virus-free plants of 8 monoembryonic cultivars of *Citrus clementina* Hort *ex* Tan. were obtained by somatic embryogenesis of the nucellus cultured in vitro. Twenty-nine percent of them had abnormal phenotypic characters that were maintained for 7 years in the greenhouse, and by budwood propagation on different rootstocks. Electrophoretic studies of the proteins disclosed differences in band intensity and distribution between normal and abnormal plants, indicating the possibility of genotypic variations. Alternative methods to obtain virus-free citrus plants are advised to reduce the high percentage of abnormalities.**

The technique of nucellus culture in vitro was developed initially to obtain true-to-type plants of monoembryonic citrus cultivars free of certain virus and virus-like diseases (2, 13, 14). This technique has been used in the Citrus Variety Improvement Program in Spain (9, 10) to recover virus-free plants of several clementine cultivars that are important for the Spanish citrus industry.

Eight self-incompatible monoembryonic mandarin cultivars were selected for this study (Table 1) (6). Flowers were hand pollinated with 'Comuna' sweet orange [*Citrus sinensis* (L.) Osb.] pollen to induce seed formation. Fruit were collected 13 to 15 weeks after pollination, and the nucellus of immature seeds was excised aseptically by removing both integuments and extracting the zygotic embryo at a heart-shaped to early cotyledonary stage of developement. The technique of embryo extraction (4, 6) minimizes breakage or damage of embryos. Only nucelli from seeds with clearly visible zygotic embryos were used.

The culture medium contained MS salts (8) and the following (in mg/liter) thiamine hydrochloride, 0.2; pyridoxine hydrochloride, 1.0; nicotinic acid, 1.0; i-inositol, 100; Bacto-malt extract, 500; sucrose, 50,000; and Bacto-agar, 10,000. The pH of the medium was adjusted to 5.7 ± 0.1, and 25-ml quantities were dispensed into 25 × 150 mm culture tubes. The medium was sterilized by autoclaving at 121°C for 5 min. One nucellus was planted in each tube, with the chalazal end embedded in the medium (4). The cultures were kept at 27° under 16 hr daily exposure to about 30 μmol $s^{-1}m^{-2}$ illumination from Sylvania Gro Lux lamps.

The embryos produced by the nucelli were recultured in fresh medium under the same environmental conditions for 4–8 weeks. Resulting plants were transplanted into pots containing an UC soil-mix modified for Citrus (1) and grown by the Navarro et al. method (12). Survival was over 90%.

Lyophilized, mature leaves sampled from the experimental plants were used for extraction of soluble proteins. The extracts were prepared by the procedure of Button et al. (3). Electrophoretic separation of protein extracts of these plants was carried out according to the method described by Iglesias et al. (5). Total soluble proteins were stained with Coomassie brilliant blue R-250.

The nucelli produced somatic embryos after 3 to 6 weeks in 10% to 30% of the cultures and callus growth in 40% to 75%. Both types of growth were initiated at the micropylar end of the nucellus. About 15% of the calluses eventually produced embryos. Some influence of the cultivar and stage of seed developement was observed on the development of the nucellus (6). The embryos, produced either directly from the nucellus or through a callus intermediary, proliferated by 'budding' (4), and several embryos usually were obtained from the same nucellus.

One hundred and sixty-five plants were obtained and grown in the greenhouse. Detailed observations of these plants disclosed 48 (29%) with abnormal phenotypic characters (Fig. 1, Table 1). The main abnormalities consisted of changes of leaf and petiole shape and size (Fig. 2). Some plants had very short internodes; 2 were thornless. One abnormal plant of 'Tardia Clementine' produced fruits that also were abnormal (Fig. 3). Abnormalities have remained through 7 years of greenhouse culture of the transplanted plants and after several graft propagations, using Troyer citrange [*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] and Cleopatra mandarin (*Citrus reshni* Hort. *ex* Tan.) rootstocks.

Table 1. Abnormal plants of Clementine cultivars obtained by nucellus culture in vitro.

Cultivar	No. of plants studied	No. of abnormal plants
Borrull	2	1
Tomatera	6	0
Oroval	13	2
Reina	16	8
Clemenfina	11	4
Fina	36	15
Tardía	32	5
Nules	49	13
Total	165	48

Fig. 1. Nucellar plants of 'Fina Clementine' obtained by nucellus culture in vitro. **Left**, normal type; **center** and **right**, abnormal types.

When more than one plant was obtained from the same nucellus, by any of the 3 processes (direct embryogenesis, through callus intermediary, or by budding of embryos), all were alike, normal or abnormal. Chromosome counts made by the aceto-carmine method revealed that the aberrant plants were diploid.

The study of leaf proteins by polyacrylamide gel electrophoresis showed differences in band intensity and distribution between normal and some of the abnormal plants of the same cultivar. Figure 4 shows the differences in the case of 'Fina Clementine.' Protein patterns of normal nucellar plants and female parents were similar.

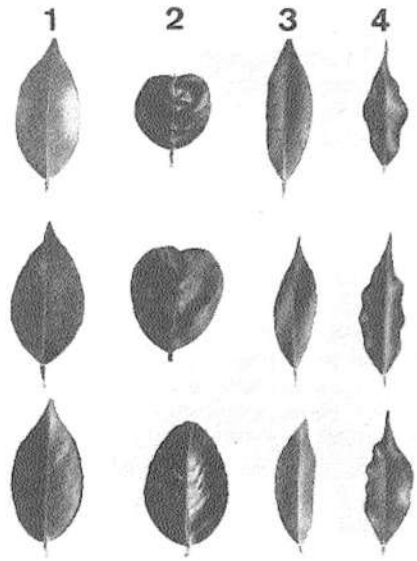

Fig. 2. Leaves of 'Fina Clementine' obtained by nucellus culture in vitro. **1**, normal; **2**, heart shaped leaf; **3**, willow leaf; and **4**, warped willow leaf.

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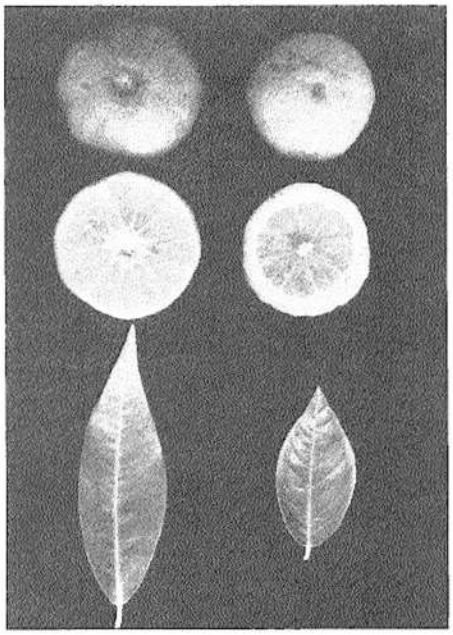

Fig. 3. Leaves and fruit of 'Tardia Clementine'. **Left**: normal; **right**, abnormal.

Protein profiles of 'Comuna' sweet orange and the Clementine cultivars were slightly different. Abnormal plants could not be identified as hybrids between Clementine cultivars and 'Comuna' sweet orange, according to their protein pattern. The morphological and biochemical observations indicate that the differences among normal and abnormal plants are genetic.

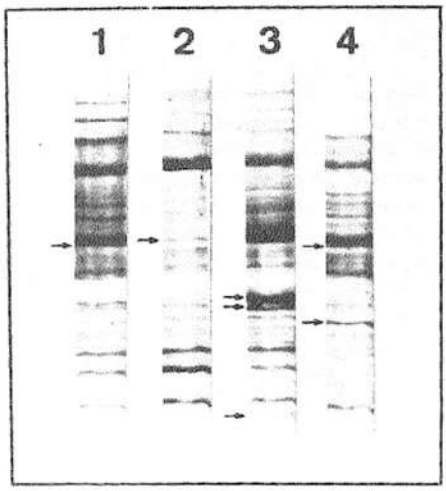

Fig. 4. Electrophoretic leaf extracts of the 'Fina Clementine' plants shown in Fig. 2. **1**, normal; **2**, warped willow leaf; **3**, heart shaped leaf; and **4**, willow leaf. Arrows show main differences in band intensity and disposition.

Although a pollen marker was not used in this study, the careful extraction of zygotic embryos and the presence of some protein bands in abnormal plants that are not present in normal Clementine cultivars or in 'Comuna' sweet orange, suggest that abnormal plants are not hybrids.

Since almost no differences in protein profiles occur among different Clementine cultivars (unpublished results), we cannot exclude the possibility that normal plants will show abnormalities when they attain fruiting stage. To ascertain this point, a field plot has been established for further observations.

Apparently, the abnormalities are not produced during in vitro culture, as it is sometimes true of other species (7). All plants obtained from the same nucellus were either normal or abnormal; in other species both normal and abnormal plants generally are produced by the same explant. Abnormal plants have been generated through the 3 embryogenetic processes previously mentioned. The time in culture was relatively short, including 4 to 8 weeks for embryo formation and 6 to 8 weeks for embryo germination and plantlet development. Recultures were made only occasionally with callus.

Some of the embryos displayed pluricotyly, anisocotyly and fasciation of cotyledons; but no correlation was found between abnormalities of plants and of embryos.

Based on our observations, we conclude that nucellus culture is not advisable for producing virus-free, true-to-type plants of the monoembryonic citrus varieties, because of the high percentage of aberrances. At this time, to avoid this problem, the in vitro shoot-tip grafting technique is recommended. It produces virus-free plants that are true-to-type and without reversion to juvenility (12). However, nucellus culture in vitro may be used to increase genetic variability in monoembryonic citrus species for obtaining new cultivars.

It is interesting that all plants recovered by in vitro somatic embryogenesis of the nucellus of polyembryonic citrus cultivars are normal (11). The different behavior of monoembryonic and polyembryonic cultivars has not been explained.

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