CELL BIOLOGY AND MORPHOGENESIS

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Improvement of *Citrus clementina* Hort. ex Tan. microspore-derived embryoid induction and regeneration

Abstract An improvement of the protocol for haploid induction through anther culture of *Citrus clementina* Hort. ex Tan. cv. Nules was achieved following the evaluation of a number of the factors affecting androgenesis. The influence of thidiazuron (TDZ) and three temperature pre-treatments (4°C, 25°C, 32°C) on the floral buds with respect to anther culture of *C. clementina* Hort. ex Tan., cv. Nules was investigated. An increased embryoid production was induced in the medium supplemented with TDZ. Pre-treatment temperatures of 4°C and 25°C were more favorable for embryo production than 32°C. Regeneration of androgenic haploid plantlets from cv. SRA 63 of *C. clementina* is reported here for the first time.

Keywords Citrus · Androgenesis · Haploid · Pre-treatment · Clementine

Abbreviations *6-BA:* 6-Benzylaminopurine · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GA₃:* Gibberellic acid · *KI:* Kinetin · *NAA:* α-Naphthaleneacetic acid · *TDZ:* Thidiazuron (*N*-phenyl-1,2,3,-thi-diazol-5-ylurea) · *ZEA:* Zeatin

Introduction

In vitro androgenesis has been reported for over 200 plant species (Dunwell 1986; Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995). Despite considerable efforts, microspore embryogenesis protocols

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have been less successful in woody plants, which are generally characterized by a long reproductive cycle, a high degree of heterozygosity and, sometimes, by self-incompatibility. Several methods have been used to obtain haploids, such as anther culture, isolated microspore culture, and wide hybridization (chromosome elimination; *bulbosum* method; Kasha and Kao 1970). In situ parthenogenesis following pollination with irradiated pollen (Ollitrault et al. 1996) or with triploid pollen (Oiyama and Kobayashi 1993) and in vitro parthenogenesis following pollination with triploid pollen (Germanà and Chiancone 2001) have also been successfully used to obtain haploids in *Citrus*.

Androgenesis through in vitro anther and isolated microspore culture is the most widely used system for haploid production. Although extensive research has been carried out on androgenesis in *Citrus* spp. and their relatives, very few successful results have been published. Haploid calli and plantlets have been recovered from *Poncirus trifoliata* L. Raf. (Hidaka et al. 1979), *Citrus madurensis* Lour. (Chen et al. 1980), and *C. clementina* Hort. ex Tan. (Germanà et al. 1994). Haploid but albino embryoids of Mapo tangelo (*C. deliciosa* × *C. paradisi*) (Germanà and Reforgiato 1997) have been reported as well as the recovery of embryoids and leafy structures of *C. limon* L. Burm. f. (Germanà et al. 1991). Haploid embryoids of *Clausena excavata* (Froelicher and Ollitrault 2000) have also been obtained.

Since the first production of haploid embryogenic calli and subsequent plantlet regeneration by anther culture of *Citrus clementina* cv. Nules was reported (Germanà et al. 1994), several studies have been carried out to improve both the frequency of microspore embryogenesis and the percentage of plantlet regeneration (Germanà and Reforgiato 1997; Germanà et al. 2000a, 2000b). In fact, before practical advantage of the haploid potential of *Citrus* breeding can be applied, fundamental research goals, such as an increase in the number of respondent genotypes, an improvement in the induction rate (the frequency of pollen grains which form embryoids), and an increase in the survival rate [the percentage of regenerated haploid

and doubled haploid (DH) plants successfully transferred from in vitro to in vivo conditions], have to be met.

The successful recovery of haploids depends on several factors, such as genotype, pre-treatment, pollen development stage, media composition, and culture conditions. The research reported here has been carried out to improve the induction rate of androgenesis in *Citrus clementina* cv. Nules. For this purpose, we investigated: (1) the effect of thidiazuron (TDZ) in the induction medium, (2) the effect on anther culture at three temperature pre-treatments (4°C, 25°C, 32°C) applied to the flower buds. Improvement in the induction rate has also allowed us to increase the number of *C. clementina* cultivars respondent to androgenesis.

Materials and methods

Plant material

Flower buds were harvested in March from 25-year-old (approximately) trees of *Citrus clementina* cvs. Nules and SRA 63 that grow at the collection orchard of the Istituto di Ricerca per la Genetica degli Agrumi, National Research Council in Lascari (Palermo, 38°N). Cultivar Nules, discovered as a bud mutation on a Fina tree, is one of the most extensively grown clementine varieties in Spain. Cultivar SRA 63 was selected at the Citrus Research Station (Station de Recherches Agrumicoles) of San Giuliano (Corsica) from a collection of Moroccan clementines (Saunt 1990).

The experiments were carried out in two consecutive years. In the first year, the experiments on the effect of TDZ and temperature pre-treatments were carried out on cv. Nules. In the second year, the trials dealt with both cultivars, Nules and SRA 63.

Pollen developmental stage

The stage of pollen development was tested in one anther per bud size using the acetic-carmine method (Sharma and Sharma 1972). The anthers were collected from flower buds at different stages of development and squashed in 1% acetocarmine in 45% acetic acid for observation under an optic microscope to identify the uninucleate stage of pollen development. This stage had previously been determined to be the most responsive for clementine androgenesis (our unpublished results). For subsequent experiments, only flower buds of the same size as those with anthers (3.5–4.0 mm in length) with microspores at the uninucleate stage were selected for culture.

Anther culture

Following the pre-treatments, the flower buds were surface-sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion in a sodium hypochlorite solution (about 0.5% active chlorine in water) for 20 min, and finally rinsed three times for 5 min each time with sterile distilled water. Petals were aseptically removed with small forceps, and the anthers were carefully dissected and placed in 6-cm-diameter Petri dishes containing 10 ml of solid medium. Between 60 and 65 anthers were placed in each dish. The subculture period was 40 days.

Culture conditions

The Petri dishes with anthers were sealed with Parafilm, incubated initially at 27±1°C for 15 days in the dark, followed by incubation under a 16/8-h photoperiod with light supplied by cool-white fluorescent lamps (Philips TLM 30 W/84) at a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1}.

Media composition

Induction media

The basal medium used was N6 Chu (1978) supplemented with Nitsch and Nitsch vitamins (1969), 18 g/l lactose, 9 g/l galactose, 5% coconut water (Sigma, St. Louis, Mo.), 500 mg/l casein, 200 mg/l L-glutamine, 0.5 mg/l biotin, and 500 mg/l ascorbic acid. The following growth regulators were added to the culture medium before autoclaving (in milligrams per liter): NAA, 0.02; 2,4-D, 0.02; KI, 1.0; 6-BA, 0.5; ZEA, 0.5; TDZ, 0.1; GA$_3$, 0.5. The pH was adjusted to 5.8 with 1 N KOH before autoclaving (20 min, 120°C). Agar (0.8% of washed agar; Sigma) was added as the gelling agent. The embryogenic haploid calli were multiplied on MS (Murashige and Skoog 1962) medium supplemented with 5% sucrose, 0.02 mg/l NAA and 0.8% agar.

Germination medium

The embryoids were germinated as soon as they reached 2–3 mm in size, either in Magenta boxes (Sigma V8505) or in test tubes with MS medium containing 3% sucrose, 1 mg/l GA$_3$, 0.01 mg/l NAA, and 0.75% agar.

Experiments

In the first year, floral buds were harvested, pre-treated at 4°C, 25°C or 32°C in darkness for 48 h and the anthers, were then cultured as described. Anthers pre-treated at 4°C were placed in the medium above described and in the same medium without TDZ to test the effect of this growth regulator. A randomized complete block design was used for the experiments. Fifteen Petri dishes with 60 explants each (anthers/Petri dish) were used per each experimental treatment (900 anthers for treatment for a total of 3,600 anthers).

In the second year, anthers of cvs. Nules and SRA 63 were cultivated in the medium supplemented with TDZ (at 0.1 mg/l) after a pre-treatment at 4°C for 48 h. Thirty Petri dishes with 65 explants each (anthers/Petri dish) were prepared for each genotype (1,950 anthers per genotype for a total of 3,900 anthers).

Data collection

Every month up to 10 months after induction, the number of anthers that did not develop, those that became swollen, and/or those that produced embryoids and calli were observed in each Petri dish. This interval was chosen because it had been observed previously that after 10 months *Citrus* anthers do not undergo further significant development.

The percentages of anthers with callus and with embryoids or embryogenic calli were calculated within each Petri dish. These values were used to calculate means. The effects of temperature pre-treatment, TDZ, and genotype on the data collected were tested by analysis of variance at the $P{\le}0.05$ level. Differences among means were tested by Student-Newman-Keuls' (SNK) Multiple Comparison test.

Plant recovery and morphological observations

Because of previously observed slow growth of plantlets in soil—presumably due to the expression of harmful recessive genes expressed in homozygosity (inbreeding depression)—small apexes (2–3 mm) of regenerated plantlets were grafted in vitro onto 20-day-old, etiolated Troyer citrange [*Citrus sinensis* (L.) Osbeck ×

Poncirus trifoliata (L.) Raf.] seedlings. The plantlets obtained were subsequently transferred to the greenhouse in pots containing sterilized peat moss, sand and soil in a 1:1:1 ration for the hardening phase.

Cytological observations

Cytological observations based on the acetocarmine method were performed weekly during the first month of culture and then every 2 weeks on the anthers in culture to observe the nuclear divisions of the microspores. Chromosome number was counted in calli and root-tip cells from regenerated embryoids and plantlets using the standard Feulgen technique (Lillie 1951). The explants were pretreated with a 0.05% (w/v) aqueous solution of colchicine for 2 h at room temperature, fixed overnight in ethanol:glacial acetic acid (3:1, v/v), and stored in 70% ethanol until they could be observed under the microscope.

Microsatellite analyses

Three microsatellites (TAA27, CAC23 and TAA41) described in Kijas et al. (1997) were analyzed to study homozygosity of the regenerated plantlets. PCR amplifications were performed as described in Kijas et al. (1997) with minor modifications using 300 ng of genomic DNA per 25 μl of reaction. Protocols for DNA extraction, polyacrylamide gel electrophoresis and silver staining of amplification products are described in Ruiz et al. (2000).

Results and discussion

Embryogenesis and regeneration of plantlets

After 1 week of culture, most of the anthers were swollen, and after 2–3 months they started to produce calli. Most calli were not morphogenic, but some of them (20 in the first year, 100 in the second one) appeared to be highly embryogenic. Morphogenic calli were friable (Fig. 1A) and white. Occasionally calli developed from two different lobes of an anther (Fig. 1B). Embryogenic calli differentiated into clumps of embryoids (Fig. 1C). Well-structured embryoids showed normal developmental patterns: globular, heart, torpedo and cotyledonary stages. Secondary embryoids developed as well.

Pluri-cotyledonary embryoids, green and spherical pseudobulbils, with or without callus, were often found. Sometimes direct gametic embryogenesis without callus formation was obtained (Fig. 1D). This was observed in 7% of the respondent anthers of Nules and in 45% of the respondent anthers of SRA 63. Plant regeneration was also obtained via direct organogenesis in 2% of the cases from compact structures produced by anthers when these structures were transferred to germination medium (Fig. 1E).

Most calli were able to regenerate for a long period by secondary embryogenesis. Counting performed after the second subculture on homozygous embryoids obtained in the second year showed that Nules and SRA 63 anthers had produced by this time a total of approximately 1,000 and 570 embryoids, respectively. That this is an improvement in haploid induction is evident when we compare these results with previous ones obtained with other androgenic and gynogenic protocols. Germanà et al. (1994) reported the production of only six haploid plantlets by anther culture of *C. clementina* cv. Nules. The production of nine haploid plantlets and two embryogenic callus lines has also been reported in clementine (*C. clementina* Hort. ex Tan.) cv. SRA 63 after in situ parthenogenesis induced by irradiated pollen of Meyer lemon (*C. meyeri* Y. Tan.) (Ollitrault et al. 1996). However, the SRA plantlets did not develop further and ultimately died. Moreover, three haploid plants were obtained from two mono-embryonic diploid (clementine and Lee)×triploid hybrid of Kawano natsudaidai (*C. natsudaidai*) in vivo crosses (Oiyama and Kobayashi 1993). The recovery of 14 haploid plantlets regenerated through gynogenesis in *C. clementina* Hort. ex Tan. cv. Nules after in vitro pollination with pollen grains of Oroblanco, a triploid cultivar of grapefruit, has been recently reported (Germanà and Chiancone 2001).

The well-structured embryoids were isolated and transferred into test tubes or in Magenta GA-7 vessels containing germination medium. The haploid embryoids germinated, and the resulting plantlets were highly vigorous as were the somatic embryoids. Because of the very high conversion rate of well-structured embryoids (96%, independentl of the cultivar used), the improvement was clear when compared with the previous results of clementine anther culture (Germanà et al. 1994). Plantlet losses were mainly due to contamination during the in vitro culture phases and during the acclimatization in vivo. The grafting in vitro of DH material onto etiolated Troyer citrange seedlings represents a successful method by which to transfer the regenerants from in vitro to in vivo (Fig. 1F). To date, 54 plants have been established in soil.

Cytological observation

Cytological observations showed the presence of multinucleated pollen grains after 1 week of culture. Chromosome counts carried out on root apexes of embryoids obtained from clementine anther culture showed a haploid set of chromosomes ($n=x=9$) (results not shown). During culture, most haploid calli diploidized spontaneously, producing DH embryoids and plantlets (Germanà 1997), and sometimes triploidized.

Microsatellite analyses

Because of the spontaneous diploidization of the haploid calli, cytological analyses could not always be used to determine androgenic plants. Microsatellites are short, tandemly repeated DNA sequences that are suitable markers for several applications in genetic analyses because of their positive features, such as co-dominant inheritance and high polymorphism. The mother plants and the regenerants of *C. clementina* cv. Nules and SRA 63 were analyzed using three microsatellites

Fig. 1 A Morphogenic, friable, white callus emerging after 3 months of culture from the inside of an anther, cv. Nules. **B** Calli emerging from two different lobes of an anther of cv. Nules. **C** Groups of haploid embryoids at different stages developing from a single SRA 63 anther in culture. **D** Embryoids without callus formation from *Citrus* anther culture. **E** Shoot regeneration from a compact structure produced by *Citrus* anther culture. **F** Doubled-haploid cv. Nules in vitro grafted onto Troyer citrange seedlings to enhance plant recovery
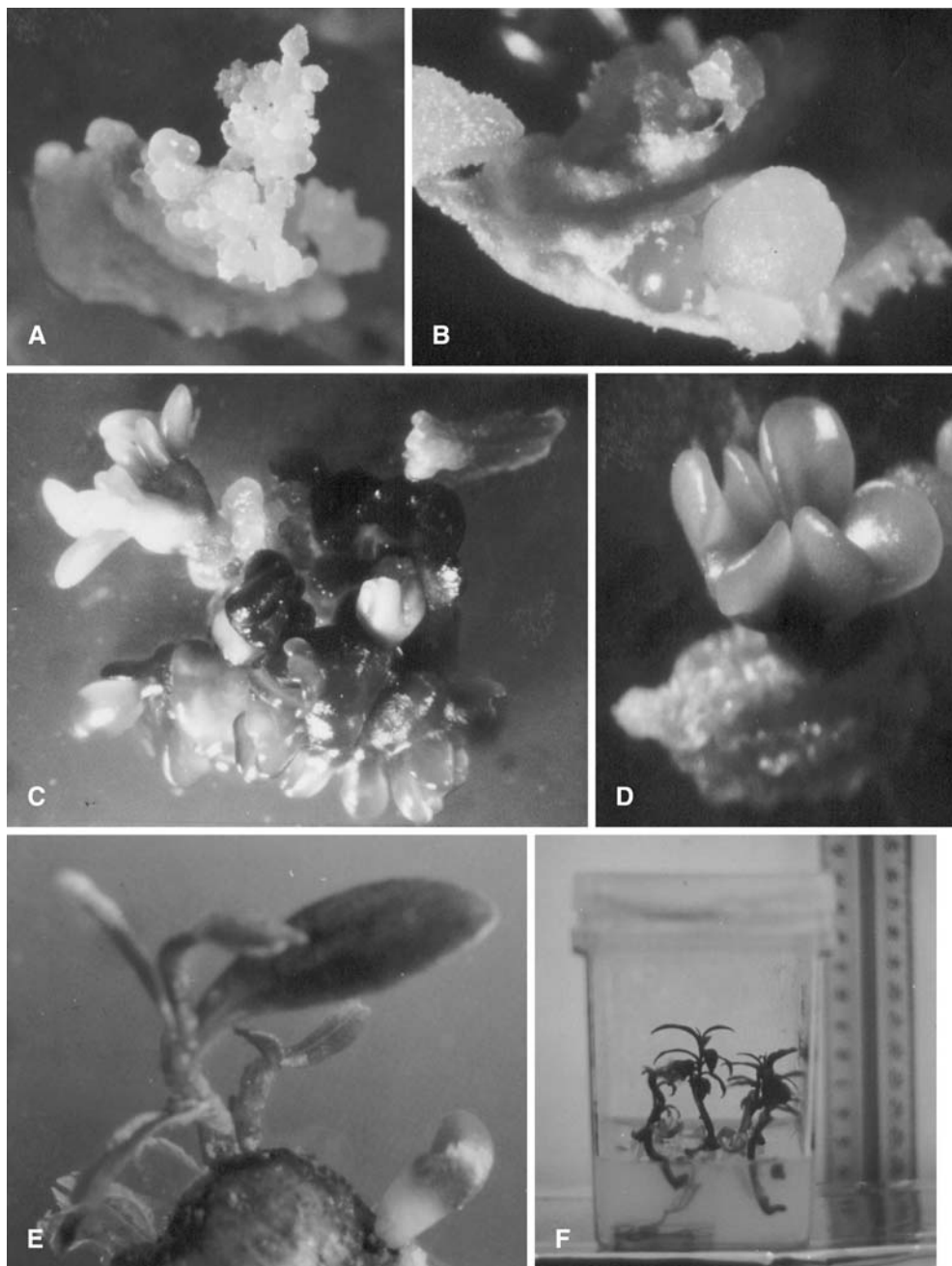
(TAA27, CAC23, and TAA41) described in Kijas et al. (1997). All of the mother plants showed two small amplification bands (indicated with an arrow in Fig. 2), while those that originated from them through anther culture had a single band at the same level, demonstrating that they retained the homozygous state (Fig. 2). Microsatellite analysis confirmed the androgenic nature of the regenerants in most of the samples examined (25 Nules from the first year, 30 Nules and 35 SRA 63 from the second year), with one exception in the first year. This heterozygous anther-derived plantlet may have arisen from: (1) somatic tissues of anther walls, (2) fusion of nuclei, (3) endomitosis, or (4) irregular microspores

formed by meiotic irregularities (D'Amato 1977; Sunderland and Dunwell 1977; Narayanaswamy and George 1982; Sangwan-Norreel 1983).

Influence of temperature pre-treatments

The effect of the temperature pre-treatments on the percentage of anthers with calli and embryoids or embryogenic calli is shown in Table 1. The percentage of anthers that did not develop was similar in all three temperature pre-treatments. The percentage of swollen anthers was higher following the 32°C pre-treatment,
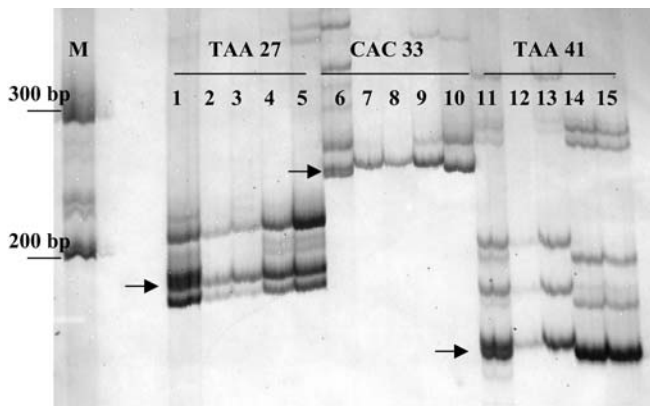
Fig. 2 Polyacrylamide gel electrophoresis of microsatellites TAA27, CAC23 and TAA41 showing the homozygous state of anther culture-derived plants. Genomic DNA was extracted from leaves of mother plants (*lanes 1, 6 and 11*) and plants derived from them through anther culture (*lanes 2–5, 7–10 and 12–15*). *M* DNA ladder (Real Escala no. 2, Durviz S.L.)

while the greatest number of anthers producing callus and embryoids or embryogenic calli was observed when the floral buds were pre-treated at 25°C. There was not significant difference between the percentages of anthers producing embryoids and/or embryogenic callus coming from floral buds pre-treated at 25°C and at 4°C. These results indicate that the highest temperature (32°C) applied to the floral buds before culture is not recommended for androgenesis in *Citrus* cv. Nules. The 48-h treatment was chosen because a longer period at high temperature (32°C) was even more detrimental for excised *Citrus* floral buds. The beneficial effect of pre-treatment at an elevated temperature for androgenesis has been reported in *Solanum melongena* L. (Rotino 1996), in *Brassica oleracea* (Keller et al. 1983), and *Solanum chacoense* (Cappadocia et al. 1984).

A number of hypotheses exist to explain the beneficial effect of a cold pre-treatment with respect to embryo induction. It seems that a cold pre-treatment increases the number of pollen grains with two equal nuclei and stimulates embryoid formation by delaying and modifying pollen mitosis or by blocking starch production, thereby dissolving microtubules (Nitsch 1977) or maintaining viability of the cultured P-grains (Heberle-Bors 1985). Cold pre-treatments are routinely used in the anther culture of many crops, and their effects are genotype-dependent (Powell 1988; Osolnik et al. 1993).

In *Citrus* anther culture, a low temperature pre-treatment (at 4–5°C for either 2 h, overnight, or for 3–6 days) has been previously used (Starrantino 1986; Ling et al. 1988; Geraci and Starrantino 1990; Germanà et al. 1991, 1994; Deng et al. 1992). In a study carried out by Chen (1985), the effect of pre-treatment at 3°C for 5–10 days on *C. madurensis* Lour. was beneficial for inducing callus and embryoids.

Influence of TDZ

The effects of TDZ on clementine Nules anther culture are shown in Table 2.

The percentage of anthers producing embryoids or embryogenic callus was statistically higher (1.8%, sixfold the control) in anthers cultivated in the medium supplemented with TDZ. Although the use of TDZ had a distinct positive effect, further investigations are necessary to test different concentrations of this cytokinin.

TDZ is one of the most active cytokinins used in woody plant tissue culture and enables micropropagation of the most recalcitrant species (Huetteman and Preece 1993). TDZ has also been employed in the anther culture of several plant species. For instance, in *Hordeum vulgare* androgenesis, the addition of 0.01 mg/l TDZ increased or decreased the number of embryos per 100 anthers or it had no effect, depending on the cultivar (Ouèdraogo et al. 1998). Conversely, in *Paeonia albiflora* anther culture, the addition of 0.0002 mg/l TDZ improved embryogen-

Table 1 Influence of three floral bud temperature pre-treatments (in darkness for 48 h) on the anther culture of *Citrus clementina* Hort. ex Tan. cv. Nules, after 10 months of culture. Each value is the average of 900 anthers per treatment (15 Petri dishes with 60 explants)

Pre-treatments	Percentages of anthers that did not develop[a]	Percentages of swollen anthers	Percentages of anthers producing callus	Percentages of anthers producing embryoids and/or embryogenic callus
4°C	8.1a	21.4b	68.6b	1.8a
25°C	9.3a	12.5c	76.1a	1.9a
32°C	10.4a	26.7a	62.5c	0.3b

[a] Values within each column followed by different letters are significantly different at $P \leq 0.05$ (SNK's test)

Table 2 Influence of TDZ addition (0.1 mg/l) to the medium on anther culture of *C. clementina* Hort. ex Tan. cv. Nules, after 10 months of culture. Each value is the average of 900 anthers per treatment (15 Petri dishes with 60 explants)

Medium	Percentages of anthers that did not develop[a]	Percentages of swollen anthers	Percentages of anthers producing callus	Percentages of anthers producing embryoids and/or embryogenic callus
No TDZ	10.4a	26.7a	62.5b	0.3b
TDZ	8.1a	21.4b	68.6a	1.8a

[a] Values within each column followed by different letters are significantly different at $P \leq 0.05$ (SNK's test)

Table 3 Comparison of the results of anther culture of cvs. Nules and SRA 63 of *C. clementina* Hort. ex Tan., after 10 months of culture. Each value is the average of 1,950 anthers per treatment (30 Petri dishes with 65 explants)

Genotypes	Percentages of anthers that did not develop[a]	Percentages of swollen anthers	Percentages of anthers producing callus	Percentages of anthers producing embryoids and/or embryogenic callus
Nules	5.7b	30.7b	59.1a	4.0a
SRA 63	1.9a	39.8a	44.1b	3.0b

[a] Values within each column followed by different letters are significantly different at $P \leq 0.05$ (SNK's test)

esis (Lee et al. 1992) as in grapevine anther culture, in which the a TDZ supplement (2.0 mg/l) enhanced somatic embryogenesis (Harst-Langenbucher and Alleweldt 1993). TDZ (0.1 mg/l) has also been successfully used in the regeneration medium of androgenetic embryoids of apple (Hofer 1995).

Influence of genotype

The results of the anther culture experiments of clementine Nules and SRA 63 carried out in the second year are shown in Table 3. With respect to the genotype influence, a better performance (a statistically higher percentage of anthers producing embryogenic calli) of Nules (4.3%) was observed when compared to SRA 63 (3.0%). The trees from which the floral buds were collected are close to each other, and they grow under identical cultural conditions. As all other factors influencing androgenesis are the same, it is possible to argue that the Nules genotype responds better than SRA 63 to these tested conditions. It has been shown in many species that genotype differences do exist with respect to androgenesis (Vasil 1980).

Widening the range of genotypes responding to androgenesis is one of the purposes of anther culture protocols of recalcitrant genera like *Citrus*. Moreover, for *C. clementina* cvs. Nules and SRA 63, which are characterized by self-incompatibility, the production of highly embryogenic haploid callus and homozygous plantlets is of great interest. Haploids from self-incompatible *C. clementina* plants would be desirable for fixing quantitative characters in completely homozygous self-incompatible diploid lines (de Nettancourt and Devreux 1977).

The number of anthers of cv. Nules producing embryoids or embryogenic calli was higher in the second year than in the first year. The different androgenic response may be due to the physiological state of the donor plants, one of the factors affecting androgenesis (Vasil 1980). The physiological condition of the donor plant, influenced by climatic and ecological conditions, should be evaluated in further experiments. This may help us to explain the reasons why the response to anther culture is so season-dependent.

The results of these experiments show that the ability to produce haploids of recalcitrant genotypes, such as *C. clementina,* can be improved investigating the factors affecting gametic embryogenesis. For this reason, more

basic studies on the sporophytic pathway of pollen grains should be carried out.

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