

# Cytomixis in Pollen Mother Cells of *Citrus* Genotypes

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## ABSTRACT

A microsporogenesis analysis performed in several *Citrus* populations revealed the presence of cytomixis (i.e., chromatin migration between meiocytes) in pollen mother cells of diploid and tetraploid genotypes. Single and double chromatin bridges between adjacent meiocytes were mainly observed at prophase I, but also in the subsequent phases of first division and in the second meiotic division. The percentage of cytomictic cells in the genotypes examined was quite variable. In addition to cytomixis, other meiotic abnormalities were observed, in both the first and second meiotic divisions. The cytomictic plants showed lower values of pollen viability compared to the control plants. A possible relationship between the occurrence of cytomixis, pollen viability and the reduced fertility of *Citrus* populations is discussed.

**Keywords:** cytogenetic, meiocytes, meiotic abnormalities, microsporogenesis, pollen viability

## INTRODUCTION

Cultivar development in *Citrus* by conventional breeding methods has been traditionally limited by its complex reproductive biology (Swingle and Reece 1967): apomixis (adventitious nucellar embryony), gametophytic and sporophytic incompatibilities, prezygotic mechanisms (inhibition of pollen germination, inadequate or arrested pollen tube growth), postzygotic mechanisms (ploidy imbalance, endosperm failure), often prevent sexual hybridization and the production of segregating zygotic populations for selection. A full understanding of the mechanisms underlying reduced fertility among and within *Citrus* species has not been achieved yet, and several factors limiting successful sexual hybridization are still obscure.

A cytogenetic study was undertaken to determine the mechanisms responsible for reduced fertility in some *Citrus* selections: the presence of several meiotic irregularities, including instances of cytomixis, were detected for the first time in pollen mother cells of *Citrus* species, at both the diploid and tetraploid levels. Cytomixis is defined as the migration of chromatin between adjacent cells through cytoplasmic connection channels, also if, in some rare cases, direct cell fusion has been observed (Bhat *et al.* 2006; Boldrini *et al.* 2006). Cytomixis occurs in a great number of plant species (Gottschalk 1970; Cheng *et al.* 1975; Omara 1976; Bellucci *et al.* 2003; Guzicka and Wozny 2004) and has mainly been observed in pollen mother cells (PMCs), but also in the tapetal cells and in the ovary cells of various plants (Koul 1990). This cytological phenomenon has been more frequently observed during the microsporogenesis of genetically unbalanced plants such as haploids, aneuploids, hybrids (de Nettancourt and Grant 1964), polyploids (Sidorchuk *et al.* 2007a) and apomicts (Mantu and Sharma 1983). Some studies show that it could be a genetically controlled mechanism involving meiotic genes and operating through a signal transduction pathway triggered by environmental stimuli (Latoo *et al.* 2006). The origin and significance of cytomixis have been extensively studied in a great number of species, but its role in evolution as well as its genetic control is still controversial and unclear. Nevertheless, cytomixis may have serious genetic consequences, such as the

formation of PMCs with anomalous chromosome numbers (Ghaffari 2006), and of aberrant microspores (dyads, triads, pentads, hexads, heptads) (Kim *et al.* 2005), pollen sterility or reduced male fertility (Soodan and Waffai 1987; Boldrini *et al.* 2006), chromosome stickiness and syncytia (Patra *et al.* 1986). All these abnormalities interfere negatively with the reproductive potential and may even modify the reproductive system (Soodan and Waffai 1987).

This research work was carried out on different *Citrus* genotypes to study the mechanisms responsible for their reduced fertility and to verify the presence of cytomixis. An interspecific tetraploid somatic hybrid was also included in order to ascertain the assumption that cytomixis seems to be more frequent in polyploid forms than in their diploid counterparts, as reported by Semyarkhina and Kuptsou (1974).

## MATERIALS AND METHODS

The following *Citrus* genotypes were used: 1) a seedless sweet orange (*C. sinensis* Osb.) diploid ( $2n=2x=18$ ) clone; 2) a nearly seedless tangerine (*C. clementina* Hort. ex Tan.) cv. 'Nules' (diploid); 3) a seedy Mediterranean mandarin (*C. deliciosa* Ten.) diploid, as control; 4) an interspecific tetraploid ( $2n=4x=36$ ) somatic hybrid between Key lime (*C. aurantiifolia* Swing.) and Valencia sweet orange (*C. sinensis* Osb.), obtained through protoplast fusion (Grosser *et al.* 1989). All the genotypes were grafted onto sour orange (*C. aurantium* L.) and grown at the same location in an experimental field station (Lascari, Palermo, Italy).

For microsporogenesis analysis, flower buds were harvested at various developmental stages and fixed whole in ethanol-acetic acid (3:1) at 4°C. After extraction from the floral buds the anthers were treated with a solution of cellulase 2% and pectinase 5% for 20 min at room temperature, stained according to the Feulgen method (hydrolysis in HCl 1N, at 60°C for 7 min and basic fuchsin for 1 h) and squashed on glass slides in a drop of 2% acetic orcein to intensify the staining of the meiotic cells. Cytological analysis of the meiotic phases was performed on a sample of 1,000 cells per genotype. Pollen was collected from the same plants as those used for meiotic analysis. Pollen viability was determined on 2,000 pollen grains per genotype on slides prepared with 2% acetic carmine and glycerin (1:1).

## RESULTS

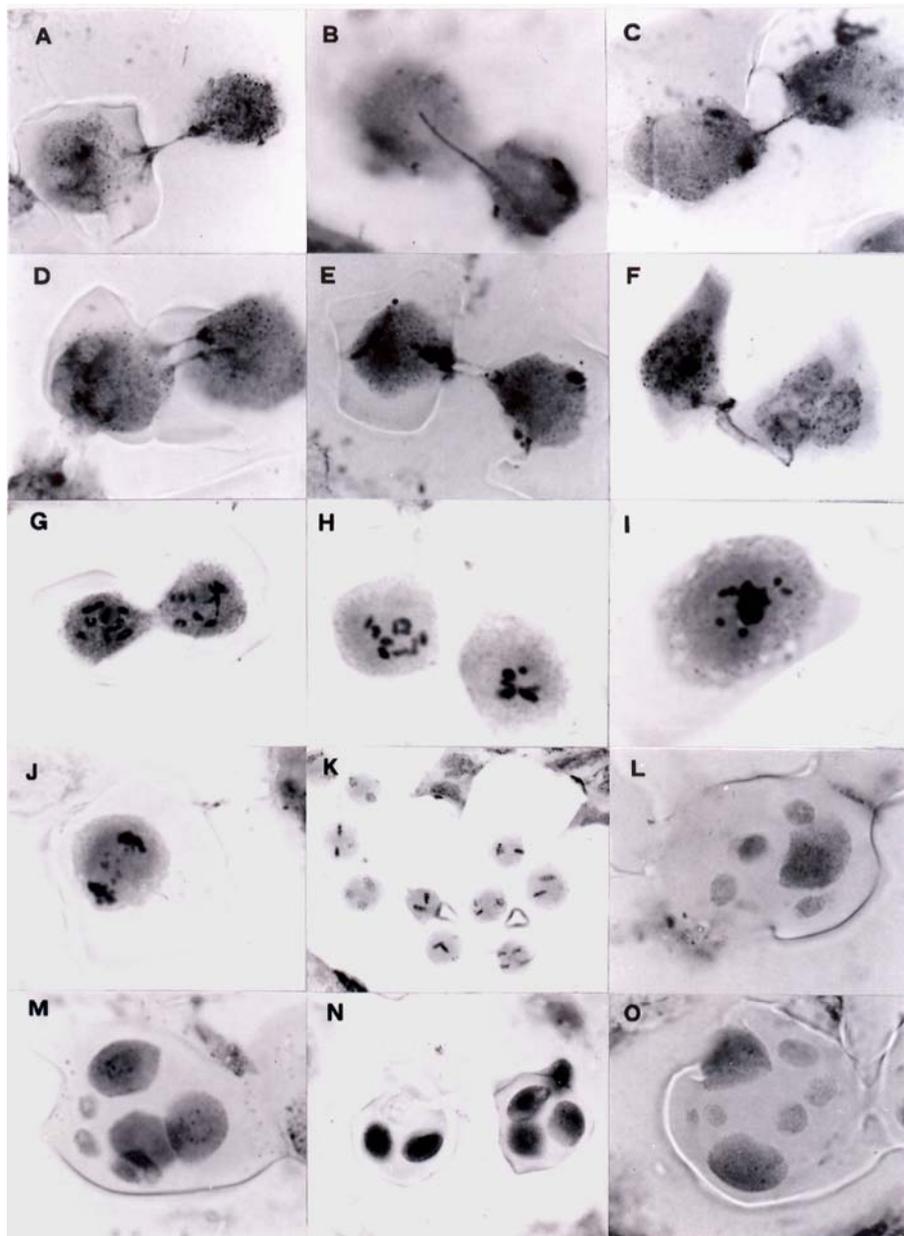
In our *Citrus* genotypes, microsporogenesis analysis revealed the presence of several meiotic abnormalities, including cytormixis, that is the migration of chromatin between meiocytes through cytoplasmic channels. This abnormality was observed at both the diploid and the tetraploid levels. Chromatin migration caused the formation of chromatin bridges which generally involved two adjacent cells, with partial or total transfer of the genetic material. This phenomenon was already visible in the first meiotic division in the PMCs of the sweet orange clone and in ‘Nules’ tangerine, both diploids, and in the tetraploid interspecific somatic hybrid “Key lime + Valencia” sweet orange (Fig. 1A-C). In sweet orange, and in ‘Nules’ tangerine, double chromosome bridges were also observed (Fig. 1D, 1E). No evidence of cytormixis was found in the control plant, the fertile diploid Mediterranean mandarin, which exhibited regular meiosis (Table 1). The percentage of intercellular connections in the first meiotic division varied from less than 3% in ‘Nules’ tangerine and in the somatic hybrid meiocytes to much higher levels in the sweet orange clone, in which 17.7% of the meiocytes showed chromatin bridges at early prophase I, and 4% at diakinesis. Chromatin transfer between PMCs was detected at a lower frequency in the second meiotic division (Fig. 1F), during which only the

tetraploid somatic hybrid meiocytes exhibited chromatin bridges at metaphase II (3.5%) and at ana-telophase II (4%).

In sweet orange and in the tetraploid somatic hybrid, we observed numerous cytoplasmic channels between meiocytes (Fig. 1G), and cells with chromatin masses distributed throughout the cytoplasm. This could indicate a probable increase in cytormixis frequency in the two genotypes. Moreover, in the somatic hybrid, we observed microsporocytes that were totally empty as a result of complete chromatin migration either into another meiocyte or occasionally outside the cell.

In addition to cytormixis, other meiotic abnormalities were observed in *Citrus* PMCs, in both the first and the second meiotic divisions. The most common of such abnormalities were those related to irregular chromosome segregation (Table 2). Diploid and tetraploid genotypes both exhibited multivalent chromosome configurations at diakinesis. Bivalents and quadrivalents were detected simultaneously at this stage. Quadrivalent formation was observed at high frequency in PMCs of the somatic hybrid (32.6%) and of the sweet orange clone (54.4%) (Fig. 1H).

Failures in chromosome pairing resulted in a high number of univalents being observed at metaphase I of the “Key lime + Valencia” somatic hybrid (Fig. 1I). The unpaired chromosomes observed at metaphase I behaved like laggards at anaphase I (Fig. 1J) went through the second divi-



**Fig. 1 Cytormixis and other meiotic abnormalities in PMCs of *Citrus*.** Meiocytes at prophase I with single (A, B, C) and double (D, E) chromatin bridges. Single chromatin bridge in second division (F). Cytoplasmic channel between two diakineses (G). Diakinesis with 7 II and 1 IV (H). Metaphase I with 6 univalents (I). Anaphase I with laggards (J). Metaphases II with abnormal spindle orientation: parallel, tripolar, V-shaped (K). Pentad (L). Hexad (M). Dyad and triad (N). Heptad (O). Magnification: A-J, L, M, O, 1400x; K, 600x; N, 1200x. Diploid sweet orange (A, D, G, H, M). Diploid ‘Nules’ tangerine (B, E). Tetraploid Key lime + Valencia sweet orange somatic hybrid (C, F, I, J, K, L, N, O).

**Table 1** Cytomixis and pollen viability in *Citrus* diploids (2x) sweet orange and Nules tangerine, in the tetraploid (4x) somatic hybrid and in the diploid control Mediterranean mandarin.

Genotype	Prophase I with cytomixis (%)	Diakinesis I with cytomixis (%)	Metaphase I with cytomixis (%)	Metaphase II with cytomixis (%)	Ana-telophase II with cytomixis (%)	Pollen viability (%)
Sweet orange 2x	17.7	4	/	/	/	29
Nules tangerine 2x	0.3	/	2.9	/	/	88.6
Key lime + Valencia 4x	/	/	1.4	3.5	4	29.8
Mediterranean mandarin 2x	/	/	/	/	/	75.2

**Table 2** Meiotic configurations in *Citrus* microsporogenesis.

Genotype	Diakinesis I with uni/tetravalents (%)	Metaphases I with univalents (%)	Ana-telophases I with laggards (%)	Metaphases II with laggards (%)	Ana-telophases II with laggards (%)	Tetrads (%)	Dyads (%)	Triads (%)	Polyads (%)
Sweet orange 2x	54.4	34.0	25.8	28.9	12.1	54.7	1.4	43.5	0.3
Nules tangerine 2x	6.1	24.0	12.3	4.5	3.6	68.8	/	28.9	2.2
Key lime + Valencia 4x	32.6	76.1	53.6	38.4	31.8	55.3	2.9	2.9	38.7
Mediterranean mandarin 2x	/	15.1	8.7	/	/	47.0	7.7	45.1	/

sion, eventually giving rise to polyads with microspores of a different size or, more rarely, to micronuclei at telophase II.

Spindles with abnormal orientations – parallel, tripolar, cross and V-shaped – were observed at a high frequency at metaphase II of sweet orange, ‘Nules’ tangerine and the somatic hybrid (Fig. 1K). In our case, the meiotic nuclear restitution events gave rise to dyads, monads and other anomalous sporocytes (Fig. 1L, 1M). The presence of spindles with an abnormal orientation at metaphase II and the subsequent formation of several poles at ana-telophase II in the somatic hybrid, both led to abnormal microspores. Furthermore, asynchrony was evident in all the genotypes examined: it affected up to 13% of metaphase and anaphase II in ‘Nules’ tangerine. Some cells with a polyploid chromosome number were also observed and were the result of endo-reduplication. This can lead to the formation of 2n and/or 4n gametes.

The effects of aberrant meiosis were mainly seen in the sporads produced. Only 55.3% of the sporads observed in the somatic hybrid were normal tetrads, the remainder being dyads, triads (Fig. 1N) and polyads. Dyads, triads, pentads, hexads and even heptads were also represented in the other genotypes: up to 29% triads were found in ‘Nules’ tangerine, and 43.5% in the seedless sweet orange clone (Table 2). The somatic hybrid, which showed a high frequency of univalents (up to 50% of diakinesis and metaphases I), had a great number of pentads (21.4%) and hexads (16.6%), and some sporads contained as many as seven microspores (Fig. 1O). There were also tetrads and polyads containing microspores of different sizes; an observation which was only occasionally present in the other genotypes.

The presence of dyads and triads caused by abnormal spindle orientations at metaphase/anaphase II, is evidence of the formation of unreduced gametes in *Citrus*.

The occurrence of cytomixis and other meiotic abnormalities was analyzed by comparison with pollen viability. Results were variable, but in general the plants characterized by a great number of cytotoxic microsporocytes had lower levels of pollen viability than both the plants with little or no trace of cytomixis and the control. The sweet orange clone and the somatic hybrid showed over 70% pollen sterility, while the ‘Nules’ tangerine and the control Mediterranean mandarin scored high values of pollen viability (88 and 75%, respectively). It should be noted, however, that large-size, mis-shaped pollen grains were found in great numbers in the sweet orange clone, which had the highest cytomixis frequency of all the genotypes.

## DISCUSSION

Our study has revealed the presence of cytomixis in *Citrus* genotypes characterized by different genetic basis; this phenomenon, which has never been reported in the genus *Citrus* until now, was evident in both diploid and tetraploid accessions of natural populations and of plants obtained from cell fusion, at relatively low levels.

The fact that frequency of cytomixis is higher in the first meiotic division than in the second division, in agreement with findings reported for other species (Zheng *et al.* 1987; Yen *et al.* 1993; Datta *et al.* 2005), suggests that cytomixis could be caused by aberrant meiotic chromosome segregation resulting from an anaphase delay during the mitosis preceding meiosis, and that the genes controlling meiotic chromosome segregation – like the DIF1 in *Arabidopsis thaliana* L. for example (Bhatt *et al.* 1999) – also control cytomixis regulation, probably involving cytoskeleton or callose walls biosynthesis (Jun *et al.* 2004; Sidorchuk *et al.* 2007b). Overall, the frequency of cytomixis in *Citrus* is not as high as that reported with other plant species. A frequency of 41% was reported with *Vigna glarerscen* (Sen and Bhattacharya 1988), 97% with *Prunus amygdalus* Batsch (Soodan and Waffi 1987) and 66% with *Medicago sativa* L. (Bellucci *et al.* 2003) in prophase I. On the other hand, certain plant species have been considered “cytotoxic” despite a very low frequency of cytomixis, for example *Brassica campestris* and *Brassica napus* in which frequency of cytomixis was found to range between 0.06 and 2.55% (de Souza and Pagliarini 1997).

In the somatic hybrid, totally empty microsporocytes were observed as a result of complete chromatin migration either into another meiocyte or occasionally outside the cell. Meiocytes with no chromatin are very likely to get lost during meiotic division, while those with an unusual chromatin content cause abnormal microspore development and the formation of more or less viable gametes with an unbalanced chromosome number. The formation of unreduced gametes as a result of chromatin migration between meiocytes cannot be excluded (Falistocco *et al.* 1995). Thus, cytomixis could be considered a possible source of polyploid or aneuploid plants (Srivastav and Raina 1980; Datta *et al.* 2005). The formation of 2n gametes in *Citrus* also has been reported. In fact, when the tetraploid somatic hybrid “Key lime + Valencia” was used as the pollen parent in interploid crosses with diploid lemon types, the resulting progeny was characterized by different ploidy levels, including pentaploids, as was confirmed by chromosome counts coupled with high-resolution FCM to estimate the nuclear DNA ploidy (Tusa *et al.* 1996). The presence of tetraploids and pentaploids, as proposed from Fatta Del Bosco *et al.* (1999), can be explained by the formation of unreduced

pollen in the *Citrus* somatic hybrid. Triploids and tetraploids have been found to come from 2x X 2x and 2x X 4x crosses, respectively (Esen and Soost 1972; Esen *et al.* 1979), confirming the occurrence of 2n eggs in unilateral sexual polyploidization. Additionally, tetraploids have been obtained from 2x X 2x crosses by bilateral sexual polyploidization (Russo and Torrisi 1951) through production of 2n gametes.

In addition to cytomixis, still other meiotic abnormalities were observed in *Citrus* PMCs, in both the first and the second meiotic divisions. The abnormalities were those related to irregular chromosome segregation. In particular, the high frequency observation of quadrivalent formation at diakinesis stadium in PMCs of the somatic hybrid and of the sweet orange clone suggests heterozygosity as a reciprocal translocation, as previously reported with Valencia sweet orange (Fatta Del Bosco *et al.* 1999), and/or intergenomic pairing. Failures in chromosome pairing resulted in a high number of univalents. The presence of univalents, a common feature in *Citrus* meiosis, can be explained by heterozygosity for structural changes in chromosomes or by a genetic mutation in synapsis control (Iwamasa 1969; Raghuvanshi 1969; Vardi and Spiegel-Roy 1981; Gmitter *et al.* 1992).

Furthermore, parallel, tripolar, cross and V-shaped spindles were obtained at a high frequency, giving rise to dyads, monads and other anomalous sporocytes. Spindles with abnormal orientations, especially parallel spindles, are well known in other genera, such as *Solanum* (Mok and Peloquin 1975) and *Medicago* (Tavoletti *et al.* 1991), in which they cause the formation of 2n pollen.

The occurrence of cytomixis and other meiotic abnormalities was analyzed by comparison with pollen viability. There has been much speculation on the possible correlation between cytomixis and pollen viability. For some authors, there is a strictly inverse correlation between the two (Sapre and Deshpande 1987; Boldrini *et al.* 2006). Conversely, in other species cytomicitic plants have been shown to have a high degree of pollen viability (Falistocco *et al.* 1995). Even though a strict relationship was not found between the mean values of cytomixis and pollen viability in our *Citrus* genotypes, it is reasonable to believe that the two traits are associated since the other meiotic abnormalities observed in both the first and the second meiotic divisions may have adversely affected pollen viability.

Cytomixis detection represents a further contribution to the understanding of the cytogenetic mechanisms involved in the reduced fertility of several *Citrus* populations. This is particularly important when considering the role of the genotypes analyzed in the present study. In fact, reduction in male fertility caused, as it seems, by cytomixis, could be a positive trait in *Citrus* diploid scion selection in terms of induced seedlessness, whereas the inverse relation between cytomixis and fertility is an obstacle to *Citrus* breeding at the interploid level. A lack of fertile tetraploid breeding parents carrying positive agronomic traits is actually the strongest limiting factor for *Citrus* scion breeding programs. Tetraploid accessions are used as male genitors, thus wide cytological screening is a fundamental pre-requisite for their employment. However, despite the considerable frequency of meiotic abnormalities found in our tetraploid somatic hybrid accession, the 'Key lime + Valencia' displayed sufficient pollen viability to accomplish fecundation and can, therefore, be considered a proper genitor in breeding programs for the introgression of useful traits, for ploidy manipulation and for germplasm enhancement.

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