Notas Científicas

Stomatal analysis of citrus somatic hybrids obtained by protoplast fusion

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Abstract – The objective of this work was to evaluate leaf epidermis morphological characteristics of three citrus somatic hybrids, compared to their parents. Parental and somatic hybrid young leaves were collected and processed for scanning electron microscope observations. Citrus polyploid hybrids have fewer stomata per area and these are larger compared to their diploid parental parents. No differences in internal arrangement of the stomatal cells were detected between parental plants and somatic hybrids. Additional studies may determine if these differences will influence physiological behavior of the plants in the field.

Index terms: stoma, somatic hybridization, scanning electron microscopy, polyploidy.

Análise estomática de híbridos somáticos de citros obtidos por fusão de protoplastos

Resumo – O objetivo deste trabalho foi avaliar as características morfológicas de três híbridos somáticos de citros, comparando-as com as de seus respectivos parentais. Folhas jovens dos híbridos somáticos e seus respectivos parentais foram coletadas e preparadas para observações em microscópio eletrônico de varredura. Híbridos poliplóides de citros apresentam menor número de estômatos por área, com maior tamanho individual quando comparados com aqueles das plantas diplóides parentais. Não foram observadas diferenças no arranjo interno das células estomáticas entre as plantas parentais e os híbridos somáticos. Investigações adicionais poderão determinar se essas diferenças poderão influenciar o comportamento fisiológico dessas plantas no campo.

Termos para indexação: estômato, hibridação somática, microscopia eletrônica de varredura, poliploidia.

Interspecific and intergeneric crosses are important for the transfer of genetic characteristics between species and genera, with the possibility of creating new species. However, sexual hybridization between individuals of different species generally does not produce viable hybrids due to sexual barriers (Grosser & Gmitter Junior, 1990). In a breeding program, somatic hybridization can overcome sexual incompatibility through somatic cell fusion. In contrast to sexual hybridization, after protoplast fusion, all nuclear and cytoplasmic DNA from both parentals are united in one individual. Several citrus somatic hybrids have been reported, including specific combinations for improved disease resistance (Mendes et al., 2001; Costa et al., 2003).

Observation and confirmation of somatic hybrids can be done through analysis of leaf morphology, molecular markers, such as PCR-RAPD, and determination of chromosome number (Grosser & Gmitter Junior, 1990). Associations of different techniques are common, since some of them may not definitely confirm the hybrid nature of the plantlet obtained from protoplast fusion experiments.

Variable leaf morphology such as trifoliate leaves (Grosser et al., 1988) or petiole wings (Ballve et al., 1997) are commonly used as morphological markers to identify potential hybrids. When both parentals have specific characteristics in their leaf morphology, such as the trifoliate leaf and petiole wings, and the potential hybrid has leaves with both characteristics, the hybrid character of the plant can be inferred. However, characteristic morphological traits are not always present, especially when interspecific combinations are used.

Molecular markers can be very useful if polymorphism is obtained. Among molecular markers, RAPD presents substantial advantage over isoenzymes and RFLP because it is based on DNA amplification (Ferreira & Grattapaglia, 1995).

Cytogenetic analysis of the number of chromosomes is very effective. However, citrus chromosomes are very similar among them and also very small (Guerra, 2000), making very difficult to confirm the chromosome number by regular chromosome counting techniques.

Morphological, cytological, and molecular analysis are usually performed in plants after acclimatization, 8 to 18 months after protoplast fusion. Morphological characteristics are usually difficult to distinguish in early-stage in vitro cultures. Cytological analysis are commonly performed using root tips of plants grown in the greenhouse, mainly because in vitro plantlets do not always present roots and when they do, the roots tend to grow by cell elongation, with few mitotic divisions. This characteristic makes chromosome count very difficult especially for species with small chromosomes, like citrus. Although very efficient, molecular analysis of in vitro plantlets can be limited by the amount of leaf tissue needed for DNA extraction.

The objective of this work was to evaluate the leaf epidermis morphological characteristics of three citrus somatic hybrids, compared to their parents.

Three somatic hybrids (*Citrus reticulata* cv. Cleópatra + *C. volkameriana*, *C. sinensis* cv. Rohde Red + *C. volkameriana*, and *C. sinensis* cv. Ruby Blood + *C. volkameriana*) – confirmed by molecular (RAPD) and chromosome counting analyses – and their respective parental plants (*C. reticulata* cv. Cleópatra, *C. volkameriana* and *C. sinensis* cvs. Rohde Red and Ruby Blood) were used for leaf sampling.

Young leaves were collected from the terminal portion of shoots of plants grown in the greenhouse. Five young leaves were collected from each plant and 1cm-leaf-discs were cut. The samples were fixed according to Rodriguez & Wetzstein (1998), mounted in 13 mm aluminum stubs and sputter coated with gold for 180 seconds. Sample analysis and image recording were performed in LEO 435VP scanning electron microscope, operating at 10 kV.

The analysis was done by counting the number of stomata in fields of the abaxial epidermis at a magnification of 700 times, corresponding to an area of 0.1213 mm^2. The length of the guard cells was also measured in five stomata per field. Counts and measurements were done in ten fields of each sample, with a total of five samples of each plant, totalizing 50 fields per diploid or tetraploid plant. The statistical analysis was done by ANOVA and the means were compared by Tukey test ($P>0.01$).

Observations of the leaf abaxial epidermis under the scanning electron microscope confirmed the differences in number of stomata and length of guard cells between the diploid parental plants and the respective somatic hybrids, which had been previously confirmed as tetraploid plants (Figure 1). Diploid parentals presented higher numbers of stomata per leaf area and the length of the guard cells was smaller, compared to the hybrids (Figure 2). The internal arrangement of the stomatal cells

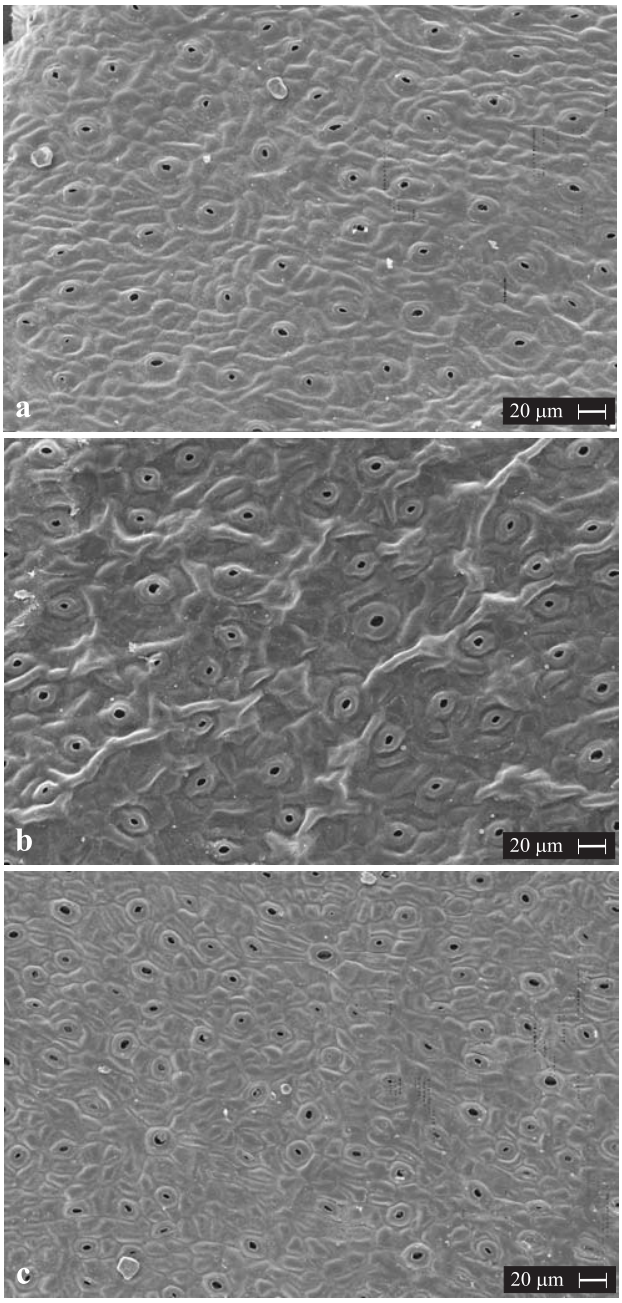

Figure 1. Scanning electron micrographs of abaxial epidermis of *Citrus* spp. diploid parental plants and the respective somatic hybrid, a) *Citrus sinensis* cv. Ruby Blood; b) *C. sinensis* cv. Ruby Blood + *C. volkameriana* and c) *C. volkameriana*.

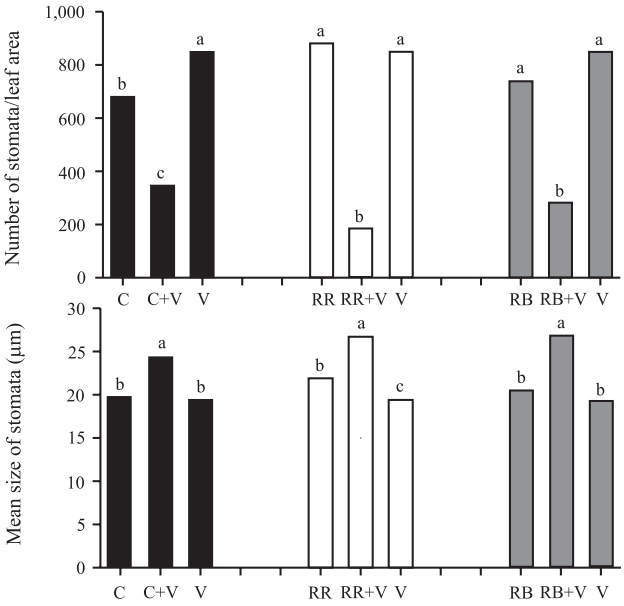

Figure 2. Number of stomata per leaf area (0.1213 mm^2) and mean size of stomata (σm) in somatic hybrids and respective parental plants. Different letters in each group of hybrid/parental show significant differences (P>0.01). (C = *Citrus reticulata* cv. Cleópatra; C+V = *C. reticulata* cv. Cleópatra + *C. volkameriana*; V = *C. volkameriana*; RR = *C. sinensis* cv. Rohde Red; RR+V = *C. sinensis* cv. Rohde Red + *C. volkameriana*; RB = *C. sinensis* cv. Ruby Blood; RB+V = *C. sinensis* cv. Ruby Blood + *C. volkameriana*).

was parallel to the guard cells for diploid parental plants and the respective somatic hybrids. Therefore, this arrangement did not allow any differentiation between the plants.

Size and frequency of stomata are affected by the ploidy level of the plant (Cutter, 1986). In polyploids, the stomata are larger and less frequent, showing a high negative correlation between these parameters. Stomatal analysis has been previously done to analyze plants with different ploidy levels with similar results, in tobacco and *Antirrhinum* sp. (Suzuki et al., 1981). Stomata were also evaluated in *Passiflora* spp. somatic hybrids as a parameter for confirmation of the tetraploid character. The methods used required the removal of the epidermis as part of the sample preparation (Dornelas, 1995; Barbosa, 1998).

The number and disposition of the stomata depends on the vegetable species (Salisbury & Ross, 1994). Differences in number, distribution, size and structure of the stomata in leaves of different species can lead the loss of water to an intensity a lot variable (Sutcliffe, 1980). Besides, those differences are also attributable to the structure of the leaf, composition of the cuticle, internal arrangement of the cell, space and the location of the vascular system.

This stomatal analysis of citrus somatic hybrids, shows that ploidy level can interfere with the number and size of stomata.

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