http://journal.unoeste.br/index.php/ca/index
DOI: 10.5747/ca.2024.v20.a537
ISSN on-line 1809-8215

Colloquium Agrariae

Submetido: 24/05/2024 Revisado: 21/10/2024 Aceito: 12/11/2024

GENETIC STABILITY BY FLOW CYTOMETRY AND STEM ANATOMY CHARACTERIZATION OF CITRIC PLANTS IN THE GRAFTING PROCESS

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Abstract

The objective of this study was to evaluate changes in the DNA content as well as in the stem anatomy of grafted citrus cultivars. In the first experiment, the DNA content of plants formed by oranges ‘Navelina’, ‘Lane Late’ and Navelate and mandarins ‘Clemenules’, ‘Ortanique’ and Okitsu’ grafted in rootstocks of citrandarins ‘Índio’ and ‘San Diego’, mandarin ‘Sunki Tropical’, rangpur lime ‘Santa Cruz’ and citrumelo ‘Swingle’ were evaluated. Leaves and roots of each cultivar in all possible scion-rootstock combinations were used, totaling 30 treatments. DNA contents were assessed by flow cytometry. Significant statistical differences were found in the DNA contents of scion leaves, which varied according to the rootstock used. In the second experiment, cross and longitudinal sections of stem were performed in the grafting region. Cultivars ‘Navelina’ and ‘Clemenules’ were evaluated in a factorial arrangement with four rootstocks, in a completely randomized design. The height of rays and vessel diameter were measured. Larger diameters of xylem vessels in the scions were observed for ‘Clemenules’ in almost all rootstocks tested. For height of rays, the results were quite variable in all combinations scion/rootstock studied, with formation and proliferation of disorganized cells in the connection of vascular elements between scion and rootstock. This study is important as it contributes to the understanding of the interactions between scions and rootstocks in citrus, revealing how these combinations can influence both DNA content and wood anatomy, providing valuable information for the selection of combinations that optimize plant development and productivity.

Keywords: wood anatomy; *Citrus* spp.; DNA.

ESTABILIDADE GENÉTICA DE PLANTAS CÍTRICAS POR MEIO DE CITOMETRIA DE FLUXO E CARACTERIZAÇÃO ANATÔMICA NO PROCESSO DE ENXERTIA

Resumo

O objetivo deste estudo foi avaliar alterações no conteúdo de DNA, bem como na anatomia da madeira de variedades cítricas de copa e porta-enxertos na idade de enxertia. Dois experimentos foram realizados com plantas enxertadas após 12 meses da enxertia. No primeiro experimento, foi avaliado o conteúdo de DNA de plantas formadas por cultivares copa de laranjeiras 'Navelina', 'Lane Late' e 'Navelate' e tangerineiras 'Clemenules', 'Ortanique' e 'Okitsu' enxertadas em porta-enxertos de citrandarins 'Índio' e 'San Diego', tangerineira 'Sunki Tropical', limoeiro 'Cravo Santa Cruz' e citrumeleiro 'Swingle'. Foram utilizadas folhas e raízes de cada uma das combinações copa/porta-enxerto possíveis, totalizando 30 tratamentos, cujos teores de DNA foram determinados por citometria de fluxo. Nessa avaliação, foram encontradas diferenças estatísticas significativas nos teores de DNA das folhas da copa, que variaram de acordo com o porta-enxerto utilizado. No segundo experimento, foi realizado um estudo anatômico do caule das plantas. Cortes transversais e longitudinais do caule foram realizados na região da enxertia. Para este ensaio, as cultivares copa 'Navelina' e 'Clemenules' foram avaliadas em esquema fatorial com quatro porta-enxertos, em delineamento inteiramente casualizado. A altura dos raios e o diâmetro dos vasos foram medidos. Os resultados dos cortes histológicos mostraram que maiores diâmetros de vasos xilemáticos das copas foram observados para a cultivar 'Clemenules' em quase todos os porta-enxertos testados. Para altura de raios, os resultados foram bem variáveis em todas as combinações copa/porta-enxerto estudadas, com formação e proliferação de células desorganizadas (calos) na ligação dos elementos vasculares entre copa e porta-enxerto. Este estudo é importante pois contribui para o entendimento das interações entre copas e porta-enxertos em citros, revelando como essas combinações podem influenciar tanto o conteúdo de DNA quanto a anatomia da madeira, fornecendo informações valiosas para a seleção de combinações que otimizem o desenvolvimento e a produtividade das plantas.

Palavras-chave: anatomia da madeira; *Citrus* spp.; DNA.

Introduction

Grafting is a common practice in citrus cultivation, traditionally used to combine the favorable traits of two genetically distinct individuals, creating a symbiotic relationship between rootstock and scion (Carvalho *et al*., 2019; Marques *et al*., 2022). While grafting has been widely studied, existing methods often focus on physiological and agronomic aspects, with limited exploration of the underlying genetic interactions and anatomical changes that occur during the grafting process.

Previous studies have analyzed nutrient uptake, fruit production, and resistance to diseases in grafted citrus plants (Sun *et al*., 2019; Carvalho *et al*., 2021). However, the impact of grafting on the genetic material and tissue compatibility between rootstock and scion remains an area requiring further investigation. This study addresses these gaps by introducing an enhanced approach that integrates advanced genetic analysis, such as DNA content evaluation through flow cytometry, and detailed anatomical assessments of graft unions. Building on the foundations of previous research, this study improves existing methods by focusing not only on traditional traits but also on the genetic and anatomical factors that may influence the long-term success of grafted plants.

The Brazilian citriculture is based on few rootstocks, especially the Rangpur lime (*Citrus limonia* Osbeck). This fact contributes to the vulnerability of plantings to pests and especially to diseases, such as the sudden death of citrus, limiting also fruit production in harvest periods (Oliveira *et al*., 2024).

Although the diversification of genetic material is considered necessary and encouraged, information on other rootstocks that allow their selection is scarce. Among the materials available for diversification are the Swingle citrumelo (*Citrus paradisi* x *Poncirus trifoliata*), a hybrid that shows resistance to CTV (citrus tristeza virus), gum disease, nematodes and cold. The Tropical Sunki mandarin (*C. sunki* (Hayata) hort. Ex. Tanaka) gives to the scion the precocity of production and late ripening of fruits. The Santa Cruz Rangpur lime (*C. limonia* Osbeck) stands out for the rusticity, adaptation to different climatic and soil conditions, and tolerance to the common strains of the Citrus Tristeza Virus complex (Carvalho *et al*., 2022). San Diego and Indio citrandarins (*C. sunki* hort ex Tanaka x *P. trifoliata*) are hybrids that have shown competitive capacity in interact to traditional rootstocks, adding advantages such as lower susceptibility to some diseases affecting citrus plants such as decline, CTV, nematodes and gum disease (Vitória *et al*., 2024).

In the union of the rootstock and scion, some physiological disorders may occur and they are characterized mainly by the rejection between tissues that compromise the welding process of the grafted parts as a result of their incompatibility (Fadel *et al*., 2019). The perfect union of scion and rootstock is necessary to ensure the transport of nutrients and photoassimilates (Rodrigues *et al*., 2023). This process is complex and involves callus formation from rootstock and scion tissues. During this process, parenchymal cells, which are potentially meristematic, multiply and retain their ability to divide even after differentiation, playing a critical role in the healing process or regeneration of lesions (Carvalho *et al*., 2019).

An anatomical evaluation in the junction region of these vessels may reveal differences between scion and rootstock as well as the indicatives of incompatibility (Hussain *et al*., 2017).

Genetic changes caused by the grafting process have been the subject of questioning for some decades. Stegemann and Bock (2009) verified the exchange of genetic information through DNA fragments in the process of grafting tobacco. Yang *et al*. (2008) identified messenger RNA exchange between rootstock and scion in grapevine grafting. Therefore, flow cytometry may be an alternative to evaluate this transfer by observing whether the rootstock exerts influence on the amount of the scion DNA and whether the scion influences the rootstock DNA.

In the search for information that can support the diversification process in Brazilian citriculture, the objective of this study was to evaluate changes in the DNA content as well as in the scion wood anatomy of citrus scion and rootstocks varieties in the grafting age.

Material and Methods

The plants used in both experiments were grafted, the bud donor plants (scions) were obtained from EPAMIG (Empresa Brasileira de Pesquisa Agropecuária do Estado de Minas Gerais) and the rootstock seeds were supplied by the Germplasm Bank of EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) Mandioca e Fruticultura. These plants were maintained in black polyethylene bags filled with commercial substrate Tropstrato®, with frequent foliar fertilization and irrigation inside the greenhouse. Combinations of orange trees and mandarins previously grafted on different rootstocks were studied, as shown in Table 1.

Table 1. Description of scion and rootstock cultivars and the combinations used in grafting.

Orange scions		**Combinations**	**Mandarin scions**	
Rootstock	**Scion**		**Scion**	**Rootstock**
'Santa Cruz' rangpur lime	'Navelina'	1	'Clemenules'	'Santa Cruz' rangpur lime
	'Navelate'	2	'Okitsu'	
	'Lane Late'	3	'Ortanique'	
'Sunki Tropical' mandarin	'Navelina'	4	'Clemenules'	'Sunki Tropical' mandarin
	'Navelate'	5	'Okitsu'	
	'Lane Late'	6	'Ortanique'	
'Índio' citrandarin	'Navelina'	7	'Clemenules'	'Índio' citrandarin
	'Navelate'	8	'Okitsu'	
	'Lane Late'	9	'Ortanique'	
'San Diego' citrandarin	'Navelina'	10	'Clemenules'	'San Diego' citrandarin
	'Navelate'	11	'Okitsu'	
	'Lane Late'	12	'Ortanique'	
'Swingle' citrumelo	'Navelina'	13	'Clemenules'	'Swingle' citrumelo
	'Navelate'	14	'Okitsu'	
	'Lane Late'	15	'Ortanique'	

For this analysis, all combinations of scion-rootstock of orange trees (15) and mandarins (15) were studied.

For the determination of the DNA content, three leaves from the scions and three actively growing root tips from the rootstocks were collected in three different plants. The samples of the experimental plant combination and those from the internal reference plant (*Raphanus sativus*) were co-chopped at the same time with razor blades in 1 mL ice-cold Marie buffer in a glass Petri-dish for the release of the nuclei. The nuclei suspension was aspirated through two layers of gauze using a plastic pipette and filtered through a 50 μm mesh. The nuclei were stained by the addition of 25 μL of propidium iodide. For each sample, 10,000 nuclei were analyzed using a logarithmic scale.

The analysis was performed on the Facscalibur (Becton Dickinson) cytometer, and the histograms were obtained using Cell Quest software and statistically analyzed using the WinMDI 2.8 software. Nuclear DNA content (pg) of the plants was estimated using the ratio between the fluorescence intensities of the G1 nuclei (nuclei in the G1 phase of the interphase) of the internal reference standard and the G1 nuclei of the experimental plant, multiplying this ratio by the amount of DNA of the reference standard (1.11 pg).

This experiment was performed in a completely randomized design, using a factorial arrangement, with 30 combinations and three replicates (leaves and roots).

These analyzes were conducted on the ‘Navelina’ orange and ‘Clemenules’ mandarin cultivars grafted on four rootstocks (‘Santa Cruz’ Rangpur lime, ‘Tropical Sunki’ mandarin, ‘Índio’ and ‘San Diego’ citrandarins). The experiment was carried out in a completely randomized design, in a 2 x 4 factorial arrangement.

The anatomical and histological analyses of the plants, as well as the measurements of rays and vessels were carried out in the Laboratory of Anatomy of the Wood of the Department of Forestry Sciences of the Federal University of Lavras. The wood of the citrus seedlings from these combinations were cut in three positions of the stem: scion, grafting point and rootstock. The cross sections were made in the three regions mentioned previously and longitudinal cuts only in the region of grafting to observe the characteristics related to the junction of the tissues of the two plants.

The specimens cut from the stems of the citrus seedlings were placed in synthetic resin (Entellan®) according to the commonly used plant microtechnology. After formation of the blocks, the material was sectioned transversely and longitudinally in a rotating microtome to obtain sections of thin thicknesses around 10-20 μm. After sectioning, the material was immersed in the following order: sodium hypochlorite; 20% ethyl alcohol; safranin; 50% ethyl alcohol; 70% ethyl alcohol; ethyl alcohol 80%; 100% ethyl alcohol; alcohol acetate 3:1; alcohol acetate 1:1; alcohol acetate 1:3; acetate 100%.

Sections were mounted on permanent glass slides and observed on Olympus® CX 31 binocular microscope with Olympus U-CMAD3 coupled camera. Images were captured using Bel View software. Measurements were performed in WinCell software.

In the cross sections of the wood, unbroken contiguous rays, in the direction of the bark, the diameter of the scion and rootstock vessels and height of the rays were measured in the longitudinal sections for each treatment. The measurements and characterization of the photomicrographs of woody materials were performed according to the IAWA Committee 1989. From the anatomical values obtained, the vessel diameter was calculated as the average of the radial and tangential diameters, thus obtaining the meaning of the vessel diameter of the scions and rootstocks of the treatments.

For both flow cytometry and anatomical assays, the obtained data were evaluated by the analysis of variance ($p \leq 0.05$) using the SISVAR (Ferreira, 2019). When significant differences were detected, the means were grouped by the Scott-Knott test ($p \leq 0.05$).

Results and discussion

In the first experiment, the flow cytometry analyses of apyrenic scions, from both orange and mandarin cultivars, showed that only the scion DNA contents were significant (Tables 2 and 3). The analysis of orange scion cultivars within the same rootstock revealed no significant differences in DNA content for the three scions grafted onto ‘Tropical Sunki’ or ‘Índio’ cultivars (Table 2). However, ‘Navelate’ grafted onto ‘Santa Cruz’ Rangpur lime exhibited lower DNA content compared to ‘Navelina’ and ‘Lane Late’ oranges. The same pattern was observed with ‘Navelina’ and ‘Navelate’ cultivars grafted onto ‘San Diego’, as well as ‘Navelina’ onto Swingle citrumelo. Therefore, scion/rootstock combinations that showed altered DNA content may lack genetic stability and could be exchanging genetic material.

Table 2. DNA content, in picograms (pg), in leaves of orange scion cultivars grafted in several rootstocks.

Rootstocks	DNA content of oranges		
	Navelina	**Navelate**	**Lane Late**
‘Santa Cruz’ rangpur lime	4,43 a A	3,96 b C	4,28 a B
‘Tropical Sunki’ mandarin	3,92 a B	4,02 a C	3,73 a C
‘Índio’ citrandarin	4,37 a A	4,34 a B	4,15 a B
‘San Diego’ citrandarin	4,36 b A	4,25 b B	4,63 a A
‘Swingle’ citrumelo	4,18 b B	4,61 a A	4,67 a A
CV (%)		4,06	

Means followed by the same lowercase letter in the line and upper case in the column belong to the same group by the Skott-Knott test ($p \leq 0.05$).

In the scion cultivars within each rootstock, all treatments exhibited significant differences. The ‘Navelina’ cultivar exhibited higher DNA content when grafted on ‘Santa Cruz’ Rangpur lime, ‘Índio’, and ‘San Diego’ citrandarins, and lower content on ‘Tropical Sunki’ mandarin and ‘Swingle’ citrumelo. For ‘Navelate’, the highest DNA content was observed on ‘Swingle’ citrumelo, followed by ‘San Diego’ and ‘Índio’. ‘Lane Late’ grafted on ‘Swingle’ citrumelo and

'San Diego' citrandarin exhibited higher DNA content, followed by 'Índio' and 'Santa Cruz' Rangpur lime.

In relation to mandarins, in the comparison of the DNA content of scions within each rootstock, we observed that 'Clemenules' grafted on 'Santa Cruz' Rangpur lime exhibited a higher DNA content (Table 3). The same was observed for 'Ortanique' and 'Clemenules' grafted on 'Tropical Sunki' mandarin and 'Clemenules' on 'San Diego' citrandarin. There were no significant differences in the DNA content of the mandarins grafted on Indio citrandarin and 'Swingle' citrumelo.

Table 3. DNA content in picograms (pg) in leaves of mandarin scion cultivars grafted in several rootstocks.

Rootstocks	**DNA content of mandarins**		
	Ortanique	**Okitsu**	**Clemenules**
'Santa Cruz' rangpur lime	3,70 b A	3,66 b A	4,03 a A
'Tropical Sunki' mandarin	3,69 a A	3,30 b B	3,95 a A
'Índio' citrandarin	3,96 a A	4,00 a A	3,78 a A
'San Diego' citrandarin	3,83 b A	3,56 b B	4,13 a A
'Swingle' citrumelo	3,97 a A	3,80 a A	3,97 a A
CV (%)		5,08	

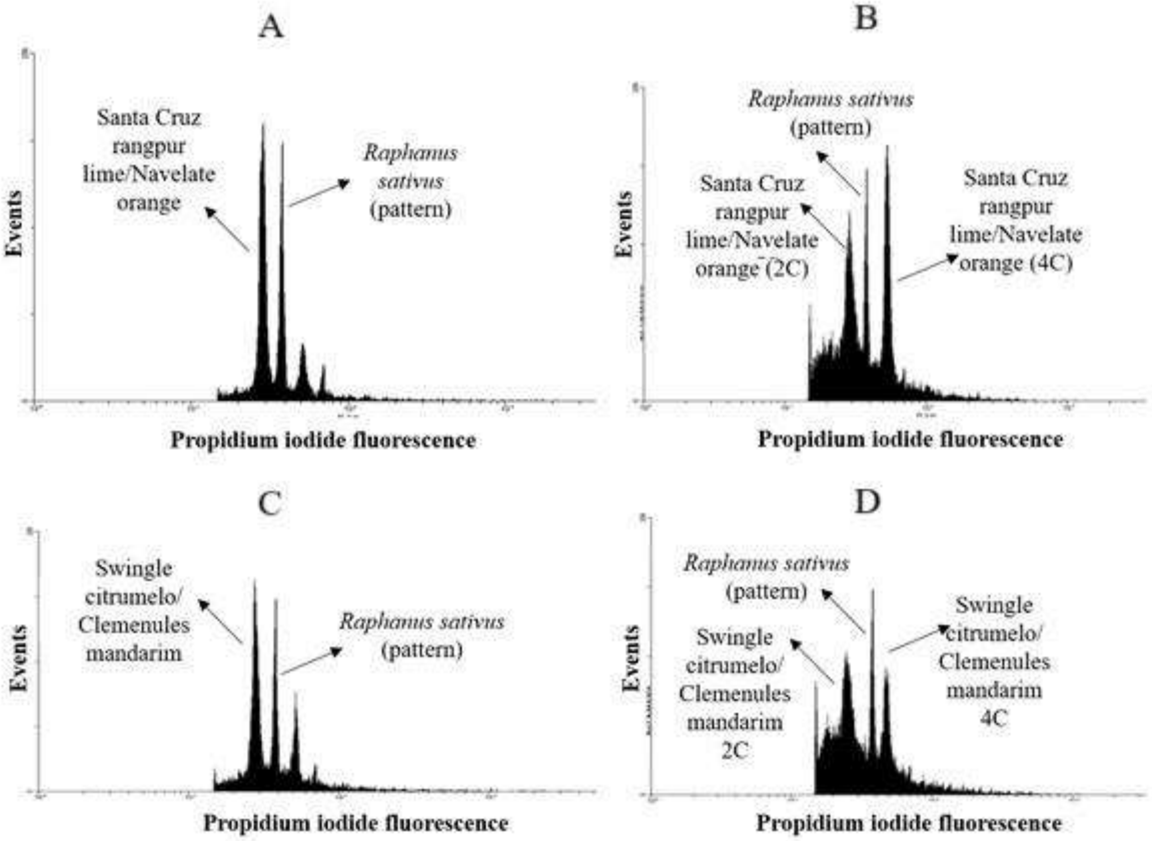
Means followed by the same lowercase letter in the line and upper case in the column belong to the same group by the Skott-Knott test ($p \leq 0.05$).

However, when the rootstock was considered within each scion, significant differences were observed only for 'Okitsu' mandarin, which showed higher DNA content when it was grafted on 'Santa Cruz' Rangpur lime, 'Índio' citrandarin and 'Swingle' citrumelo.

When the DNA content was analyzed in the roots of the grafted plants, there was no significant difference, for any treatment, of orange or mandarin. Based on the analysis of flow cytometry, it is possible to infer that the DNA content of the scions varies according to the rootstock used. However, the rootstocks did not show detectable changes in the DNA content as a function of the scions.

Moreover, for all analyzed rootstocks, we observed two DNA contents with two distinct ploidies in the tissues. The first ploidy value is possibly 2C, represented by the first peak in the histogram, and the second is a 4C (third peak). This phenomenon is known as endoreduplication or polysomaty (Figure 1).

Figure 1. Histograms of flow cytometry of the combination of 'Santa Cruz' Rangpur lime/'Navelate' orange, being analyzed the leaves of 'Navelate' (A) and the roots of 'Santa Cruz' Rangpur lime (B). The combination 'Swingle' citrumelo/'Clemenules' mandarin also was verified, being analyzed the leaves of 'Clemenules' (C) and the roots of 'Swingle' citrumelo (D). The internal reference standard was radish (*Raphanus sativus*).



Vilcherrez-Atoche (2022) state that polysomaty refers to the occurrence of different levels of ploidy in the same organ or tissue and is estimated to occur in more than 90% of angiosperms. Thus, the presence of somatic cells with different ploidies in the same organ is a result of the process of endoreduplication, i.e., the transition of nuclei by repeated cycles of DNA replication without cell division (Hittorf *et al*., 2023).

The endoreduplication observed in the rootstocks can be advantageous, since it can contribute to the adaptation to varied environmental factors, as reported by Moreira et al. (2021). It also can support the nuclear DNA against irradiation or even prevent the uneven chromosome segregation during mitosis, contributing to the plant adaptation to high salt concentrations (Paige, 2018), water deficit (Tian *et al*., 2019) and heat stress (Tossi *et al*., 2022).

Citrus rootstocks influence various plant attributes, such as bioactive compounds (Ordóñez-Díaz *et al*., 2020), mineral content (Sáenz-Pérez *et al*., 2020) and metabolite composition (Albrecht *et al*., 2019). However, in the present study, flow cytometry results suggest that this interference also occurs in the DNA content.

Until recently, it was believed that in the grafting process, the genotypes maintained their genetic integrity and their DNAs did not mix. However, Stegemann and Bock (2009) proved otherwise. The researchers grafted transgenic tobacco plants from two strains that carry different marker genes and reporters in two cell compartments, the nucleus and the plastid (Stegemann; Bock, 2009). With this assay, they were able to demonstrate that in the process of plant grafting there is an exchange of genetic information through large pieces of DNA or entire plastid genomes. In the present study, several changes in DNA content were observed from several scion/rootstock combinations, inferring that this genetic exchange may be happening. This observation has very important implications for grafting techniques and provides a possible pathway for horizontal gene transfer. This would provide a direct asexual mechanism to generate variability.

Studies have demonstrated that whole chloroplast genomes can be transferred horizontally through the scion/rootstock joint, potentially explaining the so-called ‘organelle catch events’ that occurred in evolution (Stegemann *et al*., 2012; Wang *et al*., 2024). In fact, microscopic evidence supports the idea that nuclear material may occasionally move from cell to cell in plant tissues, a phenomenon known as cytomixia (Mursalimov *et al*., 2013).

In more in-depth studies, Fuentes *et al*. (2014) have shown that in the grafting process every nuclear genome can be transferred between plant cells. The authors provided direct evidence for this process, which resulted in the emergence of a new genotype.

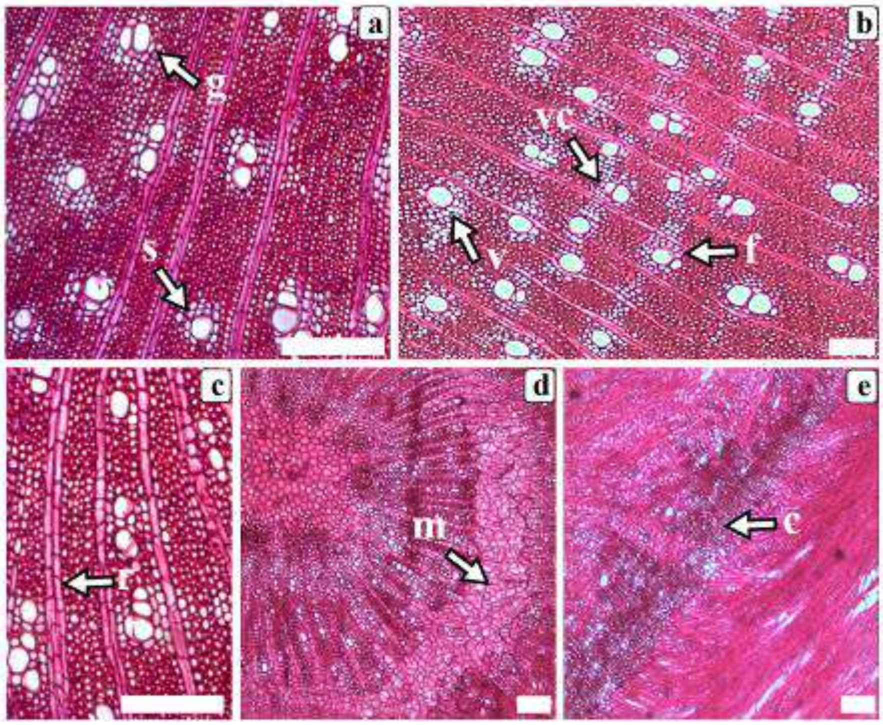
Flow cytometry analysis was performed by Fuentes *et al*. (2014), aiming to evaluate the gain or loss of DNA in grafted plants of two species of tobacco. DNA losses were observed in some plants suggesting that somatic genome instability exists, but movement of whole nuclear genomes from cell to cell and through graft and rootstock junction was also observed. According to the authors, this transfer was possible by the fusion of neighboring cells at the site of the graft, or by the migration of nuclei from cell to cell through plasmodesmas in a process similar to cytomixia, as observed in microscopic studies (Mursalimov *et al*., 2013).

In the present study, this movement of DNA seems to be quite complex, because there is the mechanism of endoreduplication happening in the rootstock cells. Therefore, the fact that the tissues of the grafts have two distinct levels of ploidy and the occurrence of repeated cycles of DNA replication may be a reference that this transfer of DNA from the rootstock to the scion is potentiated. Endoreduplication may also explain why the difference in DNA of the rootstock was not detected. Due to the difference in ploidy in the tissues, the exit or entry of small DNA content can not be detected by flow cytometry in the rootstock, but in the scion it can.

Thus, in the present study, flow cytometry analysis detected differences in the DNA content of some scion/rootstock combinations in citrus plants. These differences were detectable only in the orange and mandarin scions because the phenomenon of endoreduplication occurring in the rootstock masked this evidence.

In the second experiment, we found that all the citric combinations studied showed numerous xylematic vessels, with uniform diffuse porosity, and oval and circular shape; mostly of the solitary type with presence of some geminate ones (Figure 2).

Figure 2. Photomicrographs of stem sections in the grafting region of different citrus combinations. Cross sections of the stem revealing: a) solitary vessels (s) and geminate (g); b) patterns of occurrence of the parenchyma, being: vasicentric (v), confluent vasicentric (vc) and in bands (f); c) rays (r); d) macules (m); e) longitudinal section showing callus (c) formed at scion tissue junction sites (right side) and rootstock (left side). Bars = 100 µm.



In the analysis of the xylem vessel diameters, significant interaction was observed for both scion and rootstock (Table 4). In general, the diameter of the scion vessels showed a tendency of superiority in relation to the means of the rootstock in all the combinations. We observed that at the union line between the parts, changes occurred in the width and number of vessels, as well as in their ability to conduct water. The highest value occurred with the ‘Navelina’ orange on the ‘Índio’ citrandarin.

The qualitative anatomical description of the citrus scion/rootstock combinations may allow inferences about the efficiency of grafting in the various combinations as well as in the transport of water, minerals and photo assimilates. This may indicate possible anatomical adaptations contributing to graft compatibility, which depend on the specific citrus rootstocks used.

Table 4. Mean diameter (μm) of the scion and rootstock xylem vessels obtained in the cross section of the Navelina and Clemenules scion cultivars grafted on the 'San Diego' citrandarin, 'Santa Cruz' Rangpur lime, 'Índio' citrandarin and 'Tropical Sunki' mandarin rootstocks, after 16 months of grafting.

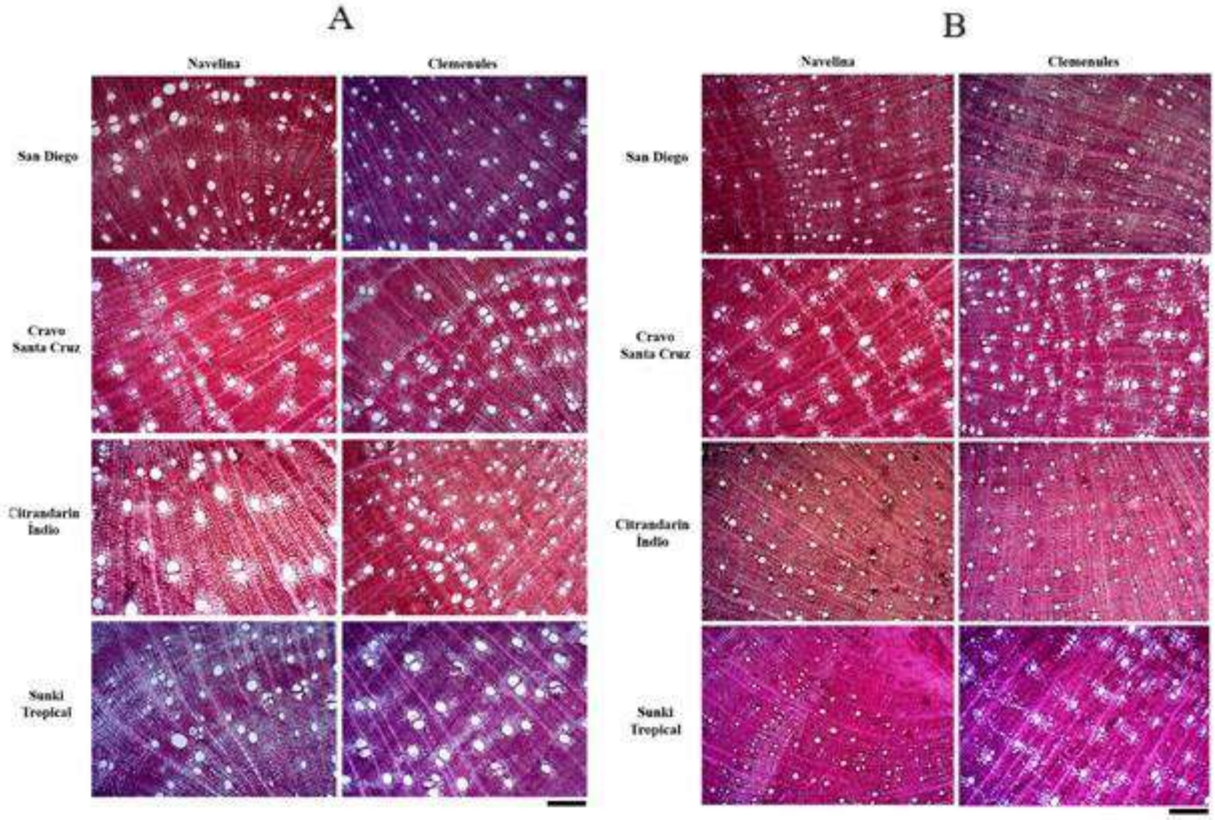
Treatments	**Diameter of the scion vessels on the different rootstocks**		**Diameter of the rootstocks vessels on the different scions**	
	Navelina	**Clemenules**	**Navelina**	**Clemenules**
'San Diego' citrandarin	38,74 aB	38,09 aB	31,82 bB	36,32 aA
'Santa Cruz' rangpur lime	35,66 bB	42,44 aA	37,58 aA	37,72 aA
'Índio' citrandarin	48,64 aA	40,71 bA	33,10 aB	30,67 aB
'Tropical Sunki' mandarin	37,88 bB	42,68 aA	34,34 aB	30,33 bB
CV (%)	17,24		19,99	

Means followed by the same lowercase letter in the line and upper case in the column belong to the same group by the Skott-Knott test ($p \leq 0.05$).

The study of xylem vessels, also known as vessel elements, is important for knowledge of the water and mineral salts conduction system, especially in the case of grafted plants, since this characteristic contributes to the success of the grafting (Camboué *et al*., 2024). In addition, this factor is important for studies of graft incompatibility, since the choice of scions and rootstocks with distinct vessel elements in diameter may lead to overgrowth or dwarfism in the plants formed (Rasool *et al*., 2020). For the vessels, there was an increase in the diameter in the medulla-bark direction in the cross sections of the scion and rootstock, and at the point of grafting in the citrus combinations. Fan *et al*. (2009) also observed an increase in the diameter of the vessels in the same direction in Fagaceae, so they suggest that these variations reflect the maturation process of the vascular cambium, and in the case of grafting, the success rates.

In the region of the grafting, the photomicrographs presented in Figure 3 showed the presence of some parenchyma types (vasicentric, in bands, and vasicentric paratracheal congfluent). These tissues perform the function of storing and translocating water and solutes at close range. The cells are elongated vertically with thin walls with paratracheal parenchyma associated with vessel elements; they have the vasicentric pattern when it forms a complete sheath around the vessels and confluent when the vasicentric parenchyma of two or more contiguous vessels unite to form irregular bands. Also in this cross section of the secondary xylem, axial parenchyma is observed in bands that are associated with the vessels, forming continuous bands.

Figure 3. Cross section of scion stem (A) and rootstock stem (B): abundant fibers (dark red part), diffuse, solitary, twin and multiple radial vessels (light and oval parts), and axial parenchyma bands associated with vessels (vasocentric parenchyma, vasocentric confluent, and in bands). Medullary rays in bands. Bar = 100 μm.



Rays were observed in both cross and longitudinal sections, with uniseriate rays (a single row of cells) and multiseriate rays (two or more rows of cells), as shown in Figures 4b and 4c, respectively.

Figure 4. Photomicrographs of longitudinal sections in the grafting region of different citrus combinations. a) Ray distribution pattern; b) Unisseriate ray; c) Multisseriate ray. Bars = 100 µm (a) and 25 µm (b and c).

There was a significant effect on the scion x rootstock interaction for the height of rays, whose mean values are presented in Table 5. The highest values were observed in the combination of 'Clemenules' mandarin/'Santa Cruz' Rangpur lime (241.18 µm) and 'Navelina' orange/'Santa Cruz' Rangpur lime (198.66 µm). San Diego citrandarin and 'Tropical Sunki' mandarin rootstocks did not change the height of rays independent of the scion cultivar used.

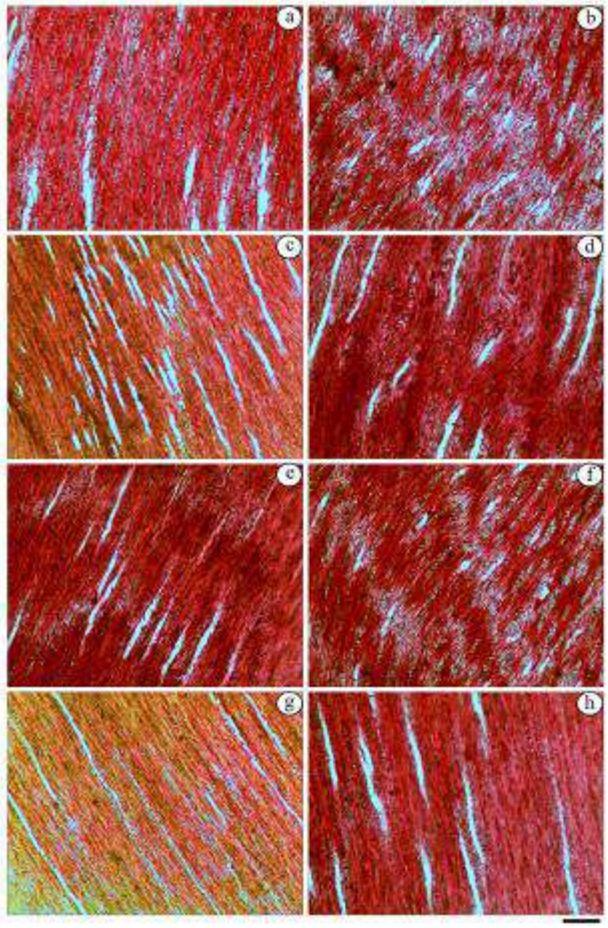
Table 5. Mean height of rays (µm) of the scion and rootstock obtained in the cross section in the different scion-rootstock combinations, after 16 months of grafting.

Treatments	Height of rays in scions		Height of rays in rootstocks	
	Navelina	Clemenules	Navelina	Clemenules
'San Diego' citrandarin	180.88 aA	177.13 aB	147.82 bB	195.11 aA
'Santa Cruz' rangpur lime	198.66 bA	241.18 aA	225.59 aA	165.70 bB
'Índio' citrandarin	179.33 aA	145.14 bC	166.49 bB	189.87 aA
'Tropical Sunki' mandarin	181.59 aA	183.60 aB	233.52 aA	154.16 bB
CV (%)	26.42		20.95	

Means followed by the same lowercase letter in the line and upper case in the column belong to the same group by the Skott-Knott test ($p\leq 0.05$).

The pattern of ray distribution of the scion/rootstock combinations is shown in Figure 5 where the rays are exposed in their longitudinal extent. It is observed that the vessels, which are cells in vertical coalescing series, form a tubiform structure of indeterminate length. Each cell that makes up the vessel is called a vessel element, and it is white; the fibers are identified with darker red coloration and the multiseriate, bisseria and uniseriate rays are distributed in the section. The presence of marrow maculae was observed in the scion stem, at the point of grafting, and in the rootstocks they were observed in the cross sections (Figure 5d). The macules are structures formed by irregularly shaped parenchyma cells with lignified walls and they are originated in response to injury to the vascular cambium, caused by biotic or abiotic damages.

Figure 5. Tangential longitudinal section showing multiseriate, bisseriate and uniseriate rays of citrus scion/rootstock combinations. a) rays of the scion of ‘Navelina’/ ‘San Diego’ combination; b) rays of the scion of ‘Clemenules’/ ‘Santa Cruz’ combination; c) rays of the scion of ‘Clemenules’/ ‘Índio’ combination; d) rays of the scion of ‘Clemenules’/ ‘San Diego’ combination; e) rays of the rootstock of ‘Navelina’/ ‘San Diego’ combination; f) rays of the rootstock of ‘Clemenules’/ ‘Santa Cruz’ combination; g) rays of the rootstock of ‘Clemenules’/ ‘Índio’ combination; h) rays of the rootstock of ‘Clemenules’/ ‘San Diego’ combination. Bar = 200 µm.



The ability to regenerate damaged parts through the multiplication of parenchyma cells in various tissues leads to the formation of attachment tissue, i.e., the healing by the union of scion and rootstock, a species-specific function. In woody plants the rays are the main responsible for this process; however other components of the cambium zone can contribute of variable form in certain species. Ray cells contribute largely to the formation of macules (Negi; Upadhyay, 2023).

It was observed the contact of the rootstock and scion cambial tissues, identifying the cambium zones with the highest possible interconnection (Figure 5e). The cells of the cambium of the two plants produced parenchyma cells forming the callus, i.e., the welding, and possibly the cells differentiated forming new cambium cells, which produced new vascular tissues, without which there is no success on the grafting (Fayek *et al*., 2022).

Although there is still clear structural disorganization, it is possible to verify the anatomical and histological details of the grafting point where there are indications of union between the rootstock and the scion (Figure 5e). This is probably due to the tissue union aspects in the grafting region, with multiplication of parenchyma cells, which establish the initial contact of the involved parts (Fayek *et al*., 2022). The grafting process takes place in a few days, and, at this early stage, there is formation of parenchyma cells at the interface of the scion, which fill the cleft at the point of grafting, constituting a callus, which associates the scion and rootstock, with subsequent contact between the involved parts.

The results obtained in this study can be used to develop innovative approaches in the management of scion/rootstock combinations in citrus, promoting sustainable practices that benefit both agricultural productivity and the conservation of natural resources, thus making a significant contribution to society.

Conclusions

The DNA content of orange and mandarin scion cultivars varied according to the rootstock used, as detected by flow cytometry. Differences were only evident in scions grafted onto rootstocks without endoreduplication activity, such as ‘Índio’ citrandarin and ‘Santa Cruz’ Rangpur lime.

All scion/rootstock combinations studied, including ‘Navelina’ orange on ‘Índio’ citrandarin and ‘Clemenules’ mandarin on ‘Santa Cruz’ Rangpur lime, exhibited numerous xylem vessels with diffuse porosity and solitary or geminate vessel patterns. The diameter of the scion vessels was generally larger compared to the rootstock, influencing water conductivity and graft efficiency.

After one year of grafting, histological analysis revealed disorganized cell proliferation (callus) in the graft union across all combinations. This was accompanied by the connection of vascular elements between scion and rootstock, crucial for the success of the grafting process.

Notably, the 'Navelina' orange/'Índio' citrandarin combination showed the highest degree of vascular integration.

The anatomical features observed in the scion/rootstock combinations, particularly the formation of parenchyma and ray cells, suggest potential adaptations that contribute to graft compatibility. The 'Clemenules' mandarin/'Santa Cruz' Rangpur lime combination, for example, displayed the highest ray height, indicating a strong potential for efficient nutrient and water transport between the grafted parts.

Acknowledgements

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Apoio à Pesquisa e a Inovação Tecnológica do Estado de Minas Gerais (FAPEMIG), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

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