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DNA index and anatomical aspects of the micrografting of dragon fruit on different rootstocks

Abstract – The objective of this work was to evaluate the viability of the micrografting of yellow dragon fruit (*Selenicereus megalanthus*) on different rootstocks, based on DNA content and anatomical analyses. The used rootstocks were: yellow dragon fruit, white dragon fruit (*Hylocereus undatus*), Saborosa (*Selenicereus setaceus*) dragon fruit, and the Cebra and Orejona red dragon fruit (*Hylocereus polyrhizus*) varieties. The experimental design was completely randomized with five treatments and four replicates of five plants. After 30 days of cultivation, the following traits were evaluated: length and diameter of the micrografts and microrootstocks; and root length, percentage of setting, and fresh mass of the micrografts. Flow cytometry analyzes were performed before and after micrografting to verify genetic stability and the occurrence of endoreduplication. In addition, histological sections were made in the micrografting region to verify the connections of vessels and tissues between the graft and the rootstock. Endoreduplication was observed in all treatments. The amount of DNA in the yellow dragon fruit micrograft increased on the red Orejona variety. The presence of vessel connections was verified between the micrografts and microrootstocks. The yellow dragon fruit was also more vigorous when grafted on Orejona. Based on DNA content and anatomical analyses, in vitro yellow dragon fruit micrografting is feasible in all used rootstocks.

Index terms: *Hylocereus polyrhizus*, *Hylocereus undatus*, pitaya, propagation, scion.

Índice de DNA e aspectos anatômicos da microenxertia de pitaia em diferentes porta-enxertos

Resumo – O objetivo deste trabalho foi avaliar a viabilidade da microenxertia de pitaia amarela (*Selenicereus megalanthus*) sobre diferentes microporta-enxertos, com base em análises de conteúdo de DNA e anatômicas. Os porta-enxertos utilizados foram: pitaia amarela, pitaia branca (*Hylocereus undatus*), pitaia Saborosa (*Selenicereus setaceus*), e as variedades Cebra e Orejona de pitaia vermelha (*Hylocereus polyrhizus*). O delineamento experimental foi inteiramente casualizado, com cinco tratamentos e quatro repetições de cinco plantas. Após 30 dias de cultivo, avaliaram-se as seguintes características: comprimento e diâmetro dos microenxertos e dos microporta-enxertos; e comprimento de raízes, percentagem de pegamento e massa fresca dos microenxertos. Realizaram-se análises de citometria de fluxo antes e depois da microenxertia, para verificação da estabilidade genética e da ocorrência de endoreduplicação. Além disso, foram feitos cortes histológicos na região da

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microenxertia, para verificação das conexões dos vasos e dos tecidos entre o enxerto e o porta-enxerto. Observou-se endoreduplicação em todos os tratamentos. A quantidade de DNA do microenxerto de pitaia amarela aumentou sobre a variedade vermelha Orejona. Verificou-se a presença de conexões de vasos entre os microenxertos e os microporta-enxertos. A pitaia amarela também foi mais vigorosa quando enxertada sobre Orejona. Com base nas análises de conteúdo de DNA e anatômicas, a microenxertia de pitaia amarela in vitro é viável em todos os porta-enxertos utilizados.

Termos para indexação: *Hylocereus polyrhizus*, *Hylocereus undatus*, pitaya, propagação, enxerto.

Introduction

Dragon fruit belongs to the Cactaceae family and originates from subtropical and tropical America (Marques et al., 2011). Due to its rusticity, exoticism, early production, and high economic return, dragon fruit has sparked the interest of producers worldwide and, consequently, in research on its production (Ibrahim et al., 2018). In the exotic fruit market, dragon fruit has become prominent because of its organoleptic characteristics (Le Bellec et al., 2006), such as a sweet and smooth flavor (Lima et al., 2013), besides its nutritional and functional properties, which make it promising for cultivation (Magalhães et al., 2019).

The term dragon fruit refers to species whose fruits are covered in scales that resemble those of a dragon (Ibrahim et al., 2018). Among these species, two belong to the genus *Hylocereus*, characterized by large and attractive-colored fruits: *Hylocereus undatus* (Haw.) Britton & Rose, which has fruits with red skin and white pulp (Le Bellec et al., 2006); and *Hylocereus polyrhizus* (Weber) Britton & Rose, which has fruits with red skin and pulp (Jamilah et al., 2011). The species *Selenicereus setaceus* (Salm-Dyck ex DC.) A.Berger ex Werderm., known as Cerrado dragon fruit or Saborosa, has red skin and whitish pulp like *H. undatus*, but smaller-sized fruits, with thorns and a sweeter taste (Junqueira et al., 2002). *Selenicereus megalanthus* [K.Schum ex. Vaupel) Moran], known as yellow dragon fruit, produces smaller fruits with thorns, has yellow skin and white pulp, and is considered more palatable than the other species (Moreira et al., 2012). However, in the field, this latter species suffers the attack of phytopathogens, such as nematodes, and shows cladodes with low vigor, i.e., thinner than those of plants of the genus *Hyloreceus* (Nascimento et al., 2020). Dragon fruit of this genus, besides showing vigorous growth, tolerates humid soils and the *Meloidogyne incognita* (Palacino, 1990) nematode, both important characteristics for a good rootstock. In addition, the Saborosa variety appears to be a potentially viable alternative for cultivation in stony, sandy, and rocky soils due to its rustic character (Junqueira et al., 2010).

As one of the propagation techniques of dragon fruit, grafting (Junqueira et al., 2010) allows the transfer of favorable characteristics, such as tolerance to biotic and abiotic factors, representing an efficient propagation method to remedy the previously cited problems. Moreover, grafting may allow increased vigor, early production, uniformity in cultivation, and increased productivity (Bettiol Neto et al., 2014). Among the grafting techniques, it is worth mentioning micrografting, which can be defined as the scion of an apical bud or stem apex of a matrix plant on a rootstock of a seedling derived from seed germination under aseptic conditions (Pahnekolayi et al., 2019). This technique has great potential for the large-scale multiplication of fruit plants (Hussain et al., 2014) and for the successful acquisition of horticultural plants resistant to soil pathogens, besides being widely used as a means of eliminating pathogens in fruit crops (Moghadam et al., 2012).

However, there may be incompatibility between scion and rootstock, which can be characterized by a poor junction of exchange rate formations or accumulation of starch around the grafting area where there is normal vascular continuity, but phloem degeneration (Aloni et al., 2010). To check the connections of the vessels and tissues between the scion and the rootstock, it is necessary to use techniques of plant anatomy, such as longitudinal histological sections in the grafting/micrografting region.

Flow cytometric analyses are performed to verify the genetic stability of the used materials, as well as the occurrence of tissue endoreduplication. With this technique, it is possible to estimate the nuclear DNA index of plants quickly, efficiently, and reliably, by comparing it with nuclei belonging to a reference standard whose DNA index is previously known (Loureiro et al., 2021). For dragon fruit, pea (*Pisum sativum* L.) is usually used as this standard (Doležel & Bartoš, 2005).

In this context, research related to grafting of dragon fruit in Brazil, which is still incipient, can contribute to the expansion of the cultivated area in the country and to an increase in income and fruit supply for producers, leading to a lower price for the consumer.

The objective of this work was to evaluate the viability of the micrografting of yellow dragon fruit on different rootstocks, based on DNA content and anatomical analyses.

Materials and Methods

The experiment was conducted at the Tissue Culture Laboratory of the Department of Agriculture of Universidade Federal de Lavras. The used seeds were from the following dragon fruit varieties: yellow (*S. megalanthus*), white (*H. undatus*), Saborosa (*S. setaceus*), and red Cebra and Orejona (*H. polyrhizus*), obtained from mature fruits purchased from Companhia de Entrepostos e Armazéns Gerais de São Paulo (São Paulo, SP, Brazil).

The dragon fruit seeds were removed and kept under benches until dry. Subsequently, they were disinfested in 70% alcohol for 1.5 min and with 40% bleach solution for 20 min, while stirred. The plant material was then washed three times with distilled and autoclaved water in a laminar flow chamber, and, afterwards, introduced in vials containing 15 mL of the culture medium. The medium used for seed germination was L2 (Phillips & Collins, 1979), plus 30 g L^{-1} sucrose and 6.0 g L^{-1} agar; pH was adjusted to 5.7±0.1 before autoclaving, which occurred at 121°C for 20 min. After inoculation, the seeds were taken to a growth room, with a light intensity of 25 µmol m^{-2} s^{-1}, temperature of 25±1°C, and photoperiod of 16 hours.

After 90 days in vials with the culture medium, the most vigorous explants were subjected to micrografting. The technique was performed inside a laminar flow chamber using a magnifying glass, tweezers, and scalpels for segment excision. The scions were obtained by excising the 0.5 cm apex of the cladodes of yellow dragon fruit, by cutting the base of the segment horizontally, and the rootstocks were obtained from 1.0 cm segments of the cladodes of white dragon fruit, the Cebra and Orejona red dragon fruit varieties, Saborosa dragon fruit, and yellow dragon fruit, by cutting horizontally the apex and the base of the segment.

The scions were put in contact with the rootstocks and inoculated in test tubes containing the same medium used for germination, plus 2.0 g L^{-1} activated carbon. The micrografted plants were then brought to the growth room, with 25 µmol m^{-2} s^{-1} light intensity, 25±1°C, and a 16-hour photoperiod, where they remained for 30 days until the treatments were evaluated.

The experiment was carried out in a completely randomized design, with five treatments, consisting of the yellow dragon fruit micrografted on all rootstocks, i.e.: on the yellow, white, Cebra red, Orejona red, and Saborosa dragon fruit varieties. The treatments consisted of four replicates of five plants each, totaling 100 plants.

After 30 days of in vitro micrografting cultivation, ten plants of each treatment were evaluated regarding: scion length and diameter, rootstock length and diameter, root length, and plant fresh matter. The length of the scion was measured from the grafting point to the plant apex, and that of the rootstocks, from that same point to the base of the stem. Diameter was determined at 1.0 cm above and 1.0 cm below the grafting point. All root lengths were measured to obtain the mean. All measurements were made using a stainless hardened caliper. Fresh matter was determined using a precision scale of three decimal places. Micrografting percentage was also obtained.

Flow cytometric analyses were performed to verify the genetic stability of the used material, by comparing the DNA contents before and after micrografting, as well as to check for the occurrence of tissue endoreduplication, a phenomenon observed in *H. undatus* but not in the other species (Menezes et al., 2012).

Before micrografting, four seedlings of each established dragon fruit genotype were sampled in vitro. After 30 days of in vitro micrografting cultivation, three plants were evaluated per treatment; for this, the scion was separated from the rootstock using a scalpel and shoots and roots were removed. Approximately 50 mg of dragon fruit cladodes were used, which were titrated in a Petri dish containing 1.0 mL ice-cold Marie buffer for the release of the nuclei. The nucleus suspension was aspirated using a plastic pipette and filtered through a 50 µm mesh. The nuclei were stained by adding 25 µL of 1.0 mg mL^{-1} propidium iodide solution to each sample. The

samples were analyzed immediately after preparation (Galbraith et al., 1983). For each sample, 10,000 nuclei were analyzed using a logarithmic scale. The analysis was performed on the FACScalibur cytometer (BD Biosciences, San Jose, CA, USA), using the CellQuest software to obtain histograms, which were analyzed statistically in the WinMDI 2.8 software. The nuclear DNA index (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 reference standard (pea) and the G1 nuclei of the sample; this ratio was multiplied by the amount of DNA of the reference standard (9.09 pg).

To verify the connections of vessels and tissues between scion and rootstock, after 30 days of in vitro cultivation, longitudinal histological sections were made in the region where the scion was inserted in the rootstock. The evaluated samples were previously fixed in 70% FAA solution, composed of formaldehyde, glacial acetic acid, and 70% ethyl alcohol (Johansen, 1940), for 72 hours, and then conserved in 70% ethanol (v/v1). Subsequently, the samples were dehydrated in an increasing alcohol gradient (80, 90, and 100%) over a 2 hour interval for each concentration. Afterwards, they were infiltrated in historesin for 24 hours and then in pure resin, finally being blocked. The sections, with a thickness of 8.0 µm, were obtained in a semiautomatic microtome and placed on slides stained with 0.05% toluidine blue solution and sealed with stained glass 500 and coverslips. Three replicates were made in each treatment. The slides were observed and photographed under the Olympus BX60 optical microscope (Olympus Optical Ltd., Tokyo, Japan), coupled to a Canon A630 digital camera (Canon Inc., Tokyo, Japan).

After data collection, the analysis of variance and Tukey's test, at 5% probability, were performed using the R statistical software (R Core Team, 2016).

Results and Discussion

A micrografting fixation of 100% was observed. In the growth analysis, only root length differed significantly between varieties (Table 1). Therefore, although the Cebra variety had a larger root system, it did not differ from those of the yellow, white, and Orejona varieties. Saborosa, however, had a shorter root length and, consequently, a more fragile root system, probably due to the fact that it is a native Brazilian species, not yet improved. Its fruits are small in size and have no commercial value; therefore, it is not suitable for use as a scion or as a rootstock. The quality of the root system of a rootstock is an extremely important requirement to ensure an adequate growth and development of any fruitful seedling. The rootstock is responsible for the root system of the newly formed plant, that is, the genetic potential expressed in the root part of the seedling derives from the rootstock; this way, the more vigorous the rootstock, the larger its root system (Zarrouk et al., 2010). In addition, since roots help fixing and obtaining nutrients (Almeida et al., 2016), larger and more numerous roots tend to lead to a better plant nutrition. Dragon fruit, being hemiepiphyte and climbing, absorbs water by its roots anchored in the soil and by its adventitious roots (Marques et al., 2011); these roots also help the plants support fixation and nutrient absorption.

In the cytometry performed before the micrografting process, three levels of ploidy represented by three

Table 1. Length and diameter of scion and rootstock during initial growth analysis, as well as root length and fresh matter of micrografted dragon fruit seedlings[(1)].

Micrografting dragon fruit	Length (cm)		Diameter (mm)		Root length (cm)	Fresh matter (g)
	Scion	Rootstock	Scion	Rootstock		
Yellow (YY)	1.46a	1.31a	2.09a	4.08a	1.45ab	0.18a
White (YW)	1.08a	1.37a	2.41a	4.59a	1.38ab	0.15a
Red Cebra (YC)	1.15a	1.28a	2.24a	4.52a	2.70a	0.14a
Red Orejona (YO)	1.65a	1.44a	2.62a	4.33a	1.87ab	0.18a
Saborosa (YS)	1.35a	1.25a	2.64a	4.35a	1.26b	0.14a

[(1)]Means followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. The treatments consisted of yellow dragon fruit (*Selenicereus megalanthus*) scions micrografted on rootstocks of: YY, yellow dragon fruit; YW, white dragon fruit (*Hylocereus undatus*); YC, Cebra red dragon fruit (*Hylocereus polyrhizus*); YO, Orejona red dragon fruit (*Hylocereus polyrhizus*); and YS, Saborosa (*Selenicereus setaceus*) dragon fruit.

peaks were observed, in addition to the peak of the reference standard (Table 2). This means that there are cells with ploidy 2C (first peak), 4C (second peak), and possibly 8C (third peak). However, the third peak was not considered in the statistical analysis, since it did not appear in all samples. The obtained result confirms that in vitro dragon fruit plants have at least two distinct levels of ploidy, and it is possible to infer that there is an endoreduplication phenomenon, which was verified for all studied species.

The DNA index at peak 1 and 2 of yellow dragon fruit was higher than those of all the other species, being followed by Saborosa (Table 2). It is possible to observe that peak 2 has approximately twice the DNA index than peak 1, evidencing two ploidy (2C and 4C).

The yellow and Saborosa dragon fruit have close DNA indexes since they are of the same genus; the same occurs with the white and Cebra and Orejona red dragon fruit varieties of the genus *Hylocereus*.

Regarding the endoreduplication phenomenon, it is characterized by cells with various ploidy within a tissue and has been reported in white-fleshed dragon fruit (Menezes et al., 2012). In the present study, the phenomenon was also observed in yellow, Saborosa, and Cebra and Orejona red dragon fruit. Endoreduplication has advantages related to the increase in ploidy level, which can increase plant productivity and quality (Comai, 2005). It also speeds up the metabolism of the plant and improves its physiological functions under biotic and abiotic stress conditions by improving its resistance (Li et al., 2019).

When the scions were compared after micrografting, it was noted that the DNA index was higher in both peaks 1 and 2 for the treatment yellow dragon fruit on the Orejona red variety, which differed from the others (Table 3). When comparing the rootstocks, the DNA index of peak 1 and 2 was higher for the treatments yellow dragon fruit on the yellow and on the Saborosa varieties, which did not differ from each other.

These results are indicative that the rootstock may influence the amount of scion DNA. It can be assumed that DNA fragments migrate from the scion to the rootstock and vice versa, as already observed in smoke plants (Stegemann & Bock, 2009). However, in the present study, the differences in the DNA index were more pronounced in the scion than in the rootstock, which remained almost unchanged.

The histograms showed three ploidy levels (diploid, tetraploid, and octaploid), represented by the three peaks of the sample, with peak 3 being smaller than the others (Figure 1); this means that there are fewer octaploid cells in the analyzed samples. The histograms also showed a peak of the pea reference standard that was used to calculate the indices of the DNA index. All treatments had the same three ploidy levels (endopolyploidy), regardless of the used rootstock.

The rootstock histograms showed four sample peaks, that is, four ploidy levels (Figure 2). However, all treatments had the same level of endopolyploidy, regardless of the micrograft used.

Table 2. DNA index (ID) of dragon fruit cladodes analyzed on a flow cytometer[(1)].

Dragon fruit species	Peak 1 (ID) (2C)	Peak 2 (ID) (4C)
Yellow (*Selenicereus megalanthus*)	5.37a	10.48a
White (*Hylocereus undatus*)	2.23c	4.67c
Red Cebra (*Hylocereus polyrhizus*)	2.12c	4.46c
Red Orejona (*Hylocereus polyrhizus*)	2.25c	4.67c
Saborosa (*Selenicereus setaceus*)	4.81b	9.38b

[(1)]Means followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. 2C, diploid cells; and 4C, tetraploid cells.

Table 3. DNA index (ID) of dragon fruit cladodes analyzed on a flow cytometer after micrografting of scions on rootstocks[(1)].

Dragon fruit scion	Peak 1 (ID) (2C)	Peak 2 (ID) (4C)
Yellow (YY)	4.34b	8.56b
White (YW)	4.77b	9.18b
Red Cebra (YC)	4.44b	8.60b
Red Orejona (YO)	6.13a	11.91a
Saborosa (YS)	4.18b	8.09b
Rootstock		
Yellow (YY)	4.60a	9.12a
White (YW)	2.13b	4.52b
Red Cebra (YC)	2.07b	4.35b
Red Orejona (YO)	2.19b	4.59b
Saborosa (YS)	4.39a	8.62a

[(1)]Means followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. The treatments consisted of yellow dragon fruit (*Selenicereus megalanthus*) scions micrografted on rootstocks of: YY, yellow dragon fruit; YW, white dragon fruit (*Hylocereus undatus*); YC, Cebra red dragon fruit (*Hylocereus polyrhizus*); YO, Orejona red dragon fruit (*Hylocereus polyrhizus*); and YS, Saborosa (*Selenicereus setaceus*) dragon fruit. 2C, diploid cells; and 4C, tetraploid cells.

According to Chevalier et al. (2011), the endoreduplication process probably happened during evolution to benefit organ and plant development, with possible functional roles considering the different plant, organ, and cellular physiologies of each species. This phenomenon frequently occurs during

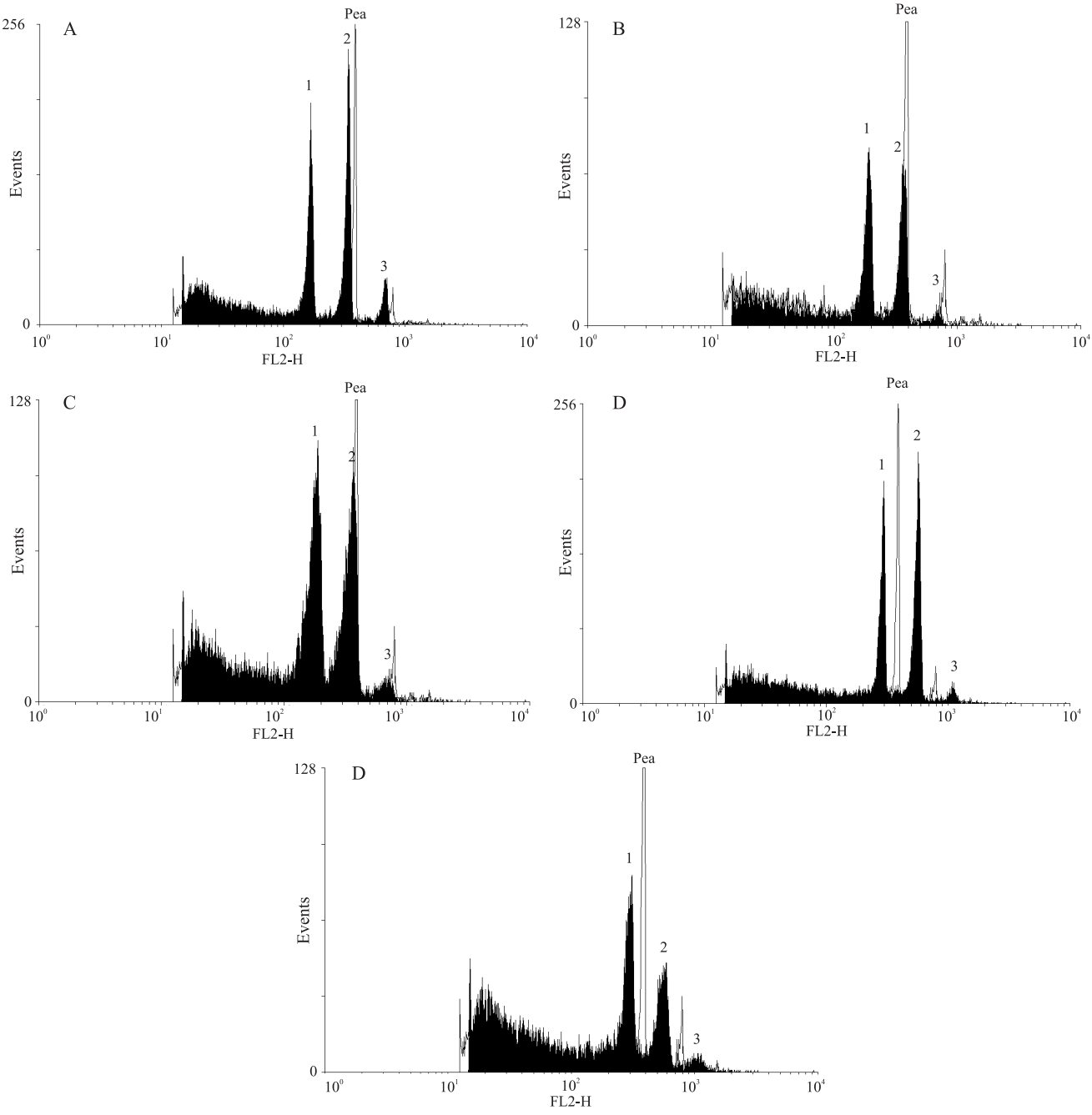

Figure 1. Flow cytometry histograms of yellow dragon fruit (*Selenicereus megalanthus*) scions, as well as of the pea (*Pisum sativum*) reference standard, micrografted on rootstocks of the following dragon fruit varieties (five treatments): A, yellow dragon fruit; B, white dragon fruit (*Hylocereus undatus*); C, red Cebra dragon fruit (*Hylocereus polyrhizus*); D, red Orejona dragon fruit (*Hylocereus polyrhizus*); and E, Saborosa dragon fruit (*Selenicereus setaceus*).

the differentiation of cells with a highly specialized morphology, as is the case of the dragon fruit cladode, which is a modified stem to store large amounts of water (Menezes et al., 2012).

The histological sections showed that the scions are less thick than the rootstocks and are slightly displaced (Figure 3). Moreover, it was observed that the vascular tissues established continuity between the scion and

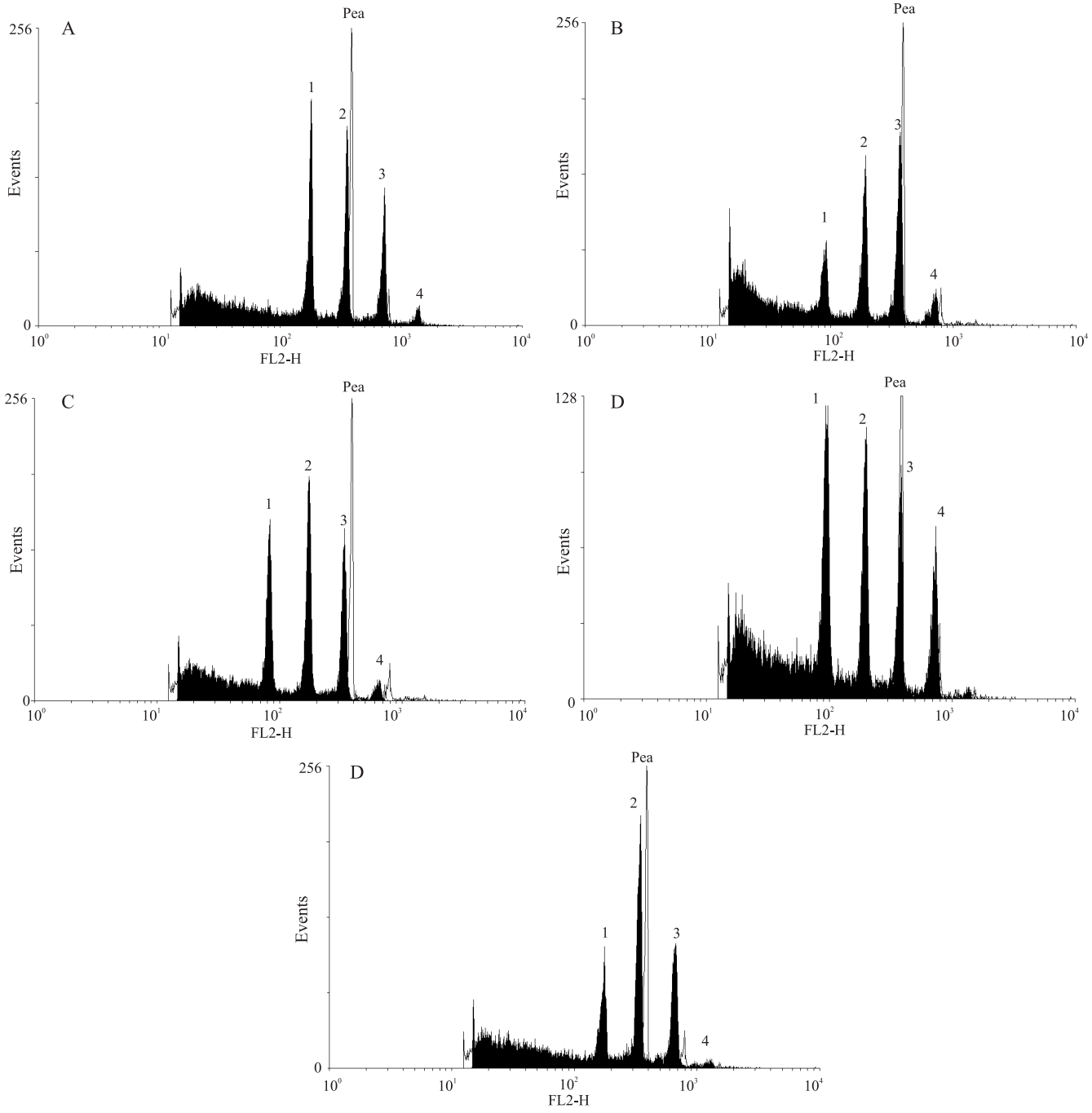

Figure 2. Flow cytometry histograms of the dragon fruit rootstocks used for the five treatments, as well as of the pea (*Pisum sativum*) reference standard, in which yellow dragon fruit (*Selenicereus megalanthus*) scions were micrografted on: A, yellow dragon fruit; B, white dragon fruit (*Hylocereus undatus*); C, red Cebra dragon fruit (*Hylocereus polyrhizus*); D, red Orejona dragon fruit (*Hylocereus polyrhizus*); and E, Saborosa dragon fruit (*Selenicereus setaceus*).

rootstock. It was also possible to note a sprout from the scion, showing the passage of water and rootstock nutrients, as well as a thin necrotic layer in the union of the scion and rootstock, which may be a scar from the cut.

For the purpose of joining the micrograft parts, there is no need for the juxtaposition of vascular tissues or for the same thickness of cladodes, but there is a need for proximity between the conducting vessels, which favors access to nutrients and water, especially during

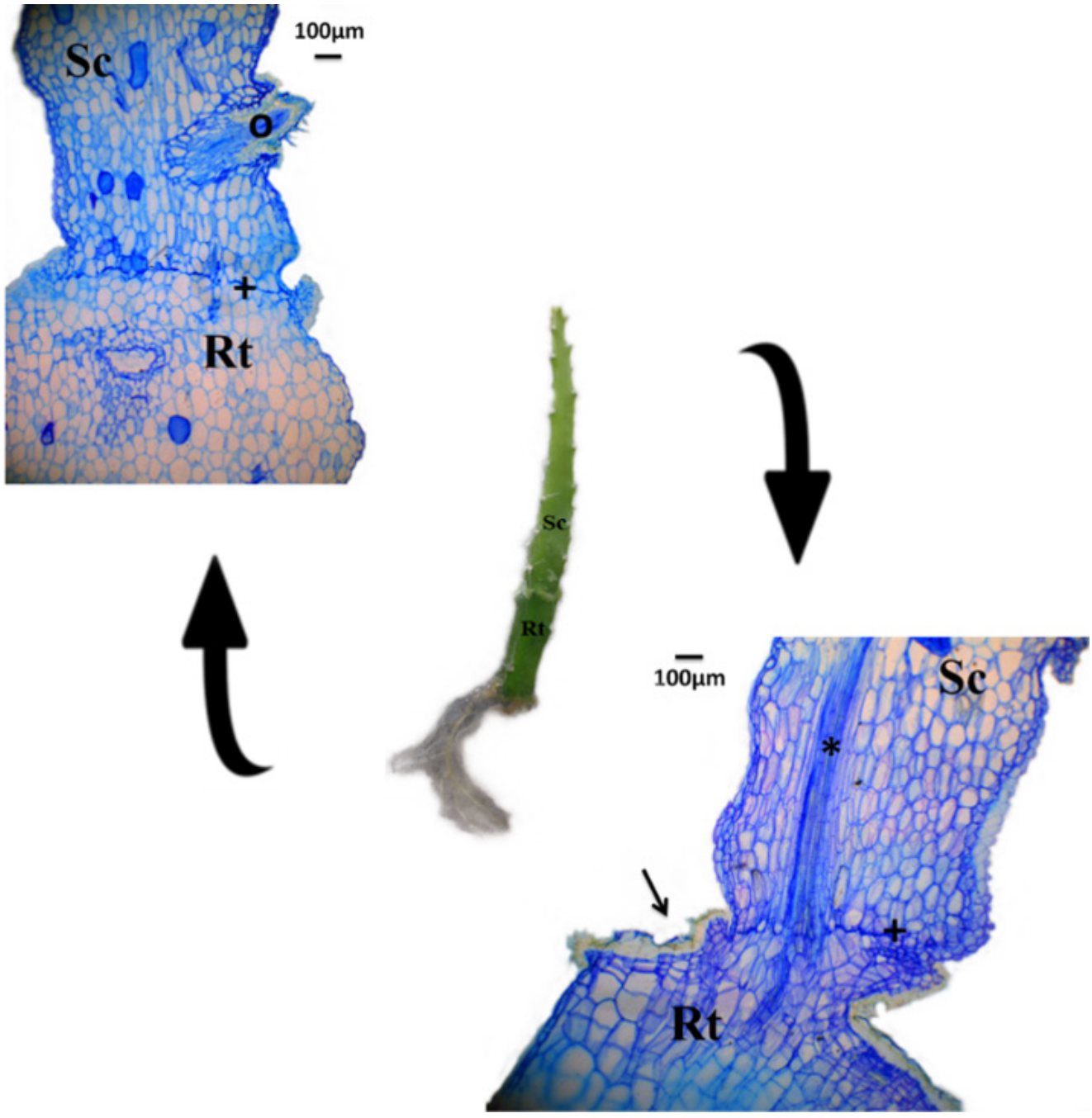

Figure 3. Micrograft and photomicrograph under an optical microscope of the longitudinal section of the micrografting region between yellow dragon fruit (*Selenicereus megalanthus*) scion (Sc) and rootstock (Rt), showing conductive vessels (*), a callus in the region of anatomical insertion and cut (→), shoot (o), and necrotic layer (+).

the initial development phases after micrografting (Ribeiro et al., 2015).

The process of vascular tissue differentiation occurs by scion induction (Balbi et al., 2019). To establish vascular continuity, a fusion between plant parts derived from the contact between the rootstock and scion is necessary (Balbi et al., 2019), being considered a successful histological marker of micrografting (Yin et al., 2012). Fan et al. (2015) reported the recovery of vascular tissues 11 days after micrografting of tomato (*Solanum lycopersicum* L.) plants, observing more developed scions and higher degrees of differentiation, which evidences the importance of the role of the scion in the induction of vascular connections. In the absence of cellular connections in the initial stages of scion development, cell-to-cell transport is the only possible form of nutrition for the scion (Ribeiro et al., 2015). Micrografting studies on apples (*Malus* spp.) revealed that, during the initial micrografting phase, when there are no vascular connections, the water used by the scion is provided by rootstock-derived exudates in response to an incisional lesion (Darikova et al., 2011).

Callus formation plays a key role in the adhesion of the scion and its initial nutrition, and the death of cells external to the callus results in the formation of cicatricial tissue, which favors the isolation of the micrografting region, probably reducing water losses by the tissues in the connection region (Pina et al., 2012). Callus formation was essential for the success of the micrografting of tomato (Fan et al., 2015) and *Nicotiana attenuata* Torr. ex S.Watson (Fragoso et al., 2011).

Regarding the necrotic layer, for some authors, its formation is the first phase of micrografting (Yin et al., 2012), also occurring in young plant grafting (Pina et al., 2012). However, in the present study, as this layer only appeared in some treatments, it was considered only a consequence of the incision procedures, that is, the presence of residual cell wall material of damaged tissues and not a true phase of scion development, as reported by Ribeiro et al. (2015) in passion fruit (*Passiflora edulis* Sims) micrografting.

Based on all the analyzes carried out, dragon fruit micrografting is viable, as it allows seedlings to have a longer and more vigorous root system. Endoreduplication – a phenomenon with a great number of advantages, such as speeding up plant metabolism – was observed both in the scion and in the rootstock. In addition, anatomical studies have shown excellent results in relation to healing and callus production (Gentile et al., 2017).

Conclusions

1. Based on DNA content and anatomical analyses, yellow dragon fruit (*Selenicereus megalanthus*) in vitro micrografting is feasible on all used rootstocks.

2. Yellow dragon fruit is more vigorous when micrografted on the red Orejona variety (*Hylocereus polyrhizus*).

3. There is endoreduplication in all scion and rootstock combinations used.

4. The DNA index is reduced in the yellow dragon fruit micrografted on the yellow, white (*Hylocereus undatus*), Saborosa (*Selenicereus setaceus*), and red Cebra (*Hylocereus polyrhizus*) rootstocks, but is increased on the red Orejona one.

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