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Mini-grafting of adult *Passiflora edulis* Sims f. *flavicarpa* Deg. scions onto vegetatively propagated adult rootstocks of *P. mucronata* Lam.

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Abstract

The mini-grafting is a nondestructive vegetative propagation method based on grafting apical segments onto adult donor plants-derived rootstocks. Here, we aimed at evaluating the mini-grafting of shoot tips derived from adult *Passiflora edulis* f. *flavicarpa* plants (yellow passion fruit) onto vegetatively propagated rootstocks of *P. mucronata* (sandbank passion fruit). Different shoot tip lengths and the fastening material were assayed. A randomized block experimental design was set up following a 2 × 3 factorial scheme [shoots: 8-12 and 3-7 cm × fastening materials (circular clip, "V" shaped clip, and Parafilm®)] totaling six treatments with four repetitions of eight plants each. The following characteristics were evaluated: graft setting (%); graft and rootstock diameters (mm); graft diameter/rootstock diameter ratio; cellular division in the graft region and starch presence in the graft and rootstock. Parafilm® provided better adhesion (89.57%) compared to circular (76.03%) and "V" shaped clips (68.74%). The attachment was favored by rootstocks with 8-12 cm shoots (90.27%), compared with those of the 3-7 cm (65.96%). The presence of starch grains in the parenchymatic cells of the graft suggests the translocation of photo-assimilates from physiologically active mature leaves of *P. edulis* f. *flavicarpa* to the stem apex. Additionally, the hydrolysis and mobilization of the starch associated and the endogenous auxin levels in this meristematic region may have been responsible for improving the success of the mini-grafting.

Keywords: Anatomy, Fasteners, Passion fruit, Shoots, Starch.
Abbreviations: CABMV_*Cowpea aphid-borne mosaic virus*; FB100_Flora Brasil 100; FB200_Flora Brasil 200; BRS_Brasil; DAM_days after mini-grafting; DAG_days after grafting; *PIN*_PIN-formed protein family of auxin transporters; IAA_indole-3 acetic acid; DR5::GUS_Auxin responsive promoter DR5 reporter system; PAL_phenylalanine ammonia lyase; GD/RD_diameter of the graft/diameter of the rootstock; FAE_formalin, acetic acid and ethanol.

Introduction

In Brazil, *Passiflora edulis* Sims f. *flavicarpa* Degener, *P. edulis* Sims f. *edulis*, and *P. alata* Curtis are species used for commercial and industrial purposes. Other species like *P. mucronata* Lam are well-known for their ornamental beauty, year-round shiny leaves, white flowers, and long flowering periods (Meletti et al., 2011). Additionally, *P. mucronata* is resistant to the bacteria of branches and leaves (Junqueira et al., 2005) and to *Fusarium oxysporum* f. sp. *passiflorae* and *Fusarium solani* (Oliveira et al., 2013; Veloso et al., 2014). *P. mucronata* appears as an important alternative to face disease control if grafted as a rootstock with *P. edulis*, since many genotypes of the latter are highly susceptible to illnesses, despite the description of a *P. edulis* f. *flavicarpa* genotype as resistant to *F. oxysporum* f. sp. *passiflorae* (Silva et al., 2013). Other species, such as *P. giberti*, *P. quadrangularis*, *P. nitida*, and *P. foetida*, are reported to be resistant to both *Fusarium solani* and *F. oxysporum* f. sp. *passiflorae* isolates (Veloso et al., 2014; Preisigke et al., 2015). In the *Passiflora* genus, grafting presents a reliable approach to study the transmission of positive signs involved in flowering induction in *P. edulis* (Nave et al., 2010). As a propagation method, grafting may greatly contribute to the *P. edulis* f. *flavicarpa* species by combining with rootstocks that are resistant to soil illnesses, mainly *Fusarium*. In this, particular, novel technologies have been developed and improved for the *Passiflora* genus, such as the *ex vitro* micro-grafting aiming the elimination of the *Cowpea aphid-borne mosaic virus* (CABMV) in *Passiflora edulis* f. *flavicarpa* plants (Ribeiro et al., 2008). Likewise, Pereira et al. (2009) carried out interspecific *ex vitro* micro-graftings of stem apexes of *P. edulis* f. *flavicarpa* onto hypocotyls of *P. alata*, *P. cincinnata*, *P. edulis*, and *P. setacea*. The mini-grafting in passion fruit, as proposed by Alexandre et al. (2013), consists of grafting adult plant-derived stem apexes onto young rootstocks. The use of fastening materials for the grafted region is highly desirable to stimulate the effective union between the scion and the rootstock. The use of adhesive tape to wrap the area of the graft in *P. edulis* f. *flavicarpa* cvs.

FB100 and FB200 on *P. edulis*, provided 98% and 94% attachment, respectively (Roncatto et al., 2011), whereas a 63.3% attachment rate was reported on the use of a "V" shaped clip in BRS Yellow Giant hybrid self-grafted (Leão et al., 2011). As sessile organisms, plants depend on the mobilization of photo-assimilates from biosynthesis sites (source areas) to tissues that use them as energy (sink areas) for growth and development. The transport of sugars through the phloem may occur between distant organs, but normally a drainage is supplied by photo-assimilates through the closest sources. Therefore, rootstocks that possess bigger shoots with expanded, more chronologically mature leaves may have better production of photo-assimilates, which can effectively translocate to closer growing tissues, as compared to rootstocks with smaller shoots and younger leaves. The aim of this work was to evaluate the mini-grafting of shoot tips of different lengths derived from adult *P. edulis* f. *flavicarpa* plants onto vegetatively propagated rootstocks of *P. mucronata*, using different fasteners, in a non-destructive basis for the donor graft mother plant.

Results and Discussion

Anatomy of the mini-grafting union

The fasteners, "V" shaped clip (gray) (Fig. 1A), circular clip (yellow) (Fig. 1B) and Parafilm® (Fig. 1C) have different characteristics, wherein that which completely seals the grafting region is Parafilm®. This may have largely contributed to the higher survival rates and efficiency in mini-graftings (Fig. 1D).

Longitudinal sections of the grafts at 21 days after mini-grafting (DAM) revealed a close physical contact of scion and the rootstock by means of circular clips, "V" shaped clips, and Parafilm®, though leading to callogenic responses from parenchyma cells (Figs. 1G and J; H and K; and I and L, respectively). However, the callogenesis was unevenly distributed throughout the scion-rootstock interfaces, most probably as a consequence of the different morphologies in cross sections: a pentagonal one in *P. edulis* f. *flavicarpa* and a round one in *P. mucronata*. This is attributed to a relatively reduced contact time (21 days) between the bionts.

Interestingly, cell proliferation took place in all treatments (Figs. 2A and B; 3D, E, and F; and 4A, B, and C). Despite being sometimes notably low, even though cell proliferation rates were sufficient to support and ensure water, nutrients and phytohormones transport to the grafting region partners, keeping the tissues alive. Sometimes, necrotic tissue layers were noticed, which resulted from the tissue cutting injury during the mini-grafting process (Figs. 2C and D; 3A and C; and 4D and E), though the plants have survived. This behavior can be related to the reduced stimulation time to induce callogenesis, which was only 21 days. These plants remained alive probably due to the starch reserves present in the parenchymatic cells of the grafts (Figs. 5A, C, E, G, I, and K).

The union of the mini-grafting is initially formed by the division of cells of the callus, originally from the graft and the rootstock, which further differentiate to form the vascular cambium. Three consecutive stages occur in the successful grafting process: the adhesion between graft and rootstock cells, callus cell proliferation, and vascular differentiation in the grafting interphase (Hartmann et al., 2011). Indeed, such abovementioned events were verified at 21 DAM (days after mini-grafting), which confirms the success of the grafting between *Passiflora edulis* f. *flavicarpa* and *P. mucronata*. However, Salazar (2013) verified that, in *P. edulis* Sims. grafted onto *P. edulis*, *P. giberti*, and *P. mucronata*, full differentiation and vascular reconnection took place only at 250 DAG (days after grafting). Interestingly, Wang et al. (2014) observed, during grafting in *Arabidopsis thaliana* (L.) Heynh, that auxin had stimulated the reconnection of the vascular tissues on the third DAG, and that the expression patterns of *PIN* family genes may have affected the graft-union development by controlling the auxin flow. The hypocotyledonary grafting of *P. edulis* f. *flavicarpa* on *P. coccinea* and *P. edulis* f. *flavicarpa* presented, after one month, regenerated vascular tissues and, despite not being exactly juxtaposed, there was reorganization for connection, which did not preclude plant growth (Nogueira Filho et al., 2010a). Studies conducted at 8 and 30 DAG (*P. edulis* f. *flavicarpa* x *P. cincinnata*) evidenced, at first, a partial cellular multiplication close to the upper region of the slot, whereas that multiplication had already occurred in the lower region. After 30 days, the graft slot was completely filled by the callus and it was possible to verify regions with cellular differentiation (Zucareli, 2011). The division retaining capacity of adult parenchyma cells plays a key role in the healing or regeneration of injuries such as grafting. This process is possible given their inherent capacity to be reprogrammed to the meristematic cytological status, which differentiates them from other cellular types (Scatena and Scremin-Dias, 2006). The new parenchymatous cells grow both in the graft and in the rootstock filling in the space between the slot and the graft (Pina and Errea, 2005). Here, the dividing cells belong to the medullar parenchyma of the graft, to xylem parenchyma cells and cambial ones as well (Fig. 3B). This fact is related to the fact that cells derived from active growing areas of the graft are somehow younger, and much more responsive to injuries due to a potentially greater division rates with respect to those of the rootstock; additionally, this tissue has a high endogenous auxin content which induces the mitotic process along with the cytokinin (Taiz and Zeiger, 2013), thus positively impacting effective healing in the graft region.

Shoot lengths and type of fasteners in the fixation of the mini-grafting

The attachment of the mini-graft (*P. edulis* f. *flavicarpa* x *P. mucronata*) was favored by rootstocks of *P. mucronata* that presented 8-12cm shoots, if compared to the 3-7 cm shoots 65.96% (Table 1A). Larger shoots present higher numbers of expanded leaves with photosynthetic capacity and higher photo-assimilate production, besides having young leaves and apical meristem (source of auxins), all decisive in leading to successful grafting. Apical meristems and young leave have high concentrations of free auxins since they are the main locations of biosynthesis of this phytohormone (Taiz and Zeiger, 2013). Ahkami et al. (2013), analyzed the spatial distribution of IAA (indole-acetic acid) in *Petunia hybrida* stem and verified higher concentrations in young leaves close to the stem, but the levels decreased furthest from the apical region. This suggests that the length of shoots on the cuttings is beneficial for grafting. According to Gauch and Dugger (1953) the auxins are transported to the base of the stem by means of the action of boron and form an ionizable borum-carbohydrate complex in which the ion transports the carbohydrate and the auxin. In *Arabidopsis thaliana* the greater staining intensity of DR5::GUS system in the grafted area indicates higher IAA concentrations (Marsch-Martinez et al., 2013). Auxin gradients may explain positive attachment of the bionts in our work, by stimulating mitotic process. The Parafilm® provided a greater attachment (89.57%) of the mini-grafting between *P. edulis* f. *flavicarpa* x *P. mucronata*, compared to the circular clips (76.03%) and "V" shaped clips (68.74%) which did not differ statistically

Table 1. Characteristics evaluated in the mini-grafting of *P. edulis* f. *flavicarpa* on *P. mucronata* with 8-12 cm and 3-7 cm shoots (**A**), with the use of fasteners (**B**) and interaction among the size of shoots and fasteners (**C**) for the diameter of the rootstock after 21 days

A	Shoots (cm)		
Characteristics evaluated	8-12	3-7	
Attachment (%)	90.27 a*	65.96 b	
Diameter of the graft - GD (mm)	2.97 a	2.81 b	
Diameter of the rootstock - RD (mm)	3.08 a	3.01 a	
Ratio GD/RD	0.97 a	0.94 a	
B	Fastening material		
Characteristics evaluated	Circular clip	"V" clip	Parafilm®
Fixation (%)	76.03 b*	68.74 b	89.57 a
Diameter of the graft - GD (mm)	2.75 b	2.90 ab	3.01 a
Ratio GD/RD	0.93 a	0.97 a	0.97 a
C	Diameter of the rootstock - RD (mm)		
	Fastening material		
Shoots (cm)	Circular clip	"V" clip	Parafilm®
8-12	3.17 aA**	3.00 Aa	3.08 aA
3-7	2.78 bB	3.03 ABa	3.23 aA

*Mean values followed by the same letter, in the line, do not differ by the Tukey test at 5% probability level. **Mean values followed by the same capital letter, in the line, and lower case, in the column, do not differ by the Tukey test at 5% probability level.

(Table 1B). An explanation for the comparative lower performance of the clips, especially the "V" shaped, is that they do not fully surround the stem, though imposing an asymmetric pressure on one of the sides of the grafted area. This, may cause a misalignment of the bionts, preventing them of being perfectly juxtaposed, further leading to a poor junction of the tissues. Conversely, the circular clip fully covers the graftion section but not as efficiently as the Parafilm®, which evenly distributes physical forces to the grafting region and, somehow completely seals the graft area, preventing excessive wetting from irrigation water. The micro-grafting in hypocotyl of *Arabidopsis thaliana* by means of a silicone tube sealed with Parafilm® achieved 87% attachment, as confirmed by the growth of the calluses, growth of the main stalk and in the histology, the differentiation and vascular connection and the accumulation of lignin (Nisar et al., 2012). According to these authors, the repetitive pruning of the graft produced new stem tissues which suggests a regular flow of nutrients from the rootstock and consequently the success of the graft. The use of Parafilm® in top wedge grafting in transgenic *Jatropha curcas* scions onto wild rootstock of the same species was rather effective with 100% genetic stability between the mother plant and the grafted plant (Jaganath et al., 2014). This reinforces our data on the usefulness of the fastening materials in improving the grafting process.

In hypocotyledonar-based grafting in five combinations of genotypes of *P. edulis* f. *flavicarpa* four different types of fastening material were used: straws, metal clips, and plastic clips (Rego et al., 2012). The metal clips were not as good as the other fastening materials possibly due to the oxidation leading to a low protection of the grafted area, whereas the other fastening materials enabled 100% viable grafted plants at 60 DAG. The quality of the Parafilm® is probably related to its effectiveness in the sealing of the connecting tissue which avoids its desiccation, keeping it alive. Alexandre et al. (2013) carried out the mini-grafting technique with a sealing system like Micropore® tape in *P. mucronata* over *P. edulis* f. *flavicarpa* reaching attachment up to 80%. In top cleft grafting of *P. edulis* Sims over *P. mucronata*, Salazar (2013) reported an attachment rate of 88.33%. Transparent adhesive tape was used in simple English type grafting for the *P. alata/P edulis* f. *flavicarpa* and *P cincinnata/P edulis* f. *flavicarpa* combinations, with 91 and 51% attachment, respectively (Santos et al., 2014). The authors associate the grater lignification of the tissues of *P. cincinnata* compared to *P. alata*, with the difficulty in the vascular reconnections as the possible cause for the reduced attachment. Therefore, the lignin in this case is a biochemical marker which can be related to the low compatibility between species. According to Vieira et al. (2011), the *P. edulis* has a lower number of caps of perivascular phloematic fibers, which favors their use as rootstocks in micro-grafting. The grafting peach (*Prunus persica* cv. Chimarrita) on Japanese apricot (*Prunus mume* cv. Umezeiro) promoted slow growth and in some cases, the death of the grafted peach (Pereira et al., 2014). According to these authors, the activity of phenylalanine ammonia lyase (PAL) enzyme and the expression of genes encoding PAL in the phenilpropanoid pathway, and responsible for biosynthesis of anthocyanins, flavonoids and lignins, were highly expressed in *P. mume*. However, the grafting of *P. edulis* f. *flavicarpa* on *P. cincinnata* did not compromise the initial growth and levels of the nutrients of the commercial passion fruit plants (Zucareli et al., 2014). The difference in data reported by Santos et al. (2014) and Zucareli et al. (2014) is probably related to the ontogenetic and chronological age of the *P. cincinnata* plants as rootstock. The former used older plants and therefore a greater lignification degree lessened grafting efficiencies.Another relevant issue to evaluate the development of the grafted plants is the girth of the graft. In this work, this characteristic was favored in plants with rootstocks with 8-12 cm shoots (Table 1A), and in plants whose graft area was sealed with Parafilm® (Table 1B), with values of 2.97 and 3.01 mm, respectively. Nogueira Filho et al. (2010b) observed in hypocotyledonary grafting of *P. edulis* f. *flavicarpa*/*P. edulis* f. *flavicarpa* that the diameter of the graft was 4.06 mm at 30 DAG. The GD/RD relation did not differ statistically for both the size of the shoots (Table 1A) and the fasteners (Table 1B). For this characteristic, the closer the results are to one (1.0), the better the graft and rootstock growth diameter similarity will be, thus suggesting compatibility, and greater balance in the circulation of sap and better performance of the grafted plant.

The diameter of the rootstock with 8-12 cm shoots did not present a difference among the fasteners. However, for 3-4 cm shoots, the use of Parafilm® and the "V" shaped clip provided the greater diameter averages of the rootstock. Using the Parafilm® and the "V" shaped clip, notwithstanding the size of the shoot, the rootstocks present

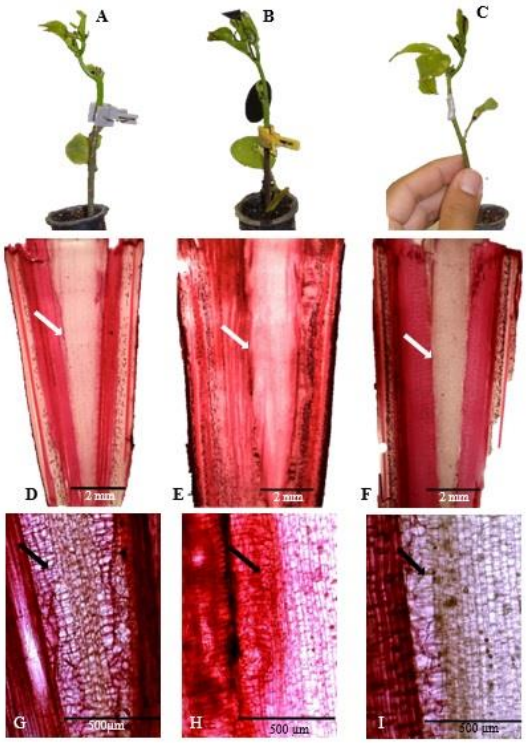

Fig 1. Longitudinal sections of the grafted area of *Passiflora edulis* f. *flavicarpa* on *P. mucronata*, with the use of a circular clip (**A**, **D** and **G**), a "V" shaped clip (**B**, **E** and **H**) and Parafilm® (**C**, **F** and **I**). Details of scion and rootstock totally connected (*white arrows*) and parenchymatic cell proliferation – **G**, **H** and **I** (*black arrows*).

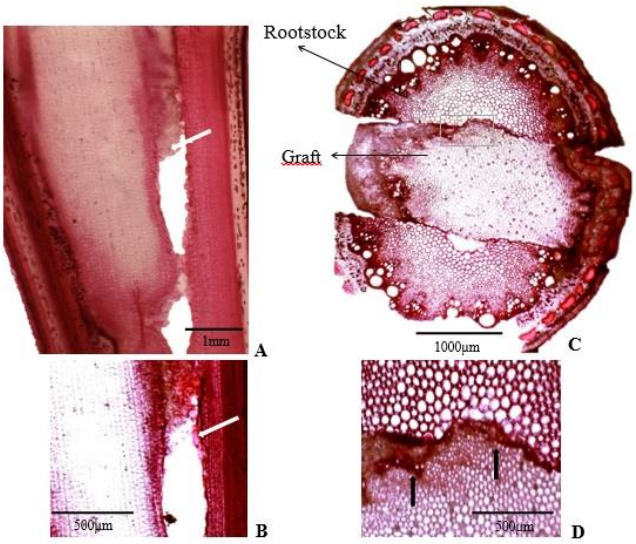

Fig 2. Transversal and longitudinal sections of the grafted area of *Passiflora edulis* f. *flavicarpa* on *P. mucronata* with the use of circular clip. Overall and detailed view of the mini-grafted region showing the partial union of tissues in the rootstock with 8-12 cm shoots (**A** and **B**). Overall and detailed view showing healing responses in rootstock with 3-7 cm shoots (**C** and **D**). Partial cellular multiplication (*white arrow*). Necrotic layer tissue (*black arrow*).

similar diameters for 8-12 cm shoots (3.08 and 3.23 mm) and 3-7 cm (3.00 and 3.03 mm), respectively. For the circular clip, the diameter of the rootstock was larger (3.17 mm) in 8-12 cm shoots at 21 DAG (Table 1C). Nogueira Filho et al. (2010b) obtained values of 4.43 and 3.65 mm in diameter in hypocotyledonary grafting of *P. edulis* f. *flavicarpa*/*P. cincinnata* and *P. edulis* f. *flavicarpa*/*P. alata*, respectively at 30 DAG.

Anatomy: starch as a biochemical marker

In the stem apexes (grafts) grains of starch were observed around the perivascular phloem fibers (Figs. 5A, C, E, I and K). Starch, as a biochemical marker, demonstrates photo-assimilates produced in physiologically mature leaves of the *P. edulis* f. *flavicarpa* species were translocated to these stem apexes. Starch is hydrolyzed to smaller molecules (sucrose) and this is the main carbohydrate transported in the majority of plants. However the auxins produced in the stem apexes and young leaves are in the free form and their translocation is intermediated by the conjugation to other composites, as for example amino acids and sugars (sucrose), in order to protect themselves from oxidant degradation (Taiz and Zeiger, 2013). This conjugated form is transported in the phloem in a polar basipetal manner which influences several physiological mechanisms, especially the healing of lesions (Taiz and Zeiger, 2013), for example, from mini-grafting.

Materials and Methods

Mini-grafting

The study was carried out in the Centro Universitário Norte do Espírito Santo, Campus São Mateus, in São Mateus/ES, using branches of adult field-grown *P. mucronata* plants gathered at Fazenda Cedro, in São Mateus/ES. The cuttings (average 10 cm) were planted in plastic tubes (55 cm^3) containing a commercial substrate (Bioplant®) to promote the rooting of the rootstocks and kept in a greenhouse for 50 days. Shoot tips (average 5 cm length) of *P. edulis* f. *flavicarpa* were collected in the municipality of Sooretama/ES, conditioned in coolers, kept hydrated and immediately sent to the Plant Propagation Laboratory. The technique used was the modified methodology of mini-grafting for passion fruit (Alexandre et al., 2013). With the aid of steel razor blades, equal-sided-wedge cuttings at the base of the graft (tip of *P. edulis* f. *flavicarpa* adult shoot branches) were made which were introduced into the split of the rootstock. To keep the bionts (graft x rootstock) closely tied either clips or Parafilm® were used. Grafted plants were conditioned and kept for 10 days in plastic bags (7 x 15 cm) in order to create a wet environment and reduce the dehydration of the graft. The experiment was kept in a greenhouse with irrigation by micro aspersion managed at four three-minute irrigations per day in three hour intervals. The attachment (%); graft diameter (GD, mm) and that of the rootstock (RD, mm), were evaluated with the aid of a digital caliper above and below the graft area, respectively, following 21 days after mini-grafting (DAM). With these two characteristic the graft diameter/rootstock diameter ratio was obtained (GD/RD).

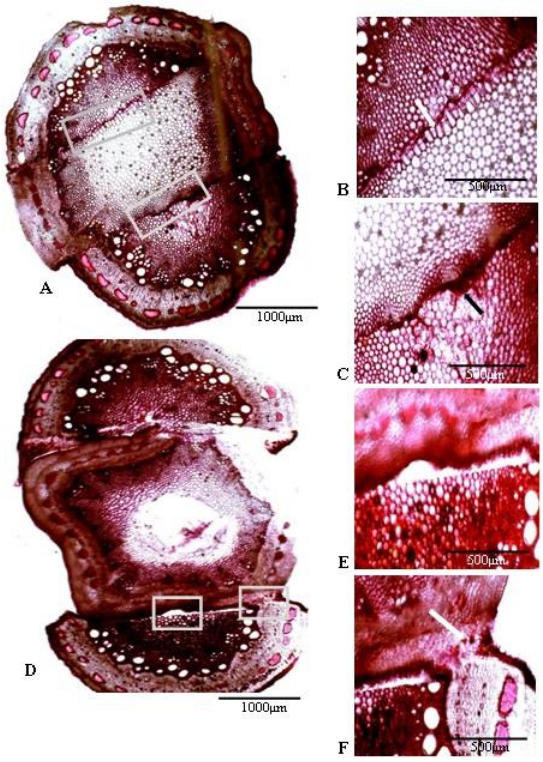

Fig 3. Transversal sections of the top wedge grafted area of P. edulis f. flavicarpa on P. mucronata with use of a "V" shaped clip. General and detailed view of the beginning of the mitosis and tissue healing response in rootstock with 8-12 cm shoot (A, B and C). General and detailed view of the cellular multiplication in rootstock with 3-7 cm shoot (D, E and F). Cellular multiplication (white arrow). Necrotic tissue (black arrow).

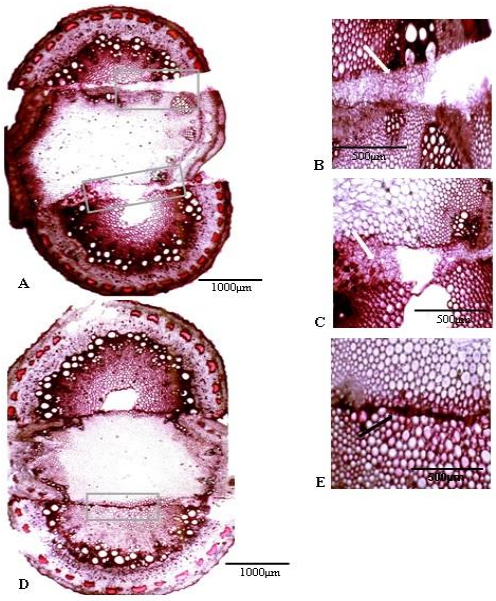

Fig 4. Transversal sections of the grafted area of P. edulis f. flavicarpa on P. mucronata with the use of Parafilm®. General and detailed view of the partial cellular multiplication in rootstock with 8-12 cm shoot (A, B and C). General and detailed view of tissue healing in rootstock with 3-7 cm shoot (D and E). Partial cellular multiplication (white arrow). Necrotic tissue (black arrow).

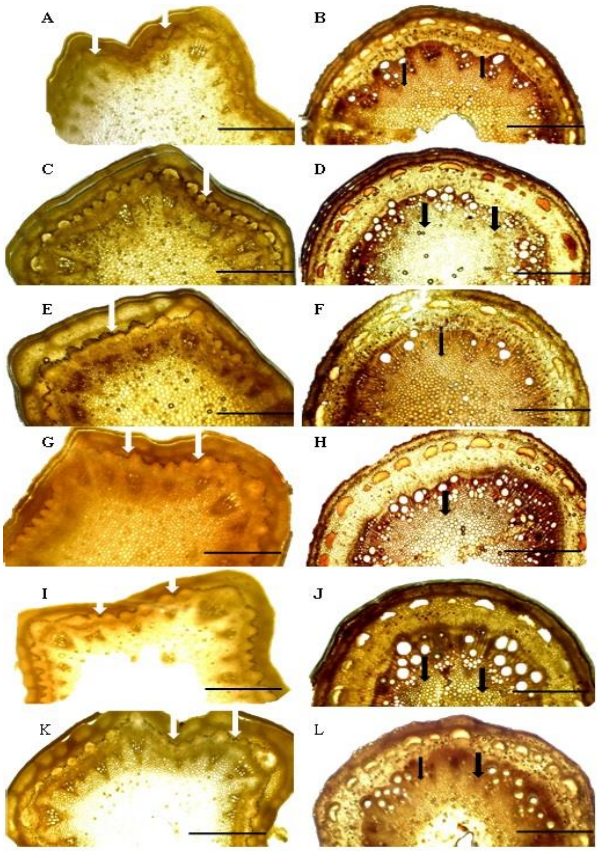

Fig 5. Transversal cuts of the graft (P. edulis f. flavicarpa) and of the rootstock (P. mucronata) with 3-7 and 8-12 cm shoots, for the evaluation of starch. Graft (A) and rootstock (B) with 3-7 cm shoots, fastened with circular clip; graft (C) and rootstock (D) with 8-12 cm shoots, fastened with circular clip; graft (E) and rootstock (F) with 8 cm shoots, fastened with "V" shaped clip; graft (G) and rootstock (H) with 8-12 cm shoots, fastened with "V" shaped clip; graft (I) and rootstock (J) with 3-7 cm shoots, fastened with Parafilm®; and graft (K) and rootstock (L) with 8-12 cm shoots, fastened with Parafilm®; Presence of amyliferous sheath (white arrows) and absence of starch (black arrow). Bar: 1000 µm.

Histological analysis

The histological analysis was made at 21 DAM. Free-hand sections, by means of steel blades, were made in the areas above and below the grafting area. The samples were stored in glass containers with an FAE (formalin, acetic acid and ethanol, 1:1:1 v/v) solution, for 48 hours followed by its replacement with 70% ethanol (Johansen, 1940). Longitudinal and transversal sections of the graft region were made in order to verify the union of the tissues; transversal to the graft and rootstock, in order to verify for starch with an iodine solution at 2% (Johansen, 1940), which, after adding one drop of iodine, were analyzed and photographed. For the identification of the tissue fusion, a section was stained with 1% safranin, the excess removed with water and the slides set with glycerin. All the sections were visualized by means of a Motic® BA 210 photomicroscope coupled to a 3MP Moticam camera.

Statistical analysis

The experimental design followed a randomized blocks in a 2 x 3 factorial scheme, the first factor comprised of 8-12 and 3-7 cm shoots that emerged sideways in the rootstocks of *P. mucronata*, and the second by 3 fasteners (connection systems: circular clip, "V" shaped clip and Parafilm®) for a total of six treatments with four repetitions of eight plants each. The data was subjected to variance analyses and the averages compared by the Tukey test at a 5% probability level using the Genes statistical program (Cruz, 2013).

Conclusions

The mini-grafting of scions derived from adult plants of *P. edulis* f. *flavicarpa* onto vegetatively propagated *P. mucronata* rootstocks represents a novel and reliable grafting approach, with advantages of not being destructive and using a rootstock resistant or immune to *F. oxysporum* f. sp. *passiflorae* and *Fusarium solani*. The attachment of the mini-grafting of *P. edulis* f. *flavicarpa* in clonal rootstocks of *P. mucronata* is greater with 8-12 cm shoots and with Parafilm®. The presence of starch in the reserve parenchyma is a biochemical marker of the compatibility in the mini-grafting.

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