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ORIGINAL ARTICLE

Efficient production of vigorous passion fruit rootstock for in vitro grafting

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Abstract

In vitro grafting is one of the promising techniques for fruit crop breeding. This study was performed to produce in vitro vigorous passion fruit rootstocks and developed a simple in vitro grafting protocol with the support of nylon microtubes—a low cost, wide availability, non-toxic and reusable material. The results showed that stem-lTCL explants (longitudinal thin cell layers) cultured on MS medium containing 0.5 mg/L BA and 0.5 mg/L NAA gave the highest shoot regeneration, and these shoots were initial materials for micropropagation of passion fruit rootstock. MSM medium containing 5 mg/L AgNPs increased shoot multiplication twofold higher than that of control and significantly reduced leaf yellowing and leaf drop during this stage. Rooting rate and quality of the plantlets were optimal on MSM medium contained with 2.5 mg/L IBA as well as their survival rate was enhanced in nursery. Nylon microtubes improved the efficiency of passion fruit in vitro grafts to 73.33% with yellow passion fruit as rootstock and purple passion fruit nodal segments as scion. In addition, the use of nylon microtubes significantly improved the in vitro growth of in vitro grafted plants resulting in increased survival rate and plantlet growth in the nursery stage. The present results provide an efficient protocol for micropropagation of yellow passion fruit for rootstock and production of purple passion fruit via nylon microtubule-mediated in vitro grafting.

Key message

An efficient protocol was established for multiplication of *in vitro* vigorous passion fruit rootstock via the application of thin cell layer culture techniques, nanotechnology and modification of the culture medium. Nylon microtubes enhanced the success rate of *in vitro* grafting and *ex vitro* grafted plant growth.

Keywords In vitro grafting · Nanotechnology · Nylon microtuble · Passion fruit · Thin cell layer culture · Vigorous rootstock

Introduction

In vitro grafting is widely known as an effective technique for effective, rapid and reliable plant rejuvenation and propagation at a commercial scale (Miguelez-Sierra et al. 2017; Pahnekolayi et al. 2019). To date, in vitro grafting methods have been continuously improved to increase the probability of success and be suitable for many crops of high economic value (Hussain et al. 2014; Gentile et al. 2017). In particular, in vitro grafting is the top recommended graft for the propagation of forest and fruit trees (Miguelez-Sierra et al. 2017) such as apples (Huang and Millikan 1980), peaches (Jonard 1986), grapes (Aazami and Hassanpouraghdam 2010), walnuts (Wang et al. 2010), almonds (Işıkalan et al. 2011), *Corylus colurna* L. (Gentile et al. 2017), rose (Pahnekolayi

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et al. 2019) and passion fruit (Ribeiro et al. 2015). Compared with field grafting, in vitro grafting was not affected by external factors such as soil, weather and disease management (Hussain et al. 2014) as well as easily improved the genetic homogeneity of both scion and rootstock (Işıkalan et al. 2011). In addition, grafting techniques generally allow extension of the ecological limits of a species through the tolerant properties of rootstocks (Richardson et al. 1996; Abad and Shekafandeh 2021). However, in vitro grafting has not yet been widely commercialized in practical production because of its low success rate (Ribeiro et al. 2015).

It is well known that the connection between the rootstock and the scion had a significant influence on the success of the graft. Therefore, in vitro grafting required meticulous manipulation and high precision (Ewens and Felker 2003). However, the small size of the scions or rootstocks made it difficult to manipulate precisely. Accordingly, several supporting materials for in vitro grafts have been used to increase the success rate such as filter paper bridges in apple in vitro grafts (Huang and Millikan 1980), elastic pads for peach in vitro grafts (Jonard 1986) and tubes silicon for prunus in vitro grafts (Gebhardt and Goldbach 1988). To the best of our knowledge, there were no detailed studies on testing supporting materials to improve in vitro grafting efficiency of passion fruit. It was well known that nylon microtubes are a material that had low cost, wide availability, flexible, heat resistance, easy sterilization, can be reused, easily assembled or disassembled (Zhang et al. 2021). In the present work, nylon microtubes were used as a support material to improve the efficiency of passion fruit in vitro grafting, thereby proposing an effective method for in vitro grafting this plant in a large scale.

Passion fruit—a tropical fruit crop with high economic value was widely grown in tropical and subtropical countries around the world (Machado et al. 2008). The color of the sour passion fruit is of paramount commercial importance; purple and yellow passion fruit (*Passiflora edulis* Sim.) are two commonly grown colors in the world (Beninca et al. 2007; Bernacci et al. 2008). Compared with purple varieties, yellow passion fruit had shown better resistance to diseases and insects such as leaf spot and wilt caused by *Fusarium oxysporum*, thrips and nematodes infestations (Dornelas and Vieira 1994; Amugune et al. 1993; Nakasone and Paull 1998). Therefore, yellow passion fruit has been widely used as rootstock for grafting with purple passion fruit (Payan et al. 1975; Thokchom and Mandal 2017). Conventionally, the yellow passion fruit is propagated primarily by seed; however, factors such as low germination rates, genetic heterogeneity and maternal spread of pathogens have reduced the effectiveness of this traditional approach (Nascimento et al. 2012). Furthermore, natural or active cross-pollination by hand is required to produce yellow passion fruit. However, the natural pollinators of passion fruit are gradually decreasing in number due to climate change, and the manual pollination process is both labor intensive and costly (Yamamoto et al. 2012). These greatly limited the number of passion fruit seeds produced (Knight and Winters 1963) and resulted in a shortage of rootstock needed for commercial passion fruit propagation.

In this study, an efficient micropropagation process of yellow passion fruit was established to produce a large number of vigorous passion fruit rootstocks for in vitro grafting. Accordingly, thin cell layer culture technique was applied to regenerate in vitro vigorous shoots (i.e. rejuvenating rootstocks); besides, some important factors affecting the regeneration, rapid multiplication and rooting of yellow passion fruit such as type of explant, plant growth regulator (PGR) and mineral concentration have been investigated. In addition, silver nitrate ($AgNO_3$) and silver nanoparticles (AgNPs) were added to the culture medium to increase the quality of passion fruit rootstocks through overcoming in vitro leaf yellowing and leaf drop phenomena. In particular, nylon microtubes have been applied as a support material for the precise coupling of rootstock and scion, thereby enhancing the success of in vitro grafting and opening up new prospects for passion fruit breeding.

Materials and methods

Initial plant materials

Leaf and stem explants from in vitro 2-month-old shoots of yellow passion fruit (*P. edulis* 'Monte Alegre') were used for the shoot regeneration experiments. In which, leaf and stem explants were cut into transverse thin cell layers (tTCL) with 1.0 mm in thickness or longitudinal thin cell layers (lTCL) with 10 mm in length and 2.5 mm in width.

Culture medium

MS medium (Murashige and Skoog 1962) and MSM medium (Monteiro et al. 2000) were used in this study. These media were supplemented with different PGR depending on the purpose of the experiments. The pH of the media were adjusted to 5.8 before autoclaving at 121 °C (1 atm) for 30 min.

Nanoparticles solution

Solutions of silver nanoparticles (AgNPs) were chemically synthesized (Chau et al. 2008) at the Institute of Environmental Technology, VAST. The synthetic precursor was $AgNO_3$, the stabilizer was β-chitosan and the reducing agent was $NaBH_4$; molar ratio $[NaBH_4]/[AgNO_3] = ¼$, the droplet rate of $NaBH_4$ was 10–12 drops/min. A homogeneous

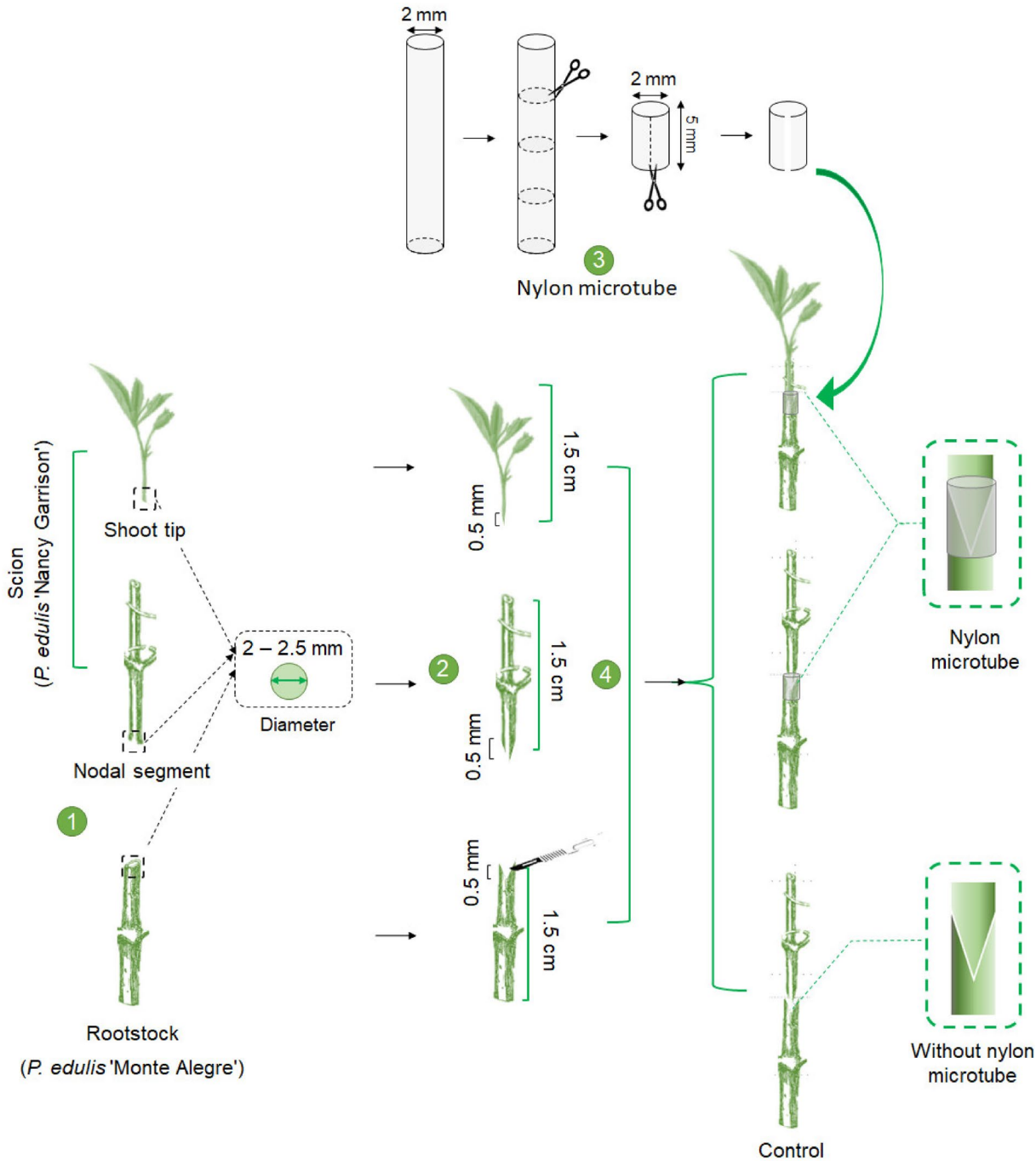

Fig. 1 Diagram illustrating 4 steps of in vitro grafting passion fruit using nylon microtubes as mediators. (1) Prepare the rootstock and scion. (2) Create a V-shaped joint. (3) Prepare nylon microtubes. (4) In vitro grafting using nylon microtubes

solution of AgNPs with an average particle size of ≤ 20 nm was obtained for use in experiments.

Nylon microtubes for in vitro grafts

Nylon microtubes (2 mm in diameter) composed of polyvinyl chloride (PVC) (Vinahankook Medical Supplies Co., JSC, Vietnam) were used in this study. The long nylon microtubes were cut into 5 mm lengths; then, the microtubes (5 mm in length) were cut along the lateral longitudinal line with scissors for easy insertion into the junction (Fig. 1); next, the cut microtubes were autoclaved at 121 °C (1 atm) for 30 min for use in the in vitro grafting experiment.

Experimental design

Adventitious shoot regeneration from in vitro leaf and stem explants

Experiments were performed to study the effect of explant type, explant cutting method and the ratio of exogenous hormones to shoot regeneration. The leaf-tTCL and leaf-lTCL explants, stem-tTCL and stem-lTCL explants were cultured on MS medium containing BA (0; 0.5; 1.0; 1.5; 2.0 mg/L) or in combination with NAA (0; 0.5; 1.0 mg/L). For control, hormone-free MS medium was used. Shoot regeneration (%), number of shoots and shoot height (cm) were recorded after 60 days of culture.

Effect of different strengths of MS medium on shoot multiplication

Based on the results of experiment 1, shoot tips (1 cm) with 1 pair of leaves were isolated from stem-lTCL explants for the shoot multiplication experiment. In order to optimize mineral nutrition for this stage, the shoot tips were cultured on strengths of MS medium (MS, ½ MS, ¼ MS, ¾ MS, MSM, ½ MSM) and added 1.0 mg/L BA, 1.0 mg/L Kin (Shekhawat et al. 2015). For control, hormone-free MS medium was used. Leaf drop (%), number of shoots, shoot height (cm), number of leaves and total chlorophyll content (nmol/cm^2) were recorded after 60 days of culture.

In which, the total chlorophyll content in leaves was measured by SPAD-502 device (Minolta Co., Ltd., Japan) as suggested by Khai et al. (2021). The measurements were performed on the second youngest leaf from the top.

Effect of AgNO3 and AgNPs on the ability to overcome leaf yellowing and leaf drop during shoot multiplication

This experiment evaluated the effects of $AgNO_3$ and AgNPs on the ability to overcome leaf yellowing and leaf drop during in vitro shoot multiplication. The shoot tips (1 cm) with 1 pair of leaves were cultured on MSM medium containing 1.0 mg/L BA, 1.0 mg/L Kin (based on the results of experiment 2) and $AgNO_3$ (0; 1.0; 2.0; 3.0; 4.0; 5.0 mg/L) or AgNPs (0; 1.0; 3.0; 5.0; 7.0; 9.0 mg/L). For control, silver ion-free shoot multiplication medium was used. Leaf drop (%), number of shoots, shoot height (cm), number of leaves and total chlorophyll content (nmol/cm^2) were recorded after 60 days of culture.

Effect of types and concentrations of auxin on in vitro rooting and acclimatization of plantlets to ex vitro conditions

This experiment was designed to optimize auxin types and concentrations on the in vitro rooting of yellow passion fruit. The shoot tips (1.5 cm) with 2 pair of leaves derived from shoot multiplication on MSM medium containing 1.0 mg/L BA, 1.0 mg/L Kin and 5.0 mg/L AgNPs were obtained as material for this experiment. The shoots were cultured on MSM medium added IBA, NAA or IAA with different concentrations (0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0 mg/L). For control, hormone-free MS medium was used. Rooting (%), number of roots, root length (cm), shoot height (cm), number of leaves, total chlorophyll content (nmol/cm^2) were recorded after 60 days of culture.

To evaluate the subsequent growth of plantlets in nursery conditions, plantlets derived from the IBA and NAA treatments were collected and inoculated into a pot containing Metro-Mix® 350 (Scottsco, Manisvill, Ohio). The plantlets were watered twice a day for the first 30 days and once a day thereafter. Survival rate (%) and plant height (cm) were recorded after 60 days of culture.

Evaluation of the effectiveness of nylon microtube-mediated in vitro grafting

This experiment was set up similar to the schematic diagram shown in Fig. 1. Two types of scions were tested, shoot tips (1.5 cm) and nodal segments (1.5 cm) of 2-month-old purple passion fruit (*P. edulis* 'Nancy Garrison') shoots cultured on hormone-free MS medium. The rootstocks were 2-month-old yellow passion fruit shoots derived from shoot multiplication on MSM medium added 1.0 mg/L BA, 1.0 mg/L Kin and 5.0 mg/L AgNPs (based on the results of experiment 3); these shoots were apex removed to homogenize into 1.5 cm as rootstock. The scions were removed from the leaves and cut into a V shape (5 mm in depth) and then fitted into the V longitudinal slot that was created on the rootstocks. The scion and rootstock were then attached using nylon microtubes; for the control, the graft was not used nylon microtubes. The explants were inoculated on MSM medium contained with 2.5 mg/L IBA (based on the results of experiment 4) for in vitro rooting. Successful in vitro grafting (%), rooting (%), number of roots, root length (cm), plant height (cm), number of leaves and total chlorophyll content (nmol/cm^2) were recorded after 60 days of culture.

Evaluation of acclimatization and subsequent growth of in vitro grafted plants

Steps to transfer in vitro grafts to the nursery were performed in the same way as described in experiment 4. Survival rate (%) and plant height (cm) were recorded after 60 days of culture.

Culture conditions

In vitro conditions: The explants were grown in culture room conditions with a temperature of 25 ± 2 °C, average humidity of 55%–60% and used fluorescent light (40–45 µmol.m^{-2}.s^{-1}) with a photoperiod of 16 h light/8 h dark.

Ex vitro conditions (nursery): The plants were grown under nursery conditions with a temperature of 18–25 °C, average humidity of 70%–75% and used natural light with 40% shading for the first month and did not shade after that.

Plant anatomy

Histological analysis was performed manually as described by Khai et al. (2021). Briefly, stem-lTCL explants were obtained every 5 days of culture for anatomy. Samples were sliced by hand with a razor blade and bleached in 10% javel for about 15 min; the sample was then neutralized in 10% acetic acid solution for 15 min; carmine iodine was used to stain the tissue for 3 min. Samples were rinsed with sterile distilled water after each step at least 3 times. Finally, the samples were placed on slides, covered with lamelle, observed and photographed under an optical microscope with magnifications × 10 and × 40.

Statistical analysis

The experiment was repeated 3 times and each replicate was 20 bottles/treatment with 3 explants/bottle (except for shoot multiplication, in vitro rooting and in vitro grafting which were 1 explant/bottle). SPSS software version 22.00 was used for statistical analysis of the data. Data were analyzed by ANOVA, and the post hoc tests were performed using Duncan's test with statistical significance established at α = 0.05 (Duncan 1995).

Results and discussion

Selection of explant type and PGR for shoot regeneration stage

The results showed that only the stem-lTCL explants regenerated shoots among the investigated samples. Accordingly, the stem-tTCL, leaf-tTCL and leaf-lTCL explants all did not react and began to necrosis after 60 days of culture; thus, in vitro leaf was not able to regenerate shoots on the investigated medium. It was shown that although the regenerative potential of stem explants was higher than that of leaf in vitro, the shoot regeneration efficiency was dependent on explant excision. The ratio of exogenous hormones also affected the shoot regeneration efficiency of yellow passion fruit

Table 1 Effect of BA and NAA on shoot regeneration from yellow passion fruit stem-lTCL after 60 days of culture

BA (mg/L)	NAA (mg/L)	Shoot regeneration (%)	No. of shoots	Shoot height (cm)
0.0	–	0.00^{i*}	0.00^{f}	0.00^{e}
0.5	–	0.00^{j}	0.00^{f}	0.00^{e}
1.0	–	58.33^{c}	0.00^{f}	0.00^{e}
1.5	–	66.67^{b}	2.33^{bc}	0.43^{c}
2.0	–	33.33^{f}	0.00^{f}	0.00^{e}
0.5	0.5	10.00^{i}	1.00^{e}	0.23^{d}
1.0	0.5	35.00^{ef}	2.67^{ab}	0.47^{c}
1.5	0.5	26.67^{g}	1.67^{d}	0.43^{c}
2.0	0.5	20.00^{h}	0.00^{f}	0.00^{e}
0.5	1.0	13.33^{i}	0.00^{f}	0.00^{e}
1.0	1.0	43.33^{d}	2.00^{cd}	0,77^{b}
1.5	1.0	71.67^{a}	3.00^{a}	1.13^{a}
2.0	1.0	38.33^{e}	1.67^{d}	0.83^{b}

*Different letters (a, b, …) in the same column represent statistically significant differences at α = 0.05 (Duncan's test)

(Table 1). Accordingly, the combination of BA and NAA gave a higher regeneration efficiency than the BA treatments. The stem-lTCL explants cultured on MS medium containing 1.5 mg/L BA and 1.0 mg/L NAA gave the rate of shoot regeneration (71.67%), number of shoots (3.00 shoots) and shoot height (1.13 cm) was significantly higher than the control (hormone-free medium) and other treatments (Fig. 2). Histological evidence showed that the subepidermal and epidermal tissue areas were the site of the formation of meristems after 5 days of culture (Fig. 2A); the formation of leaves and vascular connections to the mother tissue showed direct shoot organogenesis on the explant surface after 15 days of culture (Fig. 2B). Morphological observations showed that shoots were regenerated directly without callus formation when inoculated on MS medium containing 1.5 mg/L BA and 1.0 mg/L NAA (Fig. 2C). The shoots from this treatment showed uniform in size, vigorous and dark green leaves (Fig. 2D).

It is well known that shoot organogenesis is an important morphogenesis in plant tissue culture (Becerra et al. 2004). The efficiency of shoot regeneration depends mainly on the proportion of exogenous hormones used; whereby cytokinins alone or in combination with auxins are commonly used for shoot induction and development (Chattopadhyaya et al. 2010). In the present case, using BA resulted in significantly lower shoot regeneration than the combined treatments of BA and NAA.

In addition, the combination of BA and NAA at the appropriate ratio resulted in direct shoot regeneration from the stem-lTCL explants without callus-mediated. The available

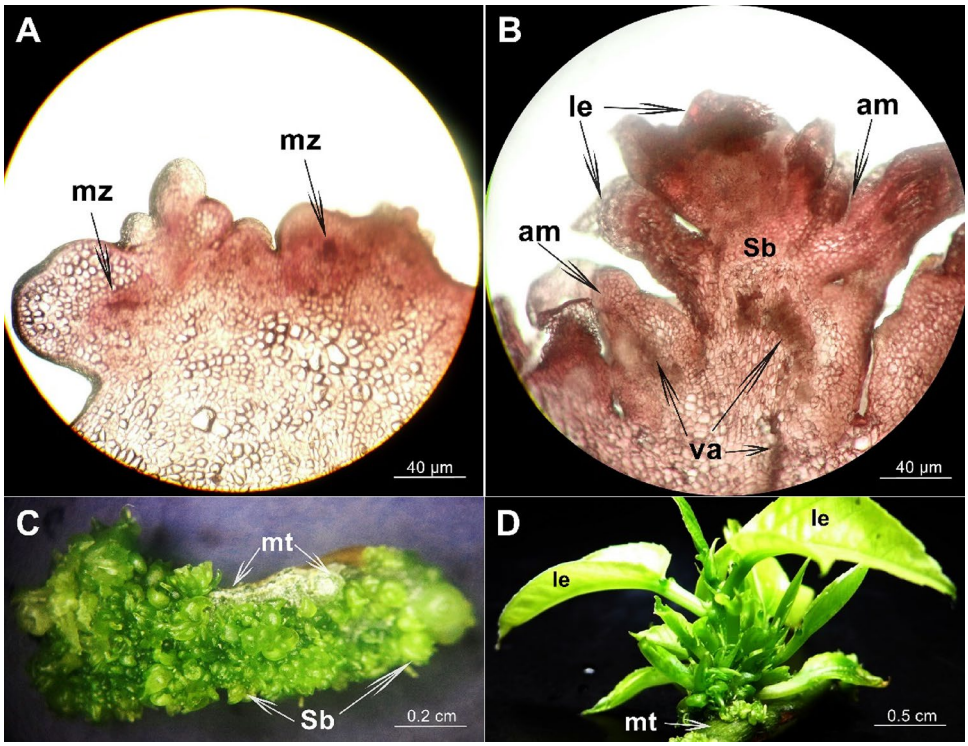

Fig. 2 Shoot regeneration from yellow passion fruit stem-lTCL on MS media supplemented with 1.5 mg/L BA and 1.0 mg/L NAA. **A.** Shoot induction after 5 days of culture. **B.** Direct shoot organogenesis after 15 days of culture. **C.** High frequency of shoot organogenesis from stem-lTCL after 30 days of culture. **D.** Morphology of regenerated shoots after 60 days of culture. Apical meristem (am), meristem zone (mz), mother tissue (mt), leaf (le), shoot bud (sb) and vascular tissue (va)

studies on the micropropagation of *P. edulis* f. *Flavicarpa* has shown that the use of BA induces callus during shoot regeneration and as a result affects the shoot regeneration coefficient (Biasi et al. 2000; Nhut et al. 2007). These results indicate that exogenous hormone ratios play an important role in the in vitro shoot organogenesis pathway.

For in vitro regeneration of the genus *Passiflora*, explant sources have been used such as apical shoots, stem segments, axillary shoots, leaves, roots and cotyledons which have been reported previously (Trevisan and Mendes 2005; Fernando et al. 2007; Palm Pinto et al. 2011; Silva et al. 2015; Pacheco et al. 2016). However, plant organs that were cut into thin cell layers are known to have the highest regenerative efficiency (Nhut et al. 2003; Singh et al. 2008). Based on its thin nature, the TCL culture system readily absorbs the nutrients and hormones required for regeneration. Our experimental results show that longitudinal stem cutting (lTCL) has a higher regeneration efficiency than transverse cutting (tTCL). Therefore, the shoot regeneration efficiency of yellow passion fruit was dependent not only on the exogenous hormone ratio but also on the explant type and excision method.

Optimizing MS medium strength on shoot proliferation

Mineral deficiency or excess is responsible for leaf yellowing and growth retardation in in vitro cultured *Passiflora* (Monteiro et al. 2000). Therefore, the concentrations of macronutrients were investigated in order to optimize for the yellow passion fruit shoot multiplication stage. The results showed that the composition and concentration of minerals in the culture medium significantly affected the number and quality of shoots after 60 days of culture (Table 2). The reduction of mineral concentration of MS medium to ¼ MS, ½ MS, ¾ MS led to a decrease in shoot growth compared with MS treatment, which was demonstrated by the parameters of shoots/explant and shoot height and number of leaves (Table 2). While changing the composition and mineral content of MS medium corresponding to MSM medium, the number of shoots/explant (4.67), number of leaves (4.67), chlorophyll content (26.83 nmol/cm^2) was higher significantly compared with other treatments. In addition, the frequency of leaf drop of MSM was significantly lower than that of other treatments; however, more than half of the shoots of this treatment had dropped leaves causing a significant reduction in shoot quality (Fig. 3A). Morphological observation showed that the shoots grown on MSM medium were uniform in size, vigorous with dark green leaves.

Common symptoms of mineral deficiencies of passion fruit can be identified visually by leaf color (yellow leaves) or chlorophyll pigment (low content) (Kantharajah and Dodd 1990). In the present case, the substitution of MS medium for MSM medium gave a positive effect on shoot multiplication efficiency and yellow passion fruit shoot quality; furthermore, leaf yellowing was overcome which corresponds to high chlorophyll content in leaves (Table 2). Compared

Table 2 Effect of different strengths of MS medium on shoot multiplication of yellow passion fruit after 60 days of culture

Medium	No. of shoots	Shoot height (cm)	No. of leaves	Total chlorophyll (nmol/cm^2)	Leaf drop (%)
MS	3.00^{bc*}	1.87^{a}	4.33^{a}	17,73^{c}	86.67^{a}
¼ MS	3.67^{b}	1.43^{b}	3.33^{b}	18.73^{b}	76.67^{bc}
½ MS	2.67^{cd}	1.30^{c}	3.00^{b}	5.37^{e}	83.33^{ab}
¾ MS	2.00^{d}	1.20^{c}	2.67^{b}	4.77^{e}	81.67^{cd}
MSM	4.67^{a}	1.47^{b}	4.67^{a}	26.83^{a}	63.33^{d}
½ MSM	3.33^{bc}	1.23^{c}	3.33^{b}	16.00^{d}	71.67^{c}

*Different letters (a, b, …) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan's test)

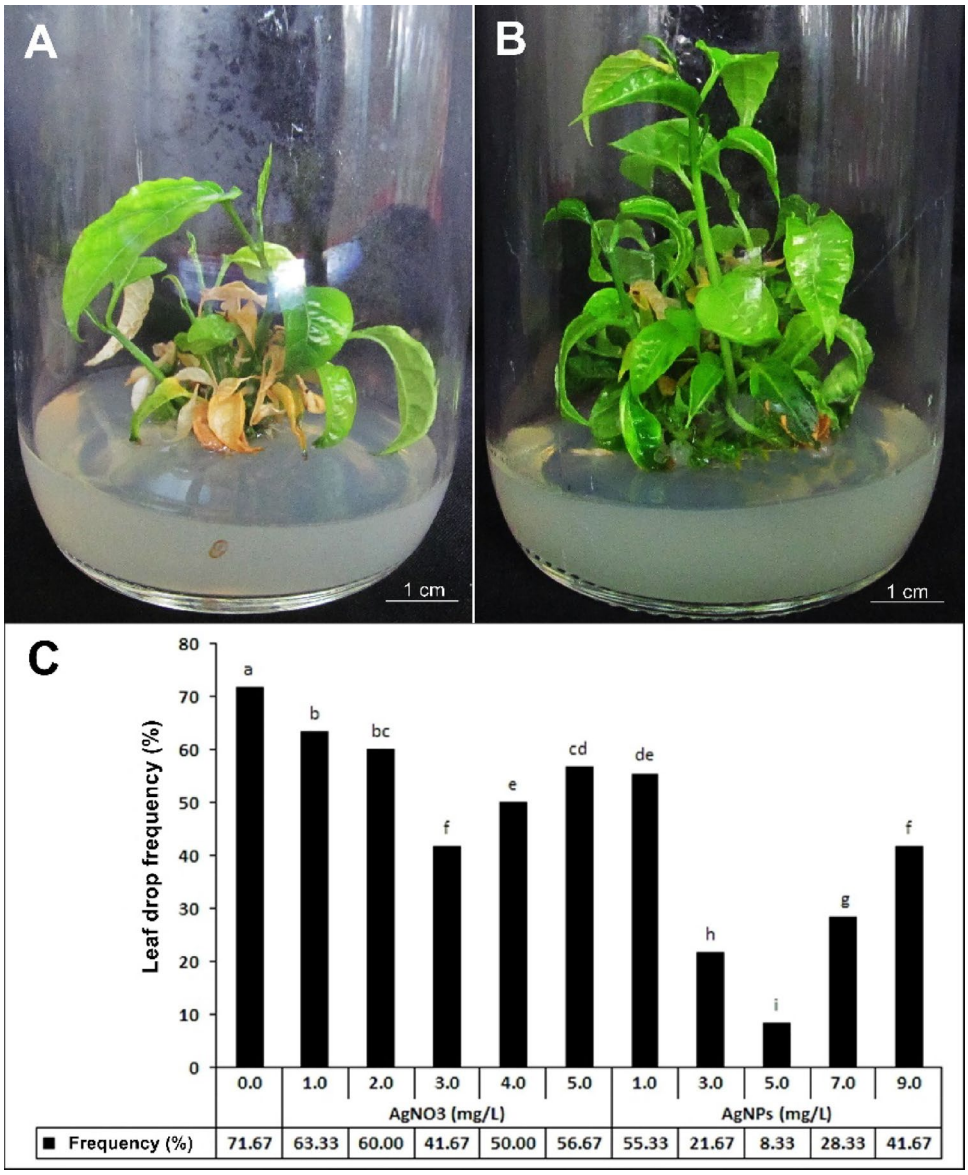

Fig. 3 Effect of AgNPs and $AgNO_3$ on shoot multiplication efficiency and inhibition of leaf drop of yellow passion fruit after 60 days of culture. **A** Control treatment. **B** Treatment supplemented with 5.0 mg/L AgNPs. **C** Comparison of leaf drop frequency between AgNPs and $AgNO_3$ treatments

with MS medium, MSM medium helps to compensate for iron and calcium mineral deficiencies and reduce possible toxicity of Chlorine (Monteiro et al. 2000). Therefore, the application of MSM medium for the shoot multiplication stage of yellow passion fruit gave a positive effect on the number and quality of shoots.

Table 3 Effect of $AgNO_3$ and AgNPs on shoot multiplication and overcoming leaf drop of yellow passion fruit after 60 days of culture

$AgNO_3$ (mg/L)	AgNPs (mg/L)	No. of shoots	Shoot height (cm)	No. of leaves	Total chlorophyll (nmol/cm^2)
0	0	4.33^{fg*}	1.57^{f}	4.33^{f}	26.40^{e}
1.00	–	5.00^{def}	1.83^{de}	5.33^{de}	27.27^{d}
2.00	–	6.33^{c}	2.13^{c}	6.00^{cd}	27.97^{c}
3.00	–	7.33^{b}	2.57^{b}	6.67^{bc}	29.40^{b}
4.00	–	4.67^{ef}	1.97^{cd}	4.67^{ef}	27.17^{d}
5.00	–	3.33^{g}	1.77^{e}	4.00^{f}	26.73^{e}
–	1.00	5.33^{de}	2.13^{c}	5.33^{de}	27.80^{c}
–	3.00	7.33^{b}	2.43^{b}	7.00^{b}	29.43^{b}
–	5.00	9.00^{a}	3.40^{a}	8.33^{a}	32.07^{a}
–	7.00	5.67^{cd}	2.53^{b}	6.33^{bc}	25.97^{f}
–	9.00	3.67^{fg}	1.80^{de}	5.33^{de}	24.17^{g}

*Different letters (a, b, …) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan's test)

Overcoming leaf drop during shoot proliferation with AgNPs and $AgNO_3$

In vitro leaf drop is one of the common phenomena that degrades the quality of tissue cultured plants. In this study, silver ions in the form of salt ($AgNO_3$) and nanoparticles (AgNPs) were used to compare the ability to overcome leaf drop in yellow passion fruit. The results showed that the addition of 5.0 mg/L AgNPs to the culture medium significantly reduced the frequency of leaf drop to 8.33%, while the treatment adding 3.0 mg/L $AgNO_3$ was 41.67% and not adding silver ion was 71.67% (Fig. 3A–C). Furthermore, the number of shoots (9.00 shoots), shoot height (3.40 cm), number of leaves (8.33 leaves) and chlorophyll content (32.07 nmol/cm^2) in the 5.0 mg/L AgNPs treatment were significantly higher than the control and other treatments (Table 3). The shoots in this treatment had uniform size and no leaf yellowing or leaf drop (Fig. 3B). However, at high concentration of AgNPs (9.0 mg/L), the effectiveness of overcoming leaf drop, number of shoots and quality of shoots were significantly reduced; similar results were observed at a concentration of 5.0 mg/L $AgNO_3$ (Table 3).

Similar results were reported in bananas at low concentrations (1.0–5.0 mg/L) of both $AgNO_3$ and AgNPs were used, which had a strong effect on shoot growth as well as increased total chlorophyll content; while high concentrations (10–200 mg/L) of $AgNO_3$ and AgNPs showed the opposite effect (El-Mahdy et al. 2019). Similar results have also been reported in citrus supplemented with $AgNO_3$ (Marutani-Hert et al. 2011), in vanilla with AgNPs as ethylene inhibitors (Spinoso-Castillo et al. 2017), in roses with AgNPs (Ngan et al. 2020) and in strawberries with AgNPs (Tung et al. 2021). The results indicate that the use of silver ions at high concentrations can easily cause plant toxicity leading to plant growth failure or even inhibition.

STS, $AgNO_3$ and AgNPs are compounds that have been widely applied to inhibit ethylene activity in micropropagation (Steinitz et al. 2010; Homaee and Ehsanpour 2015; Mahmoud et al. 2020; Ngan et al. 2020; Tung et al. 2021). At appropriate concentration, silver ions inhibit ethylene activity in plants because it displaces copper ion (Cu^{2+}) resulting in ethylene not binding to ethylene receptor (ETR1) (Kumar et al. 2009). AgNPs possess a large surface area for easy uptake and transport in plants (Ingle et al. 2008; Ngan et al. 2020), while the thiosulfate ions $S_2O_3^-$ present in STS prevents the precipitation of Ag^+ and ion chloride (Cl^-) in the culture medium (Steinitz et al. 2010). Therefore, silver ion-containing compounds have shown different effects on plants. For example, STS was superior to $AgNO_3$ in inducing rooting in *Corymbia maculata* (Steinitz et al. 2010), while STS, $AgNO_3$ and AgNPs were all equally effective in reducing finger lime leaf drop (Mahmoud et al. 2020), for potato AgNPs stimulated shoot growth in vitro better than $AgNO_3$ (Homaee and Ehsanpour 2015). In this case, the application of 5.0 mg/L AgNPs gave optimal shoot multiplication efficiency and overcame the in vitro leaf drop of yellow passion fruit than $AgNO_3$ and control. These results suggest that the application of AgNPs improved the quantity and quality of passion fruit rootstocks which can be practically applied for large-scale production. Further studies require to study, compare and clarify the mechanism of ethylene inhibitors such as cobalt nanoparticles, AgNPs, STS, $AgNO_3$, cobalt chloride or aminooxyacetic acid, etc. in the micropropagation of passion fruit and other species.

Table 4 Effect of NAA and IBA on in vitro rooting of yellow passion fruit shoots after 60 days of culture

NAA (mg/L)	IBA (mg/L)	Rooting (%)	No. of roots	Root length (cm)	Shoot height (cm)	No. of leaves	Totall chlorophyll (nmol/cm^2)
0.0	0.0	0.00^{j*}	0.00^{g}	0.00^{i}	3.47^{i}	4.00^{fg}	28.73^{c}
0.5	–	28.33^{h}	1.33^{f}	0.97^{bcd}	5.53^{de}	4.67^{ef}	27.17^{d}
1.0	–	33.33^{g}	1.67^{ef}	0.63^{efg}	3.00^{j}	2.67^{i}	25.17^{f}
1.5	–	40.00^{f}	2.67^{cd}	0.43^{gh}	6.07^{c}	4.00^{fg}	24.20^{g}
2.0	–	68.33^{b}	3.67^{b}	1.50^{a}	6.57^{a}	6.67^{b}	26.27^{e}
2.5	–	60.00^{c}	2.67^{cd}	0.77^{def}	4.87^{gh}	7.00^{b}	27.07^{d}
3.0	–	48.33^{e}	2.33^{de}	1.10^{bc}	6.73^{b}	6.33^{bc}	25.67^{f}
–	0.5	18.33^{i}	1.00^{f}	0.23^{hi}	4.57^{hi}	3.67^{gh}	25.60^{f}
–	1.0	38.33^{f}	2.67^{cd}	1.00^{bcd}	4.77^{hi}	3.00^{hi}	26.96^{d}
–	1.5	48.33^{e}	3.00^{bcd}	1.27^{ab}	5.33^{ef}	5,33^{de}	30.10^{b}
–	2.0	53.33^{d}	3.33^{bc}	0.63^{efg}	5.80^{cd}	5.67^{cd}	30.27^{b}
–	2.5	75.00^{a}	4.67^{a}	0.93^{cde}	8.10^{a}	8.67^{a}	31.73^{a}
–	3.0	56.67^{cd}	3.33^{bc}	0.50^{fgh}	5.13^{fg}	5.67^{cd}	32.10^{a}

*Different letters (a, b, …) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan's test)

Selection of auxins for rootstocks rooting and assessment of their acclimatization in the greenhouse

Effects of type and concentration of auxin on rooting ability of in vitro shoots of yellow passion fruit after 60 days of culture were recorded and shown in Table 4. The results showed that IBA gave a higher in vitro rooting efficiency than NAA while all IAA treatments did not induce rooting. Accordingly, 2.5 mg/L IBA gave higher frequency of rooting (75.00%), number of roots (4.67), shoot height (8.10 cm), number of leaves (8.67) and chlorophyll content (31.73 nmol/cm^2) compared with the control and other treatments (Table 4). Morphological observations showed that the roots formed on the medium contained in the treatment of 2.5 mg/L IBA had fairly regular sizes, long, strong and supple roots that were favorable for acclimatization. However, at high concentrations of NAA (3 mg/L) and IBA (3 mg/L) both showed significant inhibition on rooting and shoot growth (Table 4). As reported by Baker and Wetzstein (1994), high concentrations of exogenous auxins are responsible for stimulating the biosynthesis of secondary metabolites at high concentrations, which in turn may lead to inhibition of root formation. Furthermore, an excess of auxin can lead to ethylene accumulation in the culture vessel which also leads to inhibition of root induction (De Klerk 2002). Compared with other auxins, IBA stimulates root formation more effectively because IBA is structurally stable and less sensitive to auxin-degrading enzymes produced by plants (Dobránszki and da Silva 2010). For *Passiflora* species, IBA was shown to stimulate root formation more strongly than others (Prammanee et al. 2011; Ragavendran et al. 2012; Prithrivaj et al. 2015; Jafari et al. 2017). In this study, the addition of 2.5 mg/L IBA to the culture medium was suitable for the in vitro rooting stage of yellow passion fruit and it created an important premise for the subsequent in vitro grafting stage.

After the in vitro rooting stage, all plantlets from the NAA and IBA treatments were transferred to the nursery to assess the adaptability and growth of yellow passion fruit plantlets under *ex vitro* conditions. The results showed that plantlets derived from IBA-added treatments adapted and grew better than NAA treatments; while the control plants (without roots) completely died after 30 days of culture in *ex vitro* conditions (Fig. 4A, B). The tissue cultured plants derived from the treatment added 2.5 mg/L IBA achieved the highest survival rate (nearly 75%), besides the plantlets of this treatment showed significantly higher plant height than the other treatments (Fig. 4B). Morphological observations showed that the plantlets from this treatment showed vigorous growth, relatively uniform plants, dark green leaves and long internodes (Fig. 4B). Similarly, as has been reported by Jafari et al. (2017) *Passiflora caerulea* L. plantlets derived from in vitro rooting with IBA showed higher survival rates than NAA and IAA. The results show the important role of the in vitro rooting stage and the selection of the appropriate type and concentration of auxin for this stage because it directly affects the adaptability of the plantlets in the nursery (Daud et al. 2013).

The results showed that the in vitro shoots of passion fruit without roots did not survive when transferred to the soil; conversely, plantlets possessing more in vitro roots had a higher survival rate during the acclimatization stage. In summary, the addition of 2.5 mg/L IBA to the culture

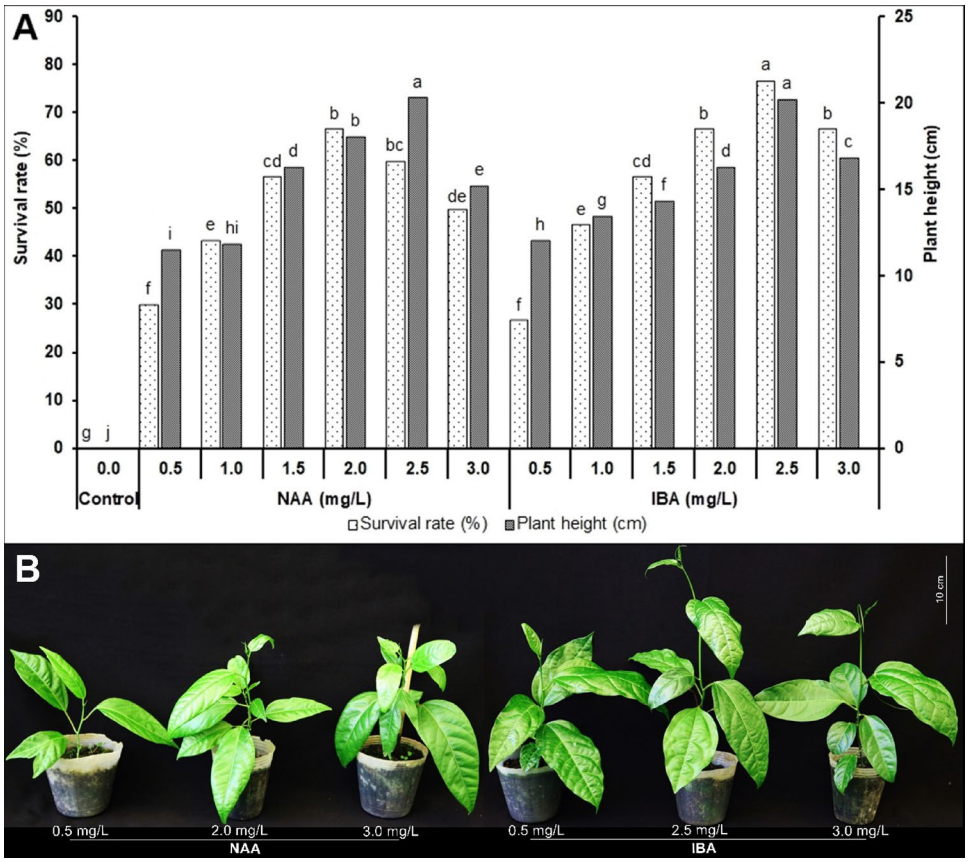

Fig. 4 Survival rate and subsequent growth of yellow passion fruit plantlets derived from culture on medium supplemented with IBA and NAA after 60 days transferring to the nursery. **A** Survival rate and plant height. **B** Subsequent growth of plantlets

Table 5 Effect of nylon microtube support and scion type on the growth of in vitro grafted passion fruit plants after 30 days of culture

Treatment	Rooting (%)	No. of roots	Root lenght (cm)	Plant height (cm)	No. of leaves	Total chlorophyll (nmol/cm^2)
nMt						
Shoot tips	33.33^{b*}	2.33^{b}	1.26^{b}	5.23^{c}	4.00^{b}	29.00^{d}
Nodal segments	41.67^{b}	3.00^{ab}	1.33^{b}	6.07^{b}	4.00^{b}	29.83^{c}
Mt						
Shoot tips	67.78^{a}	3.00^{ab}	1.87^{a}	5.90^{b}	5.00^{a}	30.60^{b}
Nodal segments	79.52^{a}	3.33^{a}	1.23^{b}	8.43^{a}	5.33^{a}	31.20^{a}

*Different letters (a, b, …) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan's test). nMt (no supporting nylon microtubes). Mt (support nylon microtubes)

medium enhanced the in vitro rooting frequency of yellow passion fruit as well as improved their survival during the nursery stage. This result is an important basis for subsequent in vitro grafting.

Nylon microtubes as an excellent support material for the connection between rootstock and scion

It is well known that the success rate of grafting depends on the connectivity of the vascular region between the scion and the rootstock (Singh et al. 2008; Ribeiro et al. 2015). However, this fusion was hindered due to the small explant size and soft texture (Aazami and Hassanpouraghdam 2010; Pahnekolayi et al. 2019) as well as the lack of support materials. In this study, nylon microtubes were initially tested as a support material for passion fruit in vitro grafts. The results showed that the in vitro grafting efficiency was significantly higher in the presence of microtubes (Mt) than in the non-microtube treatment (nMt). On the other hand, increased in vitro grafting efficiency when using nodal segments as scions instead of shoot tips was observed (Table 5, Fig. 5). Accordingly, the in vitro grafting treatment using

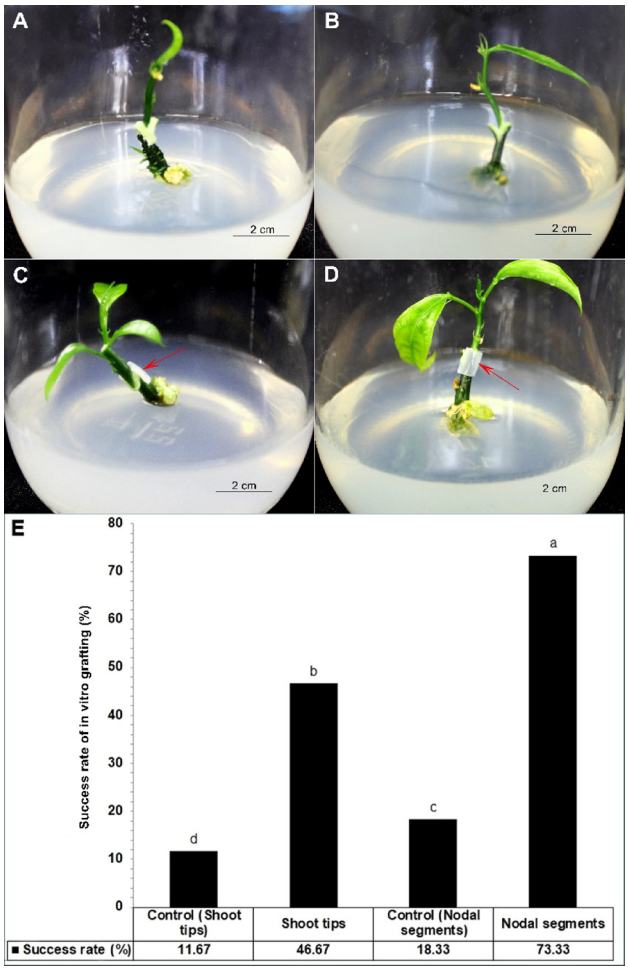

Fig. 5 Effect of nylon microtube support (Mt) and no nylon microtube support (nMt) on successful in vitro grafting of passion fruit (yellow passion fruit as rootstock and purple passion fruit as scion) after 60 days of culture. **A** In vitro grafting used nodal segments as the scion and nMt. **B** In vitro grafting used shoot tips as the scion and nMt (arrow). **C** In vitro grafting used nodal segments as the scion and Mt. **D** I*n vitro* grafting used shoot tips as the scion and Mt (arrow). **E** In vitro grafting success rate

nodal segments as scions with Mt gave the highest success rate (73.33%) compared with other treatments; in the case of nMt, the micrograft efficiency of both scions was lower than 20% (Fig. 5E). Research by Biricolti and Chiari (1994) has shown that the success rate of micrografting has reached 50% when using meristem as scion (derived from purple passion fruit *ex vitro*) grafted with cotyledon rootstock of purple passion fruit without supporting material.

The results of growth indicators showed that the micrografted plants used nodal segments as scions with Mt had the highest plant height and chlorophyll content (8.43 cm and 31.20 nmol/cm^2 respectively) (Table 5). This result indicates that the shoot tips were growing slower than the nodal segments were used as scions. Therefore, the nodal segments were used as the material for in vitro grafting, and the shoot tips were proposed as the material for the shoot multiplication stage to provide material for the subsequent in vitro grafting. Interestingly, during growth the micrografts did not induce leaf drop; it could be explained by the residual Ag^+ in the passion fruit rootstocks derived from culture on medium supplemented with 5.0 mg/L AgNPs. Although further studies are needed to quantify the Ag^+ residues in these explants, it is clear that the application of AgNPs during the shoot multiplication stage had a positive effect on the grafting stage. Ribeiro et al. (2015) reported that the vascular connection between scion and rootstock of passion fruit was established after 20 days of micrografting; while the success of grafts was observed to depend on the growth of vascular tissue between the graft and the rootstock which is fixed at the graft site by duct tape. Also, several new supporting materials have been applied to increase the success rate of in vitro grafting, such as filter paper bridges in apple (Huang and Millikan 1980), elastic pads in peach (Jonard 1986), silicon tubes in prunus (Gebhardt and Goldbach 1988), aluminum foil, parafilm, paper bridge in rose (Pahnekolayi et al. 2019). Supporting materials have the role of increasing the contact capacity at the graft site to create callus, thereby promoting the junction between the sion and the rootstock (Pahnekolayi et al. 2019). Finally, the supporting materials can easily be separated from the graft site after successful grafting (Gebhardt and Goldbach 1988).

Morphological observations showed that the Mt treatments controlled the overgrowth of callus at the graft site while the opposite was observed in the nMt treatments. In the study of Biricolti and Chiari (1994) on purple passion fruit also recorded the formation of callus on the rootstock surface after 5 days of micrografting and this affected the formation and development of shoots. Similar to the study of Navarro et al. (1975), callus completely covered 50% of micrografted meristems prevented shoot elongation. It is well known that the induction of callus promotes cell junction between the scion and the rootstock (Ribeiro et al. 2015; Pahnekolayi et al. 2019); however, excessive callus proliferation at the graft wound is synonymous with disruption of water, nutrient and hormone transport in the graft (Koufan et al. 2020). The results indicated that the support of nylon microtubes not only immobilized the scion and rootstock, but also affected the growth of callus at the graft site.

Acclimation of micrografted plants in nursery conditions

Successful micrografted plants after 30 days of culture were transferred to nursery conditions to evaluate their adaptability and growth in nursery conditions. After 60 days of transferring to the nursery (Fig. 6A), the results showed that the micrografted plants derived from the treatment of using stem as scions with Mt achieved survival rate (83.33%) and plant

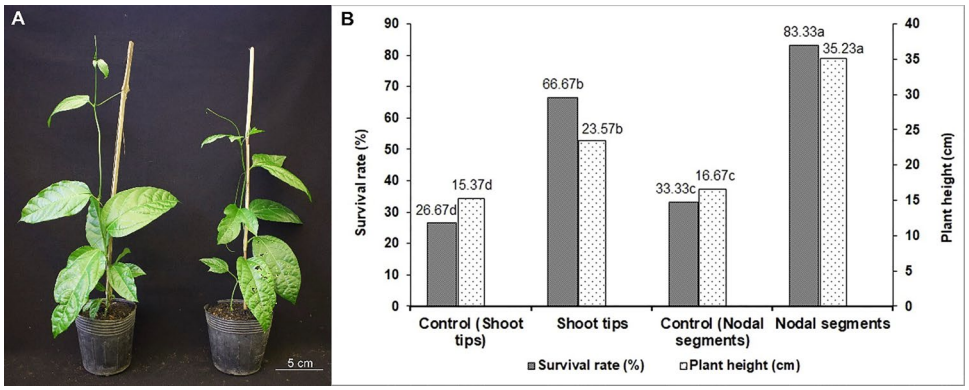

Fig. 6 Survival rate and subsequent growth of in vitro grafting passion fruit plantlets after 60 days transferring to the nursery. **A** Plantlets derived from in vitro grafting used nodal segments as the scion and Mt; in vitro grafting-derived plantlets used shoot tips as the scion and Mt (from left to right respectively). **B** Survival rate and height of in vitro grafted plantlets

height (35.23 cm) was the highest. In the case of in vitro grafts derived from the treatment with nMt showed a 35% lower survival rate and 20 cm lower plant height (Fig. 6B). These results suggested that the application of nylon microtubes in passion fruit in vitro grafts enhanced the survival of the plantlets in the nursery stage. The results of previous studies showed that the survival rate of micrografts in the *ex vitro* stage varied between species such as 100% in almond (Yıldırım et al. 2010), 85% in cherries (Bourrain and Charlot 2014); 75% in *Khasi mandarin* (Singh et al. 2019), whereas in *Kinnow mandarin*, survival rates ranged from 45.82% to 70.83% depending on the rootstock used (Chand et al. 2016). In this study, the use of nylon microtubes increased the success of passion fruit in vitro grafts leading to an improved in the quality of plantlets and enhanced their survival rate during the acclimatization stage.

Conclusions

The results of this study have developed an improved yellow passion fruit rootstock micropropagation and in vitro grafting using nylon microtubes as mediators. In the shoot rejuvenation stage, stem-lTCL explants cultured on MS medium containing 1.5 mg/L BA and 1.0 mg/L NAA gave the best shoot regeneration. MSM medium contained with 1.0 mg/L BA, 1.0 mg/L Kin and 5.0 mg/L AgNPs increased the efficiency of shoot multiplication and overcame the leaf drop in this stage. Rooting efficiency and plantlet quality were optimal on MSM medium containing 2.5 mg/L IBA as well as their survival rate was enhanced in the nursery stage. Nylon microtubes have been confirmed to be excellent supporting materials to enhance in vitro grafting of passion fruit with yellow passion fruit as rootstock and purple passion fruit nodal segments as scions. In addition, the use of nylon microtubes significantly improved the growth of in vitro grafts which resulted in an increase in their growth and survival rate in the nursery.

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Author contribution NTD, Tran Hieu and Hoang Dac Khai designed the study. Truong Hoai Phong, Nguyen Thi Nhu Mai, Do Manh Cuong, Vu Quoc Luan, Hoang Thanh Tung and Nguyen Ba Nam performed the experiments and analyzed the data. Tran Hieu, Nhut Tan Duong and Hoang Dac Khai wrote the original draft.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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