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Full Length Article

Techniques for Improving the Tissue Culture Efficiency of Purple Passion Fruit (*Passiflora edulis*)

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Abstract

Purple passion fruit (*Passiflora edulis* Sims) has gained attention in Southern China, and its planting area has increased during the last several years. Through tissue culturing, virus-free plants are produced as maternal parents for seedling production. However, there are some difficulties that affect passion fruit tissue culture efficiency, including high contamination rates in explant disinfection, low shoot proliferation, yellow or albino leaves, slow growth, and time-consuming processes. In this work, the aforementioned problems were investigated, and disinfection was optimized. Results revealed that the repeat disinfection method (0.1% $HgCl_2$ for 15 min + 0.1% $HgCl_2$ for 12 min) with a 2-d interval was the most suitable disinfection treatment for young stem segments of purple passion fruit. The addition of silver thiosulfate (STS) improved proliferation efficiency. Moreover, additional 1X iron salt was added to the bud induction and rooting medium. The regenerated shoots had a better seedling state with healthier green leaves, roots were more easily induced and better developed and the chlorophyll contents were higher. Thus, more efficient tissue culturing of purple passion fruit was achieved. © 2021 Friends Science Publishers

Keywords: Passion fruit; Tissue culture efficiency; Disinfection; Silver thiosulfate; Iron salt

Introduction

Purple passion fruit (*Passiflora edulis* sims) is a vine crop that belongs to genus P*assiflora* and family Passifloreae. This was originated in South America and now widely cultivated in tropical and subtropical areas across the world (Ulmer and MacDougal 2004; Ortiz *et al.* 2012; Silva *et al.* 2014). Passion fruit is rich in a variety of flavors that is similar with pineapple, mango, guava, banana, and several other fruits (Fu *et al.* 2005; Janzantti and Monteiro 2017). The Chinese name for passion fruit is 'Bai Xiang Guo', which means fruit with 100 kinds of aroma. Passion fruit has got attention among consumers because of its unique flavor and nutritional elements. As a result, its planting area has expanded in Southern China as it is a short-term crop with high economic returns (Huang *et al.* 2019a). Two purple varieties, 'Tainong' and 'Zixiang', and a yellow variety, 'Golden yellow', are the main varieties cultivated in China. Virus-free seedlings are in high demand in China. Through tissue culturing, virus-free, true-to-type plants can be produced as planting materials for propagation (Prammanee *et al.* 2011). Tissue culturing is also an important tool for the *ex situ* conservation of *Passiflora* germplasm (Pacheco *et al.* 2016).

However, there are some difficulties that affect passion fruit tissue culture efficiency, including high contamination rates in explant disinfection, low bud proliferation, yellow and/or albino leaves, and slow growth. Due to its complex environment and the influence of diseases and insect pests, passion fruit stem tips and segments that were used as explants from the field contain endophytic bacteria. It is difficult to completely kill endophytic bacteria by ordinary disinfection methods, so it is necessary to improve the methods, which include repeat disinfection, mixed disinfectants with different agents, antibiotic pretreatment and the addition of antimicrobial agents in culture medium (Ji *et al.* 2011). The proliferation rate of passion fruit tissue culture is 1–3 per explant and the proliferation efficiency is low and time-consuming, lasting ~6 months or longer (Tuhaise *et al.* 2019), which is far lower than seedling cutting and grafting. Therefore, the application of tissue culture seedling production was of little significance and has not been applied in seedling production.

Node and primordia regions, which are essential for maintenance and growth during cell division, are the main sites of ethylene synthesis (Jha *et al.* 2007). Less elongation and radial swelling of the stem are primary indications of higher ethylene accumulation (Danish *et al.* 2019). Ethylene

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peaks occur during meristemoid differentiation in both *P. edulis* f. *flavicarpa* and *P. cincinnata*, resulting in delayed shoot induction (Dias *et al*. 2009). Therefore, it is necessary to optimize the shoot induction and proliferation system to obtain higher proliferation efficiency. Leaf edges of tissue culture shoots become yellow or albino at the shoot elongation and rooting culture stages, then gradually spread from the edge to the whole leaf. Afterward, the leaves fall off, which affects the growth of regenerated shoots and can causes the death of the whole shoot. A previous study showed that the micro-environment in a tissue culture bottle affects the success of plant tissue cultures, including the compositions and contents of inorganic salts, carbon sources, plant growth regulators, ethylene, and CO_2 concentrations. Changes in these factors will lead to a lower Fe^{2+} content in the medium, imbalance in mineral nutrition, and slow shoot growth (Luo *et al*. 2012).

Thus, the aim of this study was to optimize the regeneration system, improve tissue culture efficiency, and enhance the rapid propagation of passion fruit using the improved explants disinfection method with the addition of ethylene inhibitors to reduce the ethylene contents in tissue culture bottles and trace elements in order to improve the seedling state of regeneration shoots.

Materials and Methods

Plant materials

Purple passion fruit var. 'Tainong' young stem segments with the shoot and stem tips were selected as tissue culture explants. Explants were surface-sterilized with 70% alcohol (v/v) for 30 s, followed by disinfection for 12–15 min and rinsed 4 times with sterilized water. Murashige and Skoog (1962) salt and vitamins with 1.0 mg/L 6-BA and 1.0 mg/L IAA were used as the basal medium for shoot induction, and half-strength MS medium with 2.0 mg/L IBA was used for rooting (Huang *et al*. 2019b). Cultures were kept in a growth chamber under 26 ± 1ºC with a 16/8 h light/dark photoperiod.

Comparison of different disinfection methods

Explants were soaked in commercial carbendazol for 5 min, rinsed under running water for 2 h, surface-sterilized with 70% alcohol (v/v) for 30 s, disinfected for 12–15 min, and rinsed 4 times with sterilized water. Four disinfection methods were used for comparison: two one-time disinfection methods (treatment 1: 0.1% $HgCl_2$ for 15 min; treatment 2: 2% NaClO for 15 min) and two repeat disinfection methods with 2 d intervals (treatment 3: 0.1% $HgCl_2$ for 15 min + 0.1% $HgCl_2$ for 12 min; treatment 4: 0.1% $HgCl_2$ for 15 min + 2% NaClO for 12 min). After 2 weeks, the contamination rates and uncontaminated survival rate were recorded. The contamination rate (%) was calculated as follows:

Contamination rate = (number of contaminated explants/total number of explants) × 100%.

The uncontaminated survival rate (%) was calculated as follows:

Uncontaminated survival rate = (number of uncontaminated and surviving explants/total number of uncontaminated explants) × 100%.

Effect of silver thiosulfate (STS) for shoot induction

To address the issue of low explant proliferation coefficients, 8 μmol/L STS was added to the shoot induction medium; induction medium without STS was used as the control. Cultures were sub-cultured once a month and shoot growth was recorded after sub-culturing 3 times. To prepare 8 m*M* STS stored liquor, 16 m*M* Silver nitrate ($AgNO_3$) was added and slowly mixed with 128 m*M* sodium thiosulfate solution ($Na_2S_2O_3$) in equal volumes. Then, the solution was filtered and sterilized with a 0.22 μM membrane. The molar concentration (mol/L) of STS was equal to the molar concentration (mol/L) of $AgNO_3$ and the final concentration of the culture medium was 8 μmol/L. The solution was not suitable for storage, thus it was prepared and used in time.

Effect of iron salt in the nutrient base for regenerated shoot growth

Considering the phenomenon of leaf yellowing and albinism in the later stages of tissue culturing, the basal induction and rooting medium were supplemented with iron salt with a total of 3 treatments: no additional salt (control), additional 1X iron salt, and additional 2X iron salt. Cultures were sub-cultured once a month and shoot, leaf, and root growth were recorded after subculturing 3 times in the same medium. Leaf chlorophyll was extracted with an acetone: ethanol (1:1) mixture and measured with an ultraviolet spectrophotometer. Absorbance values were recorded at wavelengths of 645 and 663 nm. Chlorophyll contents were calculated as follows (Yan *et al*. 2018):

$$Ca = (12.72 \times A_{663} - 2.59 \times A_{645}) \times v/ (1000 \times m),$$

$$Cb = (22.88 \times A_{645} - 4.67 \times A_{663}) \times v/ (1000 \times m),$$

$$Ca + Cb = (8.05 \times A_{663} + 20.29 \times A_{645}) \times v/ (1000 \times m),$$

Where Ca represents the chlorophyll A content, Cb represents the chlorophyll B content, Ca + Cb represents the total chlorophyll content, v represents the total filtrate volume (mL), and m represents the leaf weight (g).

Results

Effects of different disinfectants and methods

Results revealed that different disinfectants and methods exerted different effects. Treatment 2 had the highest contamination rates with more fungal contamination and a

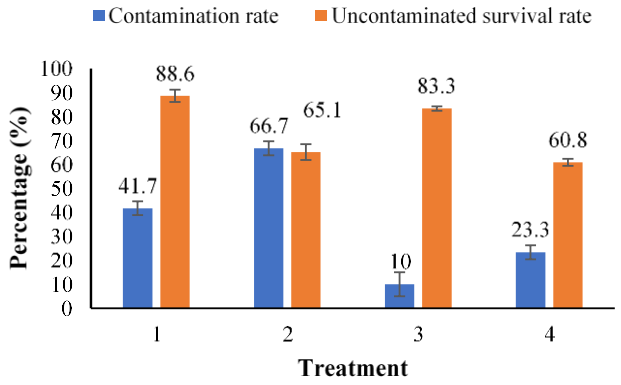

Fig. 1: Effects of different disinfection methods on purple passion fruit stem tips and segment (treatment 1: 0.1% $HgCl_2$ for 15 min; treatment 2: 2% NaClO for 15 min; treatment 3: 0.1% $HgCl_2$ for 15 min + 0.1% $HgCl_2$ for12 min with a 2-d interval; treatment 4: 0.1% $HgCl_2$ for 15 min + 2% NaClO for 12 min with a 2-d interval)

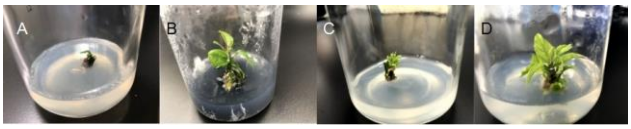

Fig. 2: Shoot induction in medium with and without silver thiosulfate (STS). **A**: Initial shoot growth in medium without STS; **B**: Multiple shoot induction in medium without STS; **C**: Initial shoot growth in medium with STS; **D**: Multiple shoot induction in medium with STS

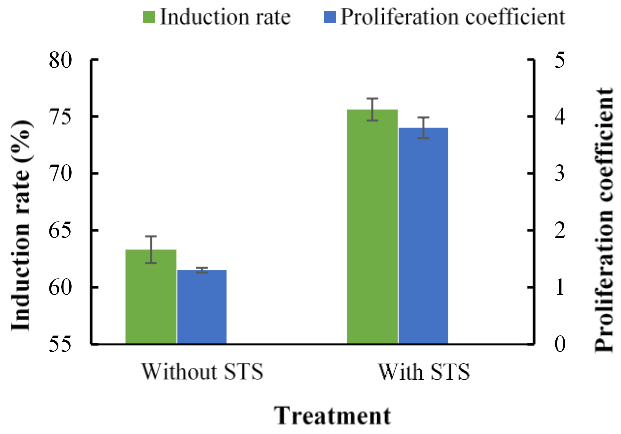

Fig. 3: The shoot induction rates and proliferation coefficients of medium with and without silver thiosulfate (STS)

small amount of bacterial contamination, which occurred 2–3 d after disinfection. Treatment 1 had the second-highest contamination rates, which occurred 4–5 d after disinfection. The contamination rates of the repeat disinfection methods with a 2-d interval were lower than the one-time disinfection methods. In the repeat disinfection methods, the uncontaminated survival rate of the $HgCl_2$ treatment was higher than the NaClO treatment, indicating that the former treatment exerted less damage to explants and was more conducive to their survival. These results indicate that the repeat disinfection method treatment 3 (0.1% $HgCl_2$ for 15 min + 0.1% $HgCl_2$ for 12 min) with a 2-d interval was the best method for disinfecting purple passion fruit stem segment and tips (Fig. 1).

Effects of STS on shoot induction

Only a few adventitious shoots were induced in medium without STS after one month. Although new shoots were induced after several subcultures, their growth was slow, elongation was not obvious, leaves were shrunken and did not extend as far as in other treatments, and the newly induced shoots turned yellow and died gradually until only one main shoot remained, resulting in weak growth and a low proliferation rate. Shoot induction and growth were better in medium with STS and adventitious shoots were induced within 3 weeks. After 2 sub-culturing cycles, the proliferation efficiency reached 3–5 per explant. The main shoot and newly induced shoots grew well and elongated normally, leaves were normal and stretched, and the leaf area was larger than medium without STS. Therefore, the addition of STS to induction medium was beneficial for shoot induction, adventitious shoot elongation, and normal leaf growth (Fig. 2 and 3).

Effects of iron salt on shoot growth and rooting

In the 3 shoot and rooting induction medium treatments, results revealed that the controls had shoot induction but the leaves were yellowing, albino, and falling off with continuous growth, which eventually led to regeneration buds left on the trunk with weak growth vigor and cultures that died. Due to the lack of or yellow/albino leaves, plant growth potential was poor and rooting was difficult. The 2X iron salt treatment resulted in shoot induction, but the leaves had a yellow-green grid in color with leaf growth, exhibiting nutrient deficiency symptoms compared to healthy leaves; the leaves also fell off, indicating that the excessive iron salt concentration was not conducive to normal leaf growth. Regenerated shoots induced rooting in the rooting stage, but the root system was weak. The 1X iron salt treatment had better result than the other treatments in the shoot induction and rooting stages with healthy green leaves. In the rooting stage, roots were easily induced, the root system was developed, and plants were robust (Fig. 4). The leaf chlorophyll contents of the 3 treatments showed that the 1X iron salt treatment was the highest, followed by 2X iron salt, while the leaf chlorophyll content in the control was the lowest (Fig. 5).

Discussion

The most commonly used disinfectants are mercuric chloride ($HgCl_2$), sodium hypochlorite (NaClO), and hydrogen peroxide (H_2O_2). Among them, NaClO has the

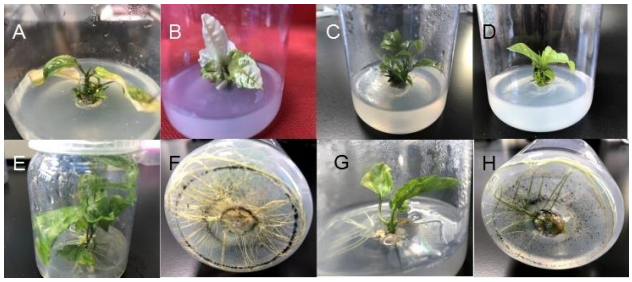

Fig. 4: Effects of iron salt on the growth and rooting of regenerated shoots (plants without additional iron salt exhibited obvious deficiency symptoms, including yellowing, abscission (**A**), and albinism (**B**). **C**: Plants with additional 1X iron salt showed robust regenerated shoot growth. **D**: Plants with additional 2X iron salt showed yellow-green grid colors on the leaves. **E**: Plants with additional 1X iron salt were robust and green. **F**: Plant roots with additional 1X iron salt were developed. **G**: Plant growth with 2X additional iron salt was weak with some yellow spots on the leaves. **H**: Plant root growth with additional 2X iron salt was weak

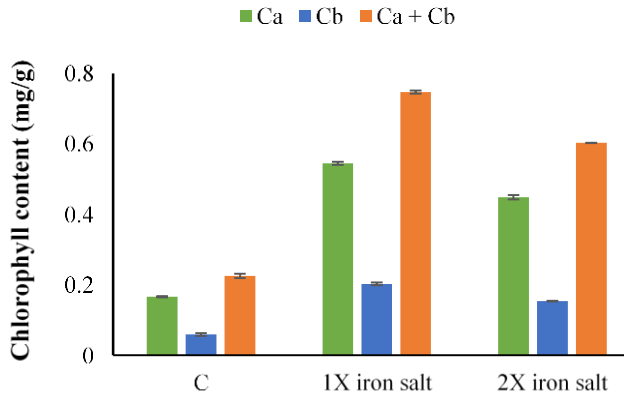

Fig. 5: Effects of iron salt on plant chlorophyll contents (Ca: chlorophyll a; Cb: chlorophyll b; Ca + Cb: total chlorophyll content; C: control)

smallest disinfection effect and its germicidal effect is worse than $HgCl_2$. Generally, increasing the sterilization time improves its germicidal efficacy. However, if the time is too long, it will lead to dehydration of explants, which is not conducive for survival. $HgCl_2$has a better disinfection effect, but high toxicity and is difficult to remove (Lin *et al.* 2013). Thus, the selection of disinfectants and treatment time need to be optimized. The repeat disinfection method has been applied in the tissue culture disinfection of grapefruit (Liu *et al*. 2017), soybean (Shan *et al.* 2013), and other plants. After sterilization with NaClO or $HgCl_2$, although most of the bacteria and fungi are killed, some spores remain due to their strong resistance to the disinfectants. After cultivation (~2 d), some spores germinate under a suitable temperature and humidity, but their resistance decreased after germination. Thus, they are more easily killed in second sterilization treatments, even if the disinfection time in the second sterilization is reduced compared to the first. This method can reduce contamination rates and greatly improve the effects of disinfection.

Auxin and cytokinin contents can promote ethylene production in plant cells and tissues in *in vitro* cultures. Ethylene peaks occur during the meristemoid differentiation period and result in delayed shoot induction (Dias *et al.* 2009). The higher ethylene production rate of *P. edulis* may limit its morphogenetic potential (Ludford 1995), causing the leaves of tissue culture seedlings to become smaller and even cause plantlets to die (Guo *et al.* 2004). To avoid the negative effects of ethylene, a porous membrane cap or the addition of ethylene antagonists in culture medium can alleviate these effects (Luo *et al.* 2012). Silver nitrate ($AgNO_3$) and STS are ethylene antagonists. Some studies showed that $AgNO_3$ can improve the shoot proliferation coefficient and rooting effects of *Picea asperata* cotyledon nodes (Venkatachalam *et al.* 2017), as well as shoot growth potential, which is better when $AgNO_3$ is added to the induction medium of *Passiflora* spp. (Trevisan and Mendes 2005; Pinto *et al.* 2010). Compared to $AgNO_3$, the silver ions in STS are more stable, easier to move in the plant vascular system, and have lower toxicity in plants; therefore, it is more suitable as an additive in plant tissue cultures (Ying and Chen 1990). Faria and Segura (1997) found that STS significantly promoted shoot induction and delayed explant senescence in *P. edulis* f. *flavicarpa* leaf tissue cultures. The addition of STS to potato tissue cultures also promoted plant growth, reduced the variation rate (Sarkar *et al.* 1999), and significantly increased the stem height, leaf area, and chlorophyll content of plantlet tissue cultures (Yuan *et al.* 2007). Generally, STS has a lower molar ratio than $AgNO_3$ and is more stable (Ying and Li 1992). In this study, STS was prepared with a molar ratio of 1:8 (sodium thiosulfate: $AgNO_3$) and the addition of STS to the medium was conducive for shoot induction, adventitious shoot elongation, and leaf growth. This study is the first to report on the tissue culture of purple passion fruit and the findings were consistent with previous studies on other species.

Iron salt is an important component of the basic medium for plant tissue culturing, which has a certain effect on plant tissue cultures and rapid propagation. Leaf chlorophyll a of photosystem II is the major source of fluorescence in green plants (Yang *et al.* 2018). In a previous experiment, purple passion fruit regenerated shoots were induced by MS basal medium and leaves were albino with abscissions, which affected shoot growth. *P. edulis* f. *flavicarpa* plantlets in MS medium showed visual symptoms of mineral deficiency (chlorosis) and reduced growth, as well as symptoms of Fe, Ca, and Mg deficiency, while plantlets grown in adjusted medium, MSM, exhibited increased concentrations of P, Ca, Mg, S, Fe, Mn, Cu, Na, and EDTA, had green leaves, and were more elongated (Monteiro *et al.* 2000). Leaf cells from plants deficient in Fe had poorly developed palisade parenchyma with reductions in chloroplast numbers. Medium with high iron content

improved these conditions. In a previous study, the survival rate of the primary culture of blueberry was improved by doubling the amount of iron in the medium (Liu *et al.* 2007), but in high-bush blueberry, high concentrations of iron salt inhibited proliferation and the chlorophyll contents of clump branches (Jiang and Yu 2009). In contrast, excessively high concentrations of iron salts tended to form a precipitation of iron phosphate, which affected the absorption of iron elements in plants and caused the loss of leaf greenness (Dalton *et al.* 1983; Hangarter and Stasinopoulos 1991). In our study, additional 1X iron salt in the shoot induction and rooting medium promoted leaf greenness and robust plantlet growth. Additionally, the chlorophyll content was higher compared with other treatments, which was consistent with previous studies. Thus, increased iron salt use should be moderated as excessive concentrations of iron salt are not conducive to plant growth and could cause nutritional imbalances.

Conclusion

The repeat disinfection method with a 2d interval reduced contamination rates and enhanced the uncontaminated survival rate. The ethylene inhibitor STS was added to shoot induction medium and increased shoot differentiation and propagation efficiency. Moreover, additional 1X iron salt was added to the shoot induction and rooting medium, which promoted regenerated shoot growth, increased chlorophyll contents, improved leaf morphology, and promoted root formation and growth. Thus, the propagation efficiency of purple passion fruit tissue culture was improved.

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Author Contributions

DH and SS planned and conducted the experiments. FM and BW interpreted the results. DC and YX analyzed the data.

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