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Research paper

Effect of various phenol inhibitors and explant size on meristem culture in the Gavieta variety of *Fragaria ananassa*

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Abstract
Strawberry (*Fragaria ananassa* cv. Gavieta) are infected with several important viral diseases. In vitro culture of the meristem-tip is a valuable method in recovering pathogen-free plants and micropropagation of such crops. In this study, the effects of meristematic dome size, medium type, and phenol inhibitors [activated charcoal, polyvinylpyrrolidone (PVP), and ascorbic acid] were investigated on the shoot tip culture of strawberry. Also the effect of plant growth regulators on shoot elongation was investigated. As the base medium, shoot tips were cultured on the MS media supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 0.2 mg/l indole-3-acetic acid (IAA). The highest regenerated shoots were obtained with the meristematic dome with two leaves. Type of medium (liquid, semi-solid, and solid) significantly affected shoot tip proliferation and the highest percentage of shoots were observed in the semi-solid medium. In the semi-solid medium all meristems were regenerated and produced more shoots with 8.55 mm length. Application of the phenol-absorbing and antioxidant compounds in the culture medium improved shoot induction and shoot performance. The best result was obtained in the medium supplemented with 500 mg/l PVP with 5 shoots, in which browning and explant death were avoided by absorbing phenolic substances from the explant.

Keywords: micropropagation, phenol-absorbing and antioxidant compounds, stem, strawberry

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Introduction

Strawberries are one of the major horticultural crops, with high economic value, that constitutes an important part of the diet of millions of people (Banaeian *et al.* 2011). Strawberries are a rich source of vitamins, minerals, phenols, and flavonoids (Debnath and Teixeira da Silva 2007). Global strawberry production has been increasing in recent years and the greatest production in the Northern Hemisphere's climate is the Mediterranean. The area under cultivation of strawberries in Iran is 4438 hectares which mainly includes Kurdistan and Golestan (FAO 2022). High-performance cultivation yielding desirable products requires healthy and disease-free plants (Nemeth 1979). Since strawberries are consumed freshly, fruit health and appearance are important parameters in the market value of this crop. Viral diseases and phytoplasmas are limiting factors in the production of crops especially strawberries, (Martin and

Tzanetakis 2006). Viruses reduce customer performance and also the quality of the plant (Sinclair and Lyon 2005). Therefore, the explant used for vegetative propagation of the crops should be virus-free (Hollings 1965). Strawberry Pisces Virus (SMOV) and Tape Vein Virus (SVBV) are two important viruses of strawberries. The only proper way to control these diseases is the use of a healthy plant source as the propagule (Thompson and Jelkmann 2003). Up to now, meristem culture is the most effective way to produce healthy and virus-free plants (Wang *et al.* 2003). Zebrowska *et al.* (2003) found that in strawberries, in vitro explants produced better plants than stolons. In addition, in vitro raised plants avoid most stresses (Zebrowska *et al.* 2003).

The plant meristem is a region of intense dividing cells that grow at the tip of the stem and root (Kartha 1985). Several factors are involved in the success of the meristem culture including the size of meristem that is isolated from the mother plant (Lizarraga and Salazar 1983), the plant genotype (Li *et al.* 2002), the culture medium (Mehrotra *et al.* 2007) and its compounds (Ngomuo *et al.* 2014).

In Fragaria sp., Zebrowska *et al.* (2003) found that plants derived from in vitro propagation behaved better under field conditions since they produced more leaves, stolons, and flowers than those propagated by stolons. In addition, the in vitro raised plants were also more resistant to leaf burn induced by frost stress (Zebrowska *et al.* 2003).

This research was conducted to study the effect of some factors (meristem size, medium, and phenol inhibitors) on meristem culture as one of the propagation methods in the micropropagation of strawberries.

Materials and Methods

Preparation of aseptic explants

The strawberry (*Fragaria ananassa*) variety (Gavieta) was provided from a strawberry greenhouse of Marand, Iran, and the experiments were carried out in the Biotechnology laboratory of Tabriz University (Figure 1). About 1-2 cm long apical shoots were cut from strawberry runners and explants were washed with tap water for 30 min. Afterwards, these explants were disinfected by dipping in 70% ethanol for 60 seconds and washed by shaking in sterile distilled water for 3 min. This was followed by surface sterilization with sodium hypochlorite 1.5% for 20 min and finally rinsing three times in sterile distilled water. Using dissecting blade, microscope and sterile forceps leaves were removed and meristem was isolated.

Meristem size for meristem culture of strawberry

According to the first experiment, three different sizes including, the meristematic dome without primordial leaves, the

Figure 1. Strawberry runners collected for preparation of explants

meristematic dome with one primordial leaves, and the meristematic dome with two primordial leaves, were isolated using a laboratory loop. They were cultured in liquid MS basal medium supplemented with 1 mg/l BAP and 0.2 mg/l IAA on an paper bridge (M form paper holder). After 14 days, explants were transferred to a fresh solid MS medium with the same composition. All cultures were incubated under 16/8 h light/dark cycle at 25 ± 2 °C by cool white fluorescent lights (30 µmol m^{-2} s^{-1} PPFD) and were subcultured every 14 days.

Medium type for shoot proliferation

In this experiment, the meristematic dome with two primordial leaves was isolated according to the results of the first experiment. The isolated meristematic domes were cultured in three types of solid, liquid, and semi-solid MS media. To prepare solid and semi-solid medium 6 g/l and 3 g/l agar were added respectively. These media contained 1 mg/l BAP and 0.2 mg/l IAA. In meristem culture in the liquid medium, a paper bridge was used to prevent the drowning of meristem in the medium. Subculturing was performed every four weeks on the same fresh medium. All cultures were incubated under 16/8 h light/dark cycle at 25 ± 2 °C by cool white fluorescent lights (30 µmol m^{-2} s^{-1} PPFD).

Activated charcoal, polyvinylpyrrolidone, and ascorbic acid treatments for improving shoot proliferation

Activated charcoal (AC), polyvinylpyrrolidone (PVP), and ascorbic acid (antioxidative agents) were applied to remove or reduced plant growth-inhibitory substances effects in strawberry meristem

culture. The mentioned three materials were used in different levels; AC (250, 500, and 750 mg/L), PVP (100, 250, and 500 mg/L), and ascorbic acid (100, 250, and 500 mg/L). These antioxidant agents were dissolved in its solvent and after filtering by 0.2 µm millipore filter were added to autoclaved medium containing 1 mg/l BAP and 0.2 mg/l of IAA. All cultures were incubated under 16/8 h light/dark cycle at 25 ± 2°C by cool white fluorescent lights (30 µmol m^{-2} s^{-1} PPFD).

Elongation of the proliferated shoots

The aseptic shoots that were obtained after 45 days of cultures were used as a source of explants for subsequent experiments. The new regenerated shoots were transferred to the shoot elongation medium. The shoot elongation medium was MS semi-solid medium supplemented with three concentrations of BAP (0, 0.2, and 0.4 mg/L) with or without GA_3.

Rooting of plantlets

Regenerated shoots were separated and placed in solid MS medium supplemented with 2 mg/l NAA and incubated under 16/8 h light/dark cycle at 25±2 °C for 4 weeks.

Acclimatization of the rooted plantlets

Four-week-old rooted plantlets were taken out from the culture tubes, thoroughly washed to remove remaining agar and then transferred to black polyethylene bags (6×10 cm; 20 microns thick) containing a steam-sterilized mixture of good garden soil and farmyard manure (FYM) in the ratio of 1:1. Plantlets were watered immediately after planting and were transferred to greenhouse with 25 ± 2 °C, relative humidity (60–70%), and 100 µmol m^{-2} s^{-1} PPF. The survival percentage of plants was recorded seven days after transfer.

Data collection and statistical analysis

Results were subjected to analysis of variance using the Statistical Analysis Program (SPSS ver. 16.0). The mean values were compared by Duncan's multiple range tests ($p \leq 0.05$). All experiments were performed in a completely randomized design with five replications. Results were statistically analyzed for variance using SPSS software (SPSS, Chicago, IL, USA), and Excel software was used for drawing graphs.

Results

Effect of apical meristem size

In this experiment, survival percentage was significantly affected by apical meristem size. It was observed that the meristem dome with two primordial leaves had the highest survival rate of shoots (100%). While the meristem dome without the primordial leaves had the lowest survival

rate (20%).

The results of analysis of variance showed that shoot number and length of regenerated shoots were significantly affected by the size of the meristematic dome. The highest average number of shoots (7.9) was related to the meristematic dome with two primordial leaves and the lowest average number of shoots (0.25) was related to the meristematic dome without initial leaves. Further, the highest mean length of shoots with 8.55 mm was observed in the meristematic dome with two pairs of initial leaves and the lowest with 0.6 mm was related to the meristematic dome without the primordial leaves (Figure 2).

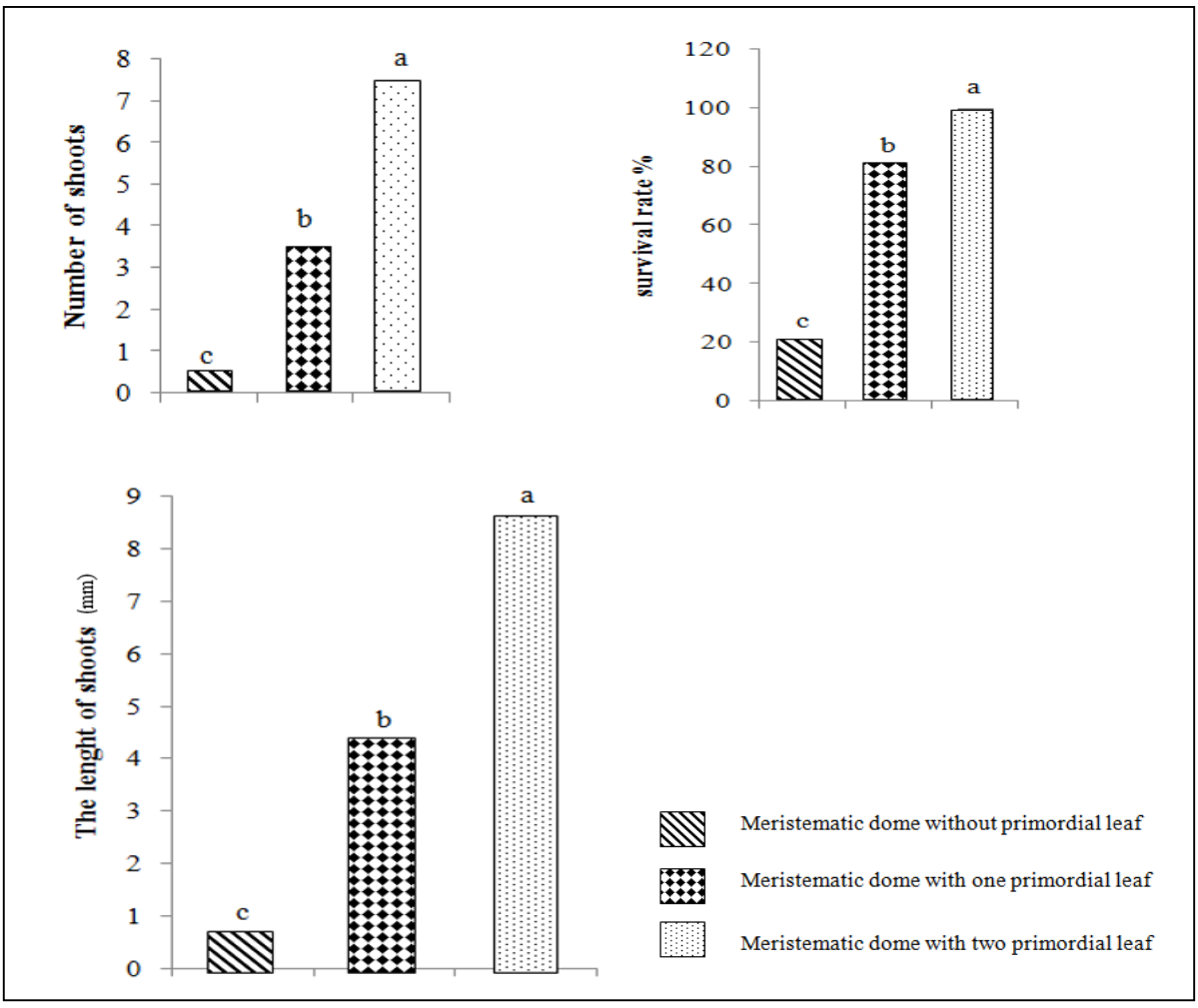

Figure 2. a) Effect of explants size in meristem culture of strawberry (*Fragaria ananassa*). Different letters above bars indicate significant differences ($p \leq 0.01$). The evaluation was performed 45 days after culture.

Effect of medium

In the second experiment, the type of culture media, as the main factor in establishing explants, was examined. According to the data, the type of medium had a significant effect on the number and length of raised shoots. In the semi-solid and solid medium, all cultured explants were regenerated and considering the survival, but there was not observed significant difference between solid and semi-solid medium. There was a significant difference in the number of primordial leaves. Comparison of the mean of the number of shoots showed the highest average number of shoots in the semi-solid medium as 8.2 shoots and the lowest number of shoots in liquid medium as 0.4 shoots per explant. The highest shoot length (10.6 mm) was related to the semi-solid medium and the lowest length (1.4 mm) was observed in the liquid medium (Figure 3).

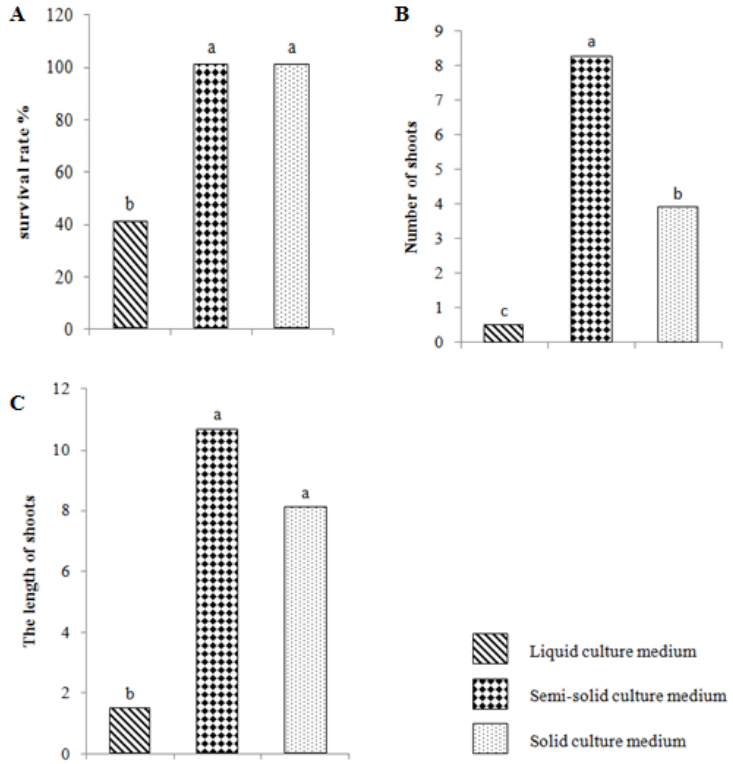

Figure 3. Effect of culture medium in meristem (with two primordial leaves) culture of strawberry (*Fragaria ananassa*). A) survival percentage; B) number of shoots. C) The length of the shoots (mm). Different letters above bars indicate significant differences ($p \leq 0.01$). An evaluation was performed 45 days after culture.

Effect of Phenol-absorbing compounds

In this experiment, shoot number and shoot length were significantly affected by PVP concentrations ($p \leq 0.01$). Comparing the average number of shoots from each meristematic dome demonstrated the

highest number of shoots (5 shoots) in the medium supplemented with 500 mg/l PVP where almost all the explants developed healthy shoots. Whilst, the lowest number of shoots were observed in the medium containing 250 and 100 mg/l AC where all the explants were necrotic. The highest shoot length was 5.6 mm, which was observed at 500 mg/l PVP. The lowest shoot was obtained by the use of activated carbon at both concentrations of 250 and 100 mg/l with no shoot development (Figures 4 and 6c).

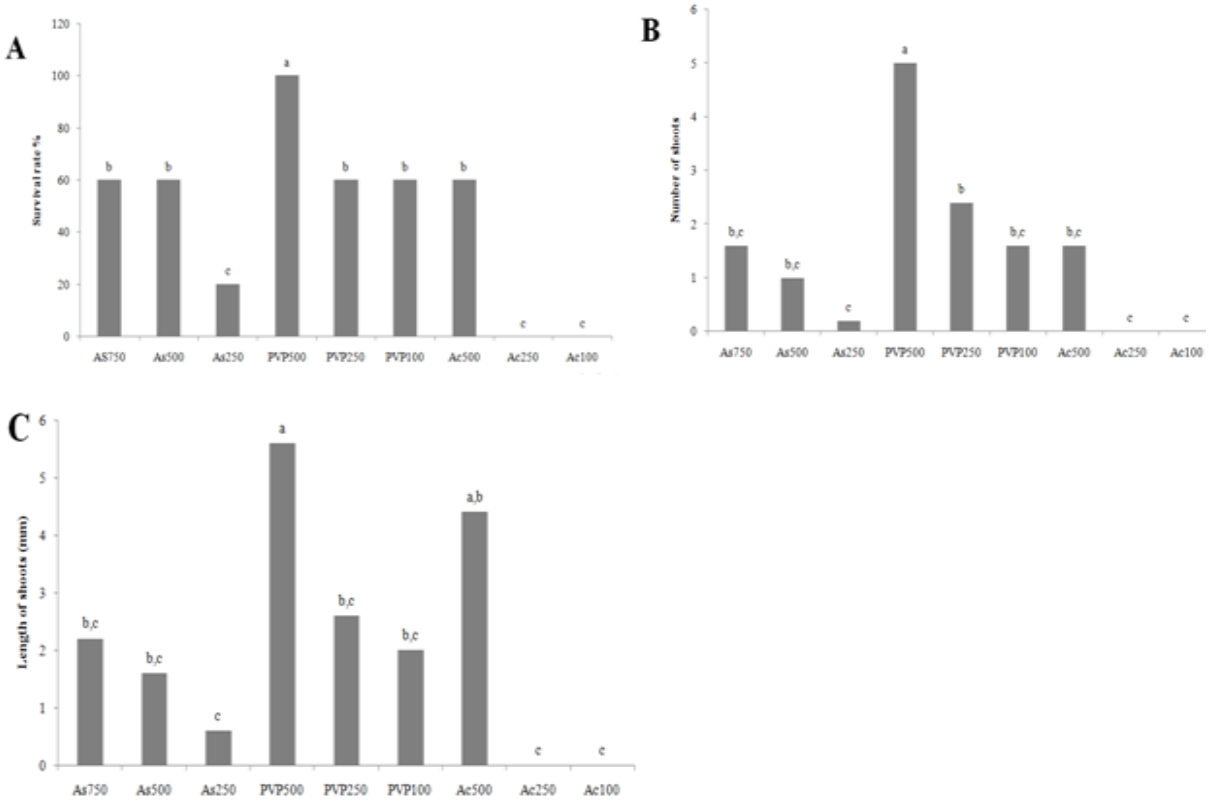

Figure 4. Effect of phenol-absorbing compounds in meristem culture of strawberry (*Fragaria ananassa*). A) survival rate; B) number of shoots. C) The length of the shoots (mm); AS: ascorbic acid; PVP: polyvinyl pyrrolidone; AC: activated charcoal. Different lowercase letters above bars indicate significant differences ($p \leq 0.01$). The evaluation was performed 45 days after culture.

Effect of growth regulators on shoot Elongation

The small shoots (4-week-old) were considered as the explants obtained from meristem culture. They were cultured in a new medium for shoot induction. Analysis of for shoot induction traits shows that the number and length of shoots significantly influenced by different types of media ($p \leq 0.01$). According to the growth of all explants, the survival rate in all treatments was considered 100%. Comparison of the mean number of shoots showed that the highest number of shoots, as 6.6, was achieved in the medium containing 0.4 mg/l BAP and 1 mg/l GA_3 (Figure 5).

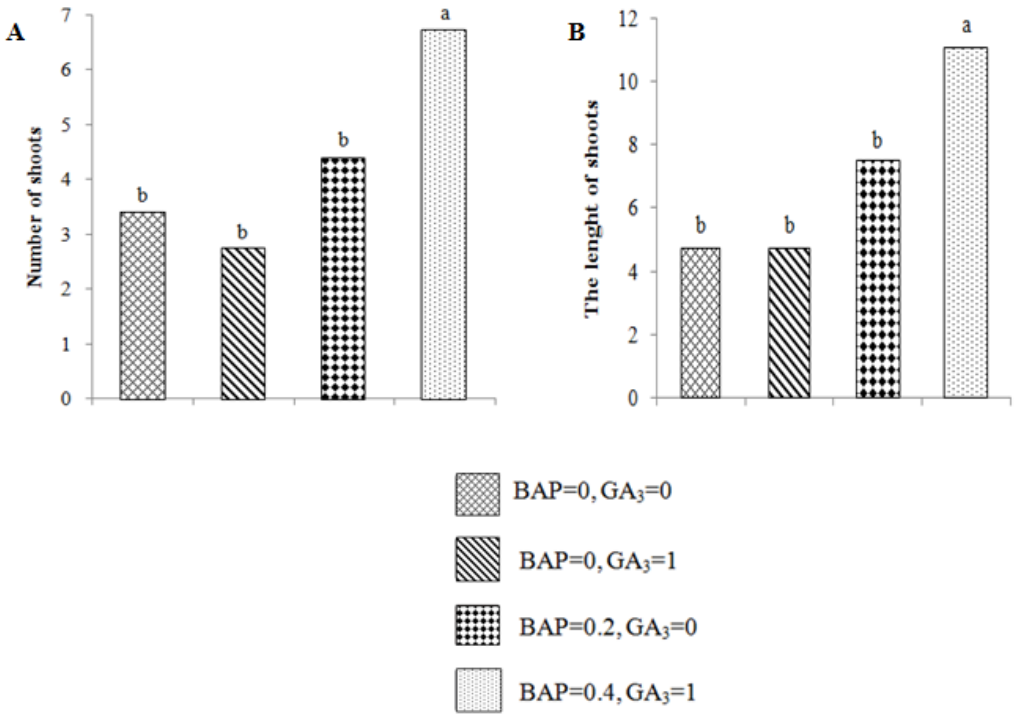

Figure 5. Effect of growth regulators on shoot induction. A number of shoots. B The length of shoots (mm). Different lowercase letters above bars indicate significant differences ($p \leq 0.01$). The evaluation was performed 45 days after culture.

Rooting and acclimatization

Rooted plantlets in MS medium with 2 mg/l NAA were taken out from culture dishes and washed thoroughly with tap water to remove the culture medium from the roots and planted to sterilized soil in polybags (Figure 7).

Discussion

Viral infection are one of the factors that affect the yield and quality of strawberries. Considering the importance of strawberry production and the annual damage caused by viral disease, the need for proper management in the production of virus-free plant material seems necessary. Meristem culture as a method of *in vitro* culture is one of the most common and effective methods of removing viral pathogens from infected plants (Torregrosa *et al.* 2001). There are several factors that influence the cultivation of meristem. In this study, the effect of three factors: the size of the meristem dome, the type of culture medium, and the effect of phenol inhibitors were examined on meristem culture of strawberry (Gavita cultivar).

In the first experiment, according to the obtained results, semi-solid medium was selected as the most suitable medium for shoot apical culture of strawberry (Figures 3 and 6b). One of the serious problems in

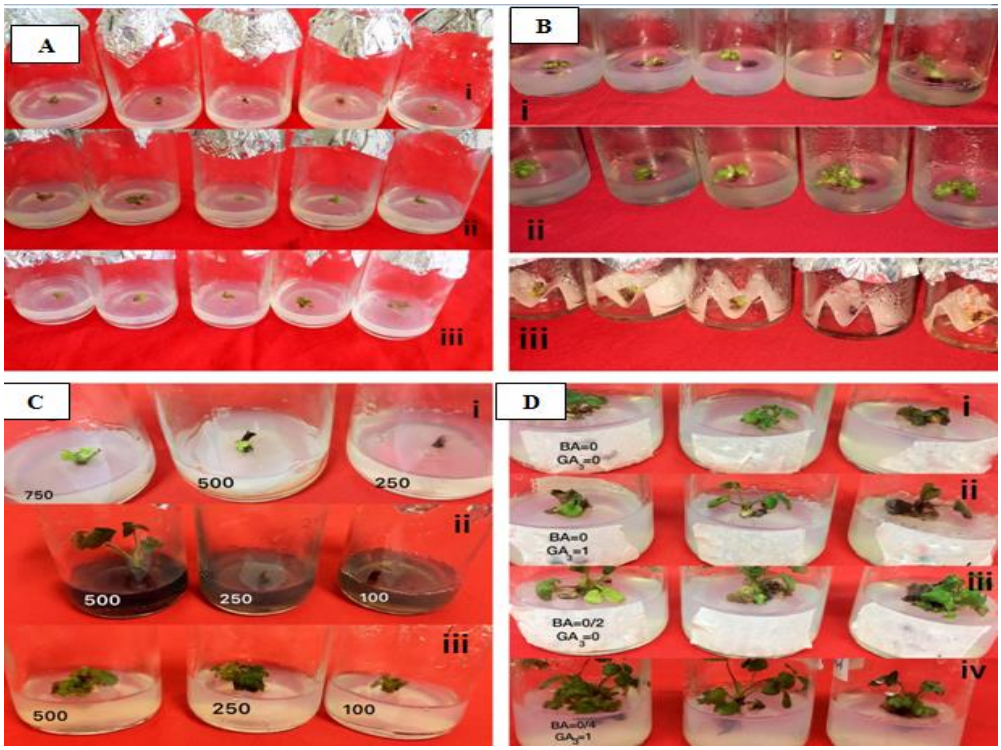

Figure 6. Different steps during experiments and *in vitro* propagation of strawberry (*Fragaria ananassa*). A) Effect of meristem size in meristem culture of strawberry. *i* the meristematic dome without primodial leaf, *ii* the meristematic dome with one primodial leaf, *iii* the meristematic dome with two piromdial leaf in MS medium with 1 mg/l BAP and 0.2 mg/l IAA. B) Effect of medium in meristem culture of strawberry.*i* meristematic dome with 2 primordial leaf cultured in solid MS medium containing 1 mg/l BAP and 0.2 mg/l of IAA, *ii* in Semi-solid MS medium, *iii* in Liquid MS medium. C) Effect of phenol-absorbing compounds in meristem culture of strawberry. *i* Meristematic dome with 2 primordial leaf cultured in Semi-solid MS medium containing 1 mg/l BAP and 0.2 mg/l of IAA, and including ascorbic acid with 3 concentration 250, 500, 750 mg/l, *ii* medium including activated charcoal with 3 contentration 100, 250, 500, *iii* medium including PVP with 3 contentration 100, 250, 500. D) Effect of growth regulators on shoot induction. *I* Semi-solid MS medium with BAP=0, GA_3=0. *ii* medium with BAP=0, GA_3=1, *iii* medium with BAP=0.2, GA_3=0. *iv* medium with BAP=0.4, GA_3=1.

Figure 7. A) rooting and B) acclimatization of plantle

the meristem culture of strawberries is browning and necrosis of the explants, caused by phenolic compounds secreted from the wound tissue of the explants (Quiroz *et al.* 2017). In these cases, regular subcultures of the explant to a new medium are often adopted to prevent the explant death. However, subculture is not a suitable solution since it increases the risk of contamination. Instead, we can take the advantage of antioxidant compounds such as AC, PVP, and phenol-absorbing compounds such as activated carbon. According to data, the use of PVP at the concentration of 500 mg/l caused the highest percentage of meristem growth induction and shoot formation in terms of both number and length.

According to the results, the ideal size of the meristem dome for shoot proliferation was a meristem dome with two primordial (Figures 2 and 6a). Our results were consistent with those of Wang *et al.* (2003) on grape meristem. There reported when 0.1 mm of meristem-tip was used, the complete seedling was not developed. While the regeneration was successful when using meristems with a size of 0.5-2 mm. The results were also in accordance with Verma *et al.* (2004) where using of 0.3 mm meristem-tips raised virus-free explants (84%). It seems that the presence of one to two initial leaves is necessary for the natural development of the whole plant from the final meristem. According to the results, the use of a meristem dome with two pairs of initial leaves has the best performance. So, we used this size of meristem dome for next experiment.

The semi-solid medium lead to the highest shoot induction compared to the liquid and solid medium (Figures 3 and 6b). It seems that the explants cultured in a semi-solid medium absorb the nutrients more easily than the solid medium. Further, the phenolic substances are not concentrated in one area and spread in the whole medium compared to the solid medium. Not to mention that the adverse effects of phenolic substances are lost in the semi-solid medium. In the liquid medium the phenolic substances secreted from the explants in contact with the paper plate do not spread efficiently in the medium and have a negative effect on shoot growth. In examining the work of other researchers, different results can be seen (Titov *et al.* 2006). The results of this experiment did not match with Vienna, where liquid medium had the highest survival with 61% (Jones and Vine 1968). Rattanpal *et al.* (2011) transferred the explants to a semi-solid medium after four weeks of incubation in the solid medium to shoot proliferation.

The use of PVP, by absorbing phenolic substances secreted from the explant, improved explant proliferation and shoot extension. In media without absorbing phenolic substances, the explants were

destroyed by browning 4-5 days after culture. The use of other phenol-absorbing compounds such as ascorbic acid and activated carbon also had positive effects on shoot proliferation. In addition to good rooting, the production of long branches was observed in the explants grown in activated carbon. The results obtained from this phase of the experiment are consistent with studies by other researchers on the positive effects of phenol-absorbing compounds on shoot proliferation (Ko *et al.* 2009).

The effect of PVP and ascorbic acid in the cultivation of strawberry meristem of *F. chiloensis* was reported, in which PVP increased plant regeneration efficiency from isolated meristems (Quiroz *et al.* 2017). In another study, the use of PVP and low temperature were expressed as the main factors to prevent phenolic secretion and explant death (Ko *et al.* 2000). It can be inferred that the treatment of the explants with antioxidants such as ascorbic acid, PVP, and phenol-absorbing compounds such as AC or culturing them with these compounds could solve the problem. The highest shoot proliferation was obtained by explants with 2 primordial leaves in semi-solid culture medium supelmented 500 mg/l PVP.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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تاثیر مهارکننده‌های فنولی مختلف و اندازه ریزنمونه در کشت مریستم توت فرنگی رقم گاویتا

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چکیده

توت‌فرنگی (*Fragaria ananassa* cv. Gavieta) با چند بیماری ویروسی آلوده می‌شود. کشت درون‌شیشه‌ای نوک مریستم یک روش ارزشمند در بازیابی گیاهان عاری از بیماری و ریزازدیادی این گونه محصولات می‌باشد. در این مطالعه، تاثیر اندازه گنبد مریستمی، نوع محیط کشت و مهارکننده‌های فنولی (زغال فعال، پلی ونیل پیرولیدون و اسید آسکوربیک) بر کشت نوک ساقه توت‌فرنگی مورد بررسی قرار گرفت. در آزمایش دیگر اثر تنظیم‌کننده‌های رشد گیاهی بر ازدیاد طول شاخساره بررسی شد. به عنوان محیط پایه، نوک ساقه در محیط کشت MS حاوی یک میلی‌گرم در لیتر ۶-بنزیل‌امین‌پورین (BAP) و ۰/۲ میلی‌گرم در لیتر ایندول-۳- استیک اسید (IAA) کشت داده شد. بزرگترین شاخساره باززایی شده با گنبد مریستمی با دو برگ به دست آمد. نوع محیط (انواع مایع، نیمه‌جامد و جامد) به طور معنی‌داری بر تکثیر نوک اندام هوایی تأثیر داشت و بیشترین میزان شاخساره در محیط نیمه جامد مشاهده شد. در محیط نیمه جامد که تمامی مریستم‌ها باززایی شدند، ساقه‌های بیشتری با طول ۸/۵۵ میلی متر تولید کردند. استفاده از ترکیبات جاذب فنل و آنتی اکسیدان در محیط کشت باعث بهبود القای شاخساره و عملکرد ساقه شد. بهترین نتیجه در محیط حاوی ۵۰۰ میلی گرم در لیتر پلی وینیل پیرولیدون (PVP) با ۵ شاخه به دست آمد، که در آن از قهوه‌ای شدن و مرگ ریزنمونه با جذب مواد فنلی از ریزنمونه جلوگیری شد.

واژه‌های کلیدی: ترکیبات جاذب فنول و آنتی اکسیدان‌ها؛ توت فرنگی؛ ریزازدیادی؛ ساقه