Original Article

# *In vitro* Production of PPV-free Sweet cherry (*Prunus avium* cv. Siahe-Mashhad) by Meristem culture and micro-grafting

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## ABSTRACT

*Plum Pox Virus* (PPV) is one of the most common viral agents in sweet cherry. This study was carried out to investigate the effect of culturing method and explant type for production of PPV-free plants in sweet cherry cv. Siahe-Mashhad, in a factorial experiment based on a completely randomized design (CRD). The first factor was the culturing method in two levels (A: Meristem culture and B: Micro-grafting) and the second factor, the type of explants in four levels (M1S1: 5mm and *in vivo* explant, M1S2: 2mm and *in vivo* explant, M2S1: 2mm and *in vitro* explant and M2S2: 5mm and *in vitro* explant). Explants of shoot tips were cultured for meristem culture as well as micro-grafting on the 'Gissela 6' rootstock and then cultured in a MS medium containing 1 $mg.l^{-1}$ BAP in controlled conditions. The results showed that among the types of explant in both survival and health plantlet indices was significant, but there was a significant difference in the culturing method only in the survival plantlet index and their interaction (at 5% level). The highest percentage of survival plantlet index (48.6%) was recorded for meristem culture when explant was 5 mm *in vitro* and the highest percentage of healthy planted index (41.6%) was obtained for meristem culture when 2 mm *in vitro* explants was used. The results of percentage of healthy plantlets in total detection of PPV using Enzyme-linked immunosorbent assay (ELISA) test showed that 30.5% of all regenerated plants in both cultures were PPV- free.

## 1. Introduction

Production of healthy, free of pathogens, and uniform plants plays an important role in the development of the horticultural industry. Sweet cherry (*Prunus avium* L.) is one of the most important horticultural products and Iran is in the third place in the world in terms of production [1]. At present, seedlings are less used for propagation of sweet cherry due to rootstock variation, rooting problems and late fruiting, while grafting is mostly used for propagation, but grafting methods also develop contaminants, especially viral ones; therefore, it is necessary to control viral agents through the production of healthy material plants. Sweet cherry is currently exposed to many viral and pseudo-viral diseases, the most important of which in Iran is the PPV, which reduces the quality and yield of fruit, abscission of buds, fruits and leaves and the appearance of systemic symptoms include chlorinated spots

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on leaves, fruits, and bright veins [2, 3].

PPV, the agent of Sharka disease, is transmitted by grafting as well as by Aphids. The disease is widespread worldwide and is the most important disease of stone fruits in the world. Due to the importance of producing healthy plants in sweet cherry trees and the significant damage caused by diseases in them, it is necessary to implement proper management in the production of virus-free plant material. Many attempts have been made to use chemical compounds and control vectors to remove viral agents in fruit trees that have not yet been successful [4]. Hence, despite the problems of propagation by seedling and other traditional methods, sweet cherry propagation by in vitro methods can lead to the production of healthy, uniform colonized plants in a much shorter time; however, in tissue culture methods, genetic stability in clone plants is affected by many factors during micropropagation, but obviously the most suitable organ that preserves the genetic characteristics of a cultivar is the bud meristems. The use of meristem culture is also one of the most effective methods to eliminate viral pathogens because the absence of vascular tissue and intercellular spaces and high speed of cell division prevents the proliferation of viral and pseudo-viral agents. The use of vegetative bud-meristem is the most common method of viral removal programs in plants, especially fruit trees [5, 6].

Major benefits of meristem culture for plants include *in vitro* clonal propagation with maximum genetic stability, high potential in removing viral, bacterial and fungal agents, storage of germplasm and keeping germplasm. Rapid, accurate propagation and safe storage of chimeras explant and transport of safe and healthy plant materials are quarantined. Meristem Culture Method for hard-rooted cultivars is also done in a micro-grafting that has all the characteristics of meristem culture[7, 8]. Commonly referred to as graft meristem culture, it is now used in plant breeding programs around the world to obtain virus-free plants for commercial production. In addition, this method is effective in reducing the risk of transmission of plant materials from one country to another; the developed micro-grafting is the last resort to eliminate viruses in plants that cannot be successfully propagated by meristem *in vitro* culture [9, 10].

Different types of meristem culture have been successfully used in order to eliminate various viruses in stone fruits: PPV in peach [11, 12], apricot [13] and PNRSV and PPV in nectarine [11] and PPV, PNRSV, PDV in plums [14], and also micro-grafting method is reported as a successful method for producing virus-free plants in some fruit tree species including ICRSV- [15] and CTV-free plants in citrus [16, 17], ACLSV-, PDV- and PNRSV-free plants in sweet cherry [18, 19] and PPV- free plants in peach [20].

Due to the importance of healthy and uniform sweet cherry orchards establishment, today it is necessary to produce PPV-free plant materials and release them to growers. Therefore, the aim of this study was to compare the efficiency of meristem culture and micro-grafting methods and to determine the best type of explant for eliminating PPV from sweet cherry (*Prunus avium* cv. Siahe-Mashhad).

## 2. Materials and methods

In order to investigate the different methods for producing virus-free plants in sweet cherry, an experiment with two factors was performed in a CRD with six replications including seven explants; 1: Culture Method, A: Meristem culture and B: Micro-grafting, and 2: Explant type, M1S1: 5mm and *in vivo* explant, M1S2: 2mm and *in vivo* explant, M2S1: 2mm and *in vitro* explant and M2S2: 5mm and *in vitro* explant.

### 2.1. Sampling of infected plants

Infected samples were collected from Khorasan Razavi Agricultural Research Center located in Golmakan during spring and summer of 2015 and 2016 and virus identification was performed by DAS-ELISA method. Leaf specimens were collected from trees with symptoms of necrotic, chlorotic and circular spots, paleness and deformity and transferred

to the laboratory to test for the presence of the virus. In order to identify and detect PPV, the DAS-ELISA- test and polyclonal virus antibodies (Leibniz-Institute DSMZ) were used. Results were read by ELISA Reader (Awareness, fax Stat - 2100) at 405 nm.

## 2.2. Preparation of explants

To obtain meristematic explants, mature shoot tips of sweet cherry trees of Siahe-Mashhad cultivar were collected from Golmakan Research Station in Mashhad with a length of 1.5 to 2 cm at the end of the growing season. After separation from the stem, the shoot tips were washed in Twin liquid for 10 minutes and then washed three times with distilled water. The explants were then immersed twice in surface 100 mL of ascorbic acid and 150 mL of citric acid for 10 minutes before surface sterilization and during culture to prevent browning of tissues. Superficial sterilization was performed with ethanol (70%) for 30 seconds and then complete disinfection using sodium hypochlorite (20%) for 10 minutes, and finally the explants were washed three times with sterile distilled water. After removing the initial leaf scales from the tip buds, meristematic explants less than 5 mm in size were isolated under a stereomicroscope (SZ6045TR, Olympus Optical Co. Ltd., Tokyo, Japan). All steps were performed under sterile conditions and under the hood [21, 22].

## 2.3. Preparation of rootstock and micro-grafting

For micro-grafting, meristematic explants of 2 and 5 mm in size as scions and stems produced from clones of Gissela 6 (*prunus avium*) with a diameter of more than 5 mm were taken out of the growth medium as a rootstock and micro-grafting was performed under stereomicroscope. In this way, in the end part of the rootstock, a cut with dimensions of one mm was made and the scion of the shoot tip alone or with leaf tops measuring two to five mm was placed in the rootstock by cleft grafting method. Then, covered with Para-film joints were closed.

## 2.4. Cultivation and maintenance of plants

Meristematic explants as well as micro-grafted plants were cultured in MS medium [23] with 1 $mg.l^{-1}$ BAP + 30 $g.l^{-1}$ sucrose + 2.8 $g.l^{-1}$ phytogel. The culture medium was used inside culture tubes of 150×25 mm, which were sterilized with membrane caps to ensure high relative humidity and were sterilized at 121 °C for 20 minutes. To prevent the interference of phenolic compounds, at first explants were kept for a week in low light conditions (100 lux) and then transferred to growth chamber in a temperature of 27 °C and light 2000 lux with a photoperiod of 16 - 8 hours of light and dark for three weeks. Plants obtained from meristem culture (after proliferation stage) as well as successful micro-grafting plants were transferred to MS culture medium with 1 mg $l^{-1}$ IBA for rooting. The resulting plants were kept *in vitro* for eight weeks and after rooting were placed in polyethylene pots containing Perlite: Peat moss (1: 1) for adaptation (Figure 1).

## 2.5. Virus detection test

To detect the virus, serological test was performed by indirect Enzyme-linked immune sorbent assay (ELISA) using multivalent antiserum, prepared from the plant pathology group of Khorasan Razavi Agricultural Research Center, with a dilution of 1: 1000. For this purpose, 0.1 g of leaves from each plant samples, negative control and positive control was extracted in three mL of extraction buffer. 75 µl of the plant extract along with 25 µl of the coating buffer was poured into the wells of the ELISA pan. Three wells were filled with extraction buffer and the pan was kept overnight at 4 °C. After draining the wells and washing with a blocking buffer, 100 µl of buffer was added to each well and kept at 37 °C for two hours. Then, the washing was done as in the previous step.

The polyunsaturated antiserum was diluted 1:1000 in the conjugate buffer and 100 µl was added to each well. At this stage, the pan was kept at 37 °C for four hours. The wash was then performed as in the previous steps and the antibody bound to the alkaline phosphatase enzyme as a secondary antibody (Goat anti-

rabbit-IgG-alkaline phosphatase) was diluted in a ratio of 1: 7500 in the Conjugate buffer to 100 µl each. The well was added and the pan was kept at 37 °C for two hours, then washed as before. In the next step, 10 mg of para-nitro phenol phosphate as a substrate of alkaline phosphatase enzyme was dissolved in 10 mL of substrate buffer and 100 µl of it was added to each well. To check the results of the ELISA test every 15 minutes for one hour, the amount of color change of the wells at 405 nm was measured with an Austrian ELISA reader (Anthos2020). Wells whose light absorption rate was more than twice the average light absorption of negative control wells (virus-free plant) were considered as positive [24].

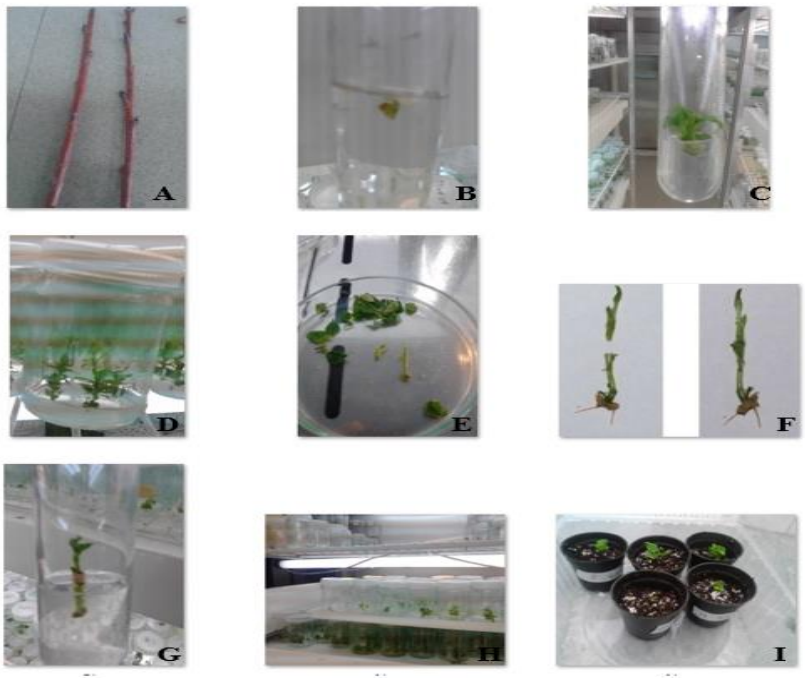


**Figure 1**. Culturing stages of sweet cherry cv. Siahe-Mashhad *in vitro* conditions; A) Preparation of meristem explant; B) Isolation and cultivation of meristem; C) Establishment of meristem as micro-scion D) Multiplication of shoot tips, E) Preparation of rootstock; F) Micro-grafting; G) Production of micro-grafted plants; H) Plant rooting: and I) Plant adaptation

## 2.6. Data analysis

Determinants in the success of meristem and micro-grafting, including the percentage of successful survival and the percentage of healthy and virus-free in each plant were measured. The data obtained from factorial experiment based on CRD design were analyzed by SAS 10 statistical software and the means were compared using DMRT at one and five percent of probability levels. Charts were drawn with Excel 2015 software.

## 3. Results

The results of ANOVA showed that there was a significant difference ($p \leq 0.01$) between explant type treatments on all indices, while between Culture Method treatments, there was only in the success rate index and in cultivar interactions in all indices. The difference was significant at 1, 5 % (Table 1).

**Table 1.** The ANOVA result for the effect of culture method and explant type on survival and health plantlet indices of Sweet cherry cv. Siahe-Mahhad *in vitro*

| S.O.V | DF | Mean Squares | |
|---|---|---|---|
| | | Health plantlet | Survive plantlet |
| **Culture Method** | 1 | 3.6 ns | 160.9* |
| **Explant Type** | 3 | 94.6** | 3275** |
| **Culture Method× Explant Type** | 11 | 3.03* | 173.5* |
| **Error** | 60 | 1.9 | 74.3 |
| **CV (%)** | - | 15.1 | 19.6 |

ns Non-significant, * ,** Significant at 5% and 1% of probability levels, respectively.

Five mm meristematic explants with *in vitro* origin had the highest amount for survival index (43.2%) while 2 mm meristem ones with *in vivo* origin had the lowest amount (22.1%). Meristem culture method (34.4%) was more successful than micro-grafting method (25%) based on survived plant production. The highest success rate for survived plant production was obtained when 5 mm explants with *in vitro* origin used in meristem culture method (48.6%) and the lowest rate was recorded when 2 mm explants of *in vivo* origin used in micro-grafting method (18.9%). Also, in all experiments, the mean survival rate was 29.6% (Table 2).

**Table 2.** The effect of culture method and explant type on survived plantlet production (%) in sweet cherry cv. Siahe-Mashhad

| Culture Method | Explant Type | | | | Mean |
|---|---|---|---|---|---|
| | M1S1 | M1S2 | M2S1 | M2S2 | |
| **Meristem culture** | 25.3 bc* | 28.5 b | 34.6 ab | 48.6 a | 34.3 A |
| **Micro-grafting** | 18.9 e | 18.3 c | 24.8 bc | 37.8 ab | 25 B |
| **Mean** | 22.1 C | 23.4 C | 29.7 B | 43.2 A | 29.6 |

M1S1: 5 mm meristem with *in vivo* origin, M1S2: 2 mm meristem with *in vivo* origin, M2S1: 2 mm meristem with *in vitro* origin, and M2S2: 5 mm meristem with *in vitro* origin.

*The mean followed by same letter (capital letters: simple effects and small letters: interaction effects) are not significantly different based on Duncan's multiple range.

In the results of healthy plants among living plants, the treatment of 2 mm explants with *in vitro* origin had the highest success rate (40.5%) and the treatment of 5 mm meristem explants with in vivo had the lowest success rate (21.8%). Among the culture methods, although there was no significant difference between meristem culture and micro grafting, the percentage of healthy plants was obtained from meristem culture (31.7%) versus micro grafting (29.2%). Among the interactions, the highest percentage of healthy plants was related to meristem culture in 2 mm explants with *in vitro* origin (41.6%) and the lowest was related to the grafting of 5 mm explants of *in vivo* origin (20.4). Also, in all alive plants, the average percentage of healthy plants was 30.5% (Table 3).

**Table 3.** The effect of culture method and explant type on Health plantlet production (%) in sweet cherry cv. Siahe-Mashhad

| Culture Method | Explant Type | | | | Mean |
|---|---|---|---|---|---|
| | M1S1 | M1S2 | M2S1 | M2S2 | |
| **Meristem culture** | 34.1 ab | 23.2 bc | 41.6 a | 28.2 b | 31.7 A |
| **Micro-grafting** | 31.7 b | 20.4 c | 39.4 a | 25.4 bc | 29.2 A |
| **Mean** | 32.9B | 21.8 D | 29.7B | 26.8 C | 30.5 |

M1S1: 5 mm meristem with *in vivo* origin, M1S2: 2 mm meristem with *in vivo* origin, M2S1: 2 mm meristem with *in vitro* origin, and M2S2: 5 mm meristem with *in vitro* origin.

*The mean followed by same letter (capital letters: simple effects and small letters: interaction effects) are not significantly different based on Duncan's multiple range.

## 4. Discussion

The results of this study showed that the success of meristem culture and micro-grafting *in vitro* conditions is affected by the type of explant in size and origin. In general, the success of survived plantlet production in micro-grafting was low compared with meristem culture, so that in some experimental units the necrosis of the grafts was very high and the grafting yield, especially in the very small meristem scions, it was zero. These poor results may be due to difficult grafting, poor rootstock and scion bonding, low physiological activity and high oxidase activity *in vitro* [8]; however, in the percentage of healthy plantlet production, the success of both methods was almost the same.

According previous studies, the explants usually used for meristem culture or micro-grafting are prepared in the standard size of less than one centimeter. However, the size of the explants may vary depending on the plant species. The frequency of survival success in meristem culture and micro-grafting increased with increasing size of explant or micro-scion, but the percentage of virus-free plantlets decreased over all. Although it also depends on the pathogen, in the type of explants in two sizes, two and five mm, it was found that explants larger than 5 mm with leaf primordia and had a greater impact on survival success than smaller 2 mm explants without leaf primordia. Probably, the presence of leaves for nutrient supply and a higher contact surface may cause more plants to survive, but this was despite the fact that 2 mm shoot tips were much better in terms of viral contamination than larger explant (Navarro, 1988). The rate of virus removal from the 5 mm shoot tips was lower than at 2 mm explants, which was consistent with Sauer's [25] report on sweet cherry.

Also, the percentage of micro-grafting success of this study was similar to the report of micro-grafting of "Siahe-Mashhad" on sour cherry seedling with micro-grafting size of 5 to 10 mm, which had the highest rate (65%)[26]. In micro-graftng of peach cultivars, the use of shoot tips on different seedling, *in vitro* conditions, although the probability of contamination was very low, with the application of meristem with two leaf primers, the success rate in micro-grafting increased significantly and contamination was still low. In apricot micro-grafting with two to five mm in shoot tips size, on seedling, a success rate of 40% was achieved [27]. In micro-grafting of almond, it was found that with increasing the size and number of leaf primordia, the success rate in grafting increased but the percentage of virus-free plants decreased [28].

## 5. Conclusion

In order to produce healthy orchards in sweet cherry, it is appropriate to use meristem culture and micro-grafting methods *in vitro* conditions. In addition to the rapid growth of a large number of new shoots, it produces healthy plants of virus-free such as PPV, due to the relatively good success of meristem culture and its integration into shoot-tip grafting with explants less than two mm in PPV-free Sweet cherry production. This study is expected to be

a successful protocol for the removal of a variety of viruses from different species of stone fruit trees.

## Abbreviation

ACLSV: *Apple Chlorotic Leaf Spot Virus*
CRD: Completely Randomized Design
DAS-ELISA: Double Antibody Sandwich - Enzyme Linked Immunosorbent Assay
DMRT: Duncan's multiple range test
ICRSV: *Indian Citrus Ringspot Virus*
PDV: *Prune Dwarf Virus*
PNRV: *Prunus Necrotic Ringspot Virus*
PPV: *Plum Pox Virus*

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## Conflict of interest

None.

## Consent for publications

The author read and proved the final manuscript for publication.

## Availability of data and material

All data generated during this study are included in this published article

## Authors' Contribution

All authors had equal role in study design, work, statistical analysis and manuscript writing.

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