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Seed culture – a successful method for avoiding polyphenolic oxidation of *Malus domestica* L. explants during micropropagation

Valbona Sota[1], Brunilda Çuko[1], Ariola Bacu[1], Efigjeni Kongjika[2]

[1]Tirana University, Faculty of Natural Sciences, Department of Biotechnology, Tirana – Albania
[2]Academy of Sciences, Section of Natural and Technical Sciences, Tirana – Albania
Email: bona_sota@yahoo.com

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Abstract

One of the new and important ways of plant propagation is micropropagation or tissue culture technique. Apple is a candidate species of micropropagation for large number of rootstocks and scion cultivars. Although, advantages of micropropagation, there are still problems associated with its commercial applications, like the browning due to the exudation of phenolics into the medium as a response to wounding at excision. The objective of the present study is to develop an efficient micropropagation protocol for two different apple cultivars (cv Golden Delicious and cv Starking) to the avoidance of polyphenic oxidation during the first stage of *in vitro* technique establishment. As primary explants are used apical buds and mature seeds, and is evaluated their efficiency during the first stage of proliferation and regeneration. Bud explants are treated by diverse methods like the use of antioxidants (ascorbic acid, citric acid, PVP), adsorbing agents (activated charcoal and PVP) and physical treatments like darkness or the frequent transfer of explants to fresh medium. The most optimal method results the use of Murashige & Skoog medium supplemented with MS vitamins and combined with cytokinin BAP (1 mg l^{-1}) and auxin IBA (0.1 mg l^{-1}), ascorbic acid (0.1%) and citric acid (0.1%) in darkness conditions. In spite of, this method is effective for only 43% of primary explants. A parallel experiment is conducted using mature seeds cultivated in MS media combined with cytokinin BAP (1 mg l^{-1}) and auxin NAA (0.1 mg l^{-1}) and in a 16/8 light/dark regime. The regeneration percentage is very high (99%) and there are observed no signs of phenolics exudation onto nutrient media. The success of the first stage of proliferation affects the optimal development of the explants in the other stages of micropropagation.

Keywords: apple micropropagation, polyphenolic oxidation, seed culture, antioxidants

Introduction

Micropropagation and meristem culture has recently become an important technique, particularly with vegetative propagated species. The main problems for establishing a successful protocol for micropropagation are associated with the browning or yellowing of nutrient medium due to the exudation of phenolics into the medium as a response to wounding at excision and shoot necrosis and mortality of the explant/cultures due to the absorption of these substances.

In order to avoid these problems, mature seeds result optimal explants for establishing the first stage of *in vitro* culture. Thereafter must be exceeded the meristems for producing virus-free plants.

The objective of the present study was to develop an efficient micropropagation protocol for two different apple cultivars using mature seeds as primary explants in order to avoid polyphenolic oxidation during the first stage of *in vitro* technique establishment.

Material and Methods

Plant materials. As initial explants were used shoot tips and mature seeds of *Malus domestica* Borkh. cv Golden Delicious, cv Starking.

Disinfection of isolated buds. Isolated buds were treated with Bavistine 0.2% for 7 min, followed by a treatment with $HgCl_2$ 0.1% for 7 min and with ethanol 70% for a min. After every treatment on laminar flow, the buds were rinsed three times with sterile and distilled water.

Nutrient medium and methods to avoid polyphenolic oxidation. For both types of explants was used MS nutrient medium supplemented with MS vitamins and combined with cytokinin BAP (1 mg l^{-1}) and auxin IBA (0.1 mg l^{-1}); sucrose 30 g l^{-1} and also agar 7%; the pH has been checked to be 5.8. Bud explants were treated by some methods in order to avoid the polyphenolic oxidation of the explants:

Methods: ***i)*** MS + ascorbic acid 0.2%, 48 hours in the darkness and twice transfers in fresh medium, ***ii)*** MS + ascorbic acid 0.2% and 72 hours in the darkness, ***iii)*** MS + polyvinyl pyrrolidone (PVP) 0.1%, ascorbic acid 0.1%, citric acid 0.1%, 48 hours in darkness and some times of transfers in fresh medium, ***iv)*** MS + active charcoal 1g l^{-1} and 24 hours in darkness, ***v)*** MS + ascorbic acid 0.1%, citric acid 0.05%, 48 hours in darkness and twice transfers in fresh medium, ***vi)*** MS medium with ascorbic acid 0.1%, citric acid 0.05% and active charcoal 1g l^{-1}, 2 days in darkness and onetime transfer to fresh medium.

Conditions of in vitro culture chamber. The test tubes are placed in the culture chamber with controlled physical conditions (temperature: 23°C, photoperiod: 16 hours with light/ 24 hours).

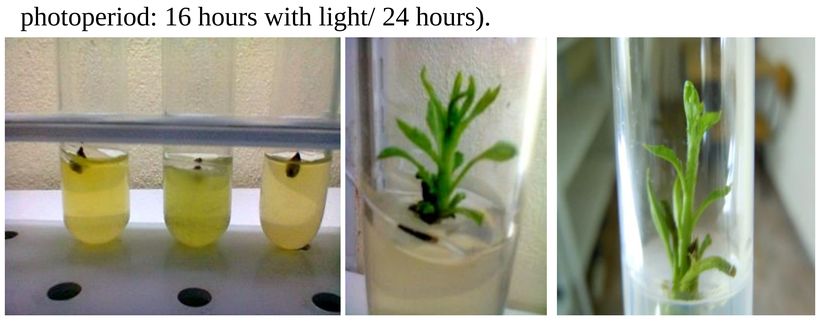
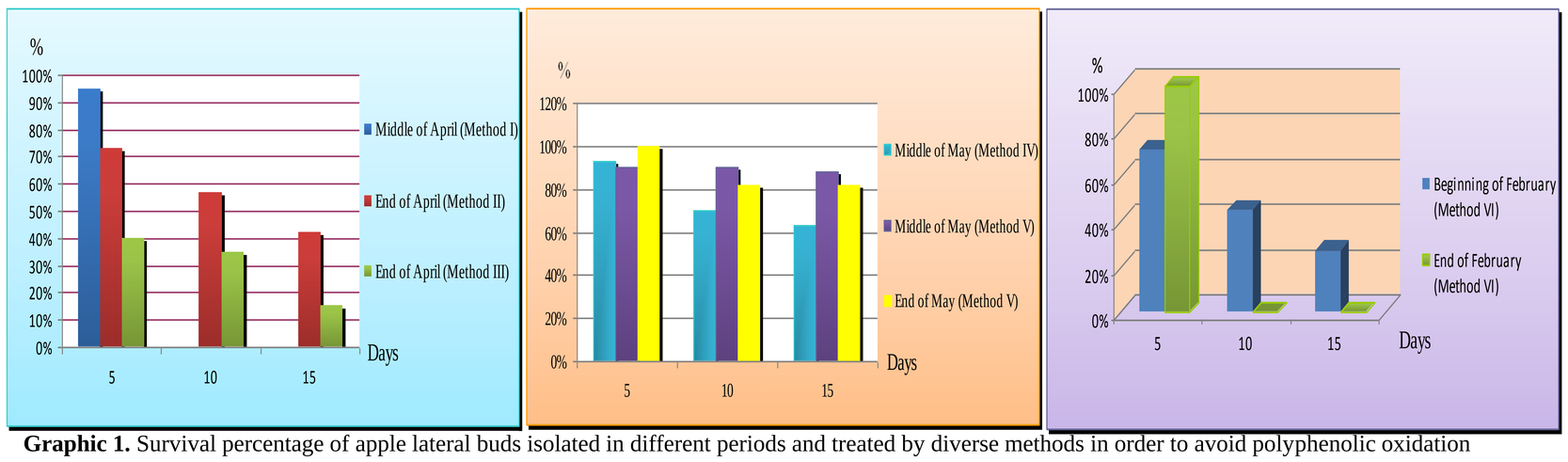


Figure 1. Development of apple buds in different stages of micropropagation

Results and Discussion

Many experiments on micropropagation of different genetic forms of apple show that except the microbial contamination, the browning of cultures due to the exudation of polyphenols are found to be the major bottleneck for the establishment of aseptic cultures. In this experiments, the yellowing of MS medium was observed immediately 2 – 3 hours after buds inoculation (Fig. 1).

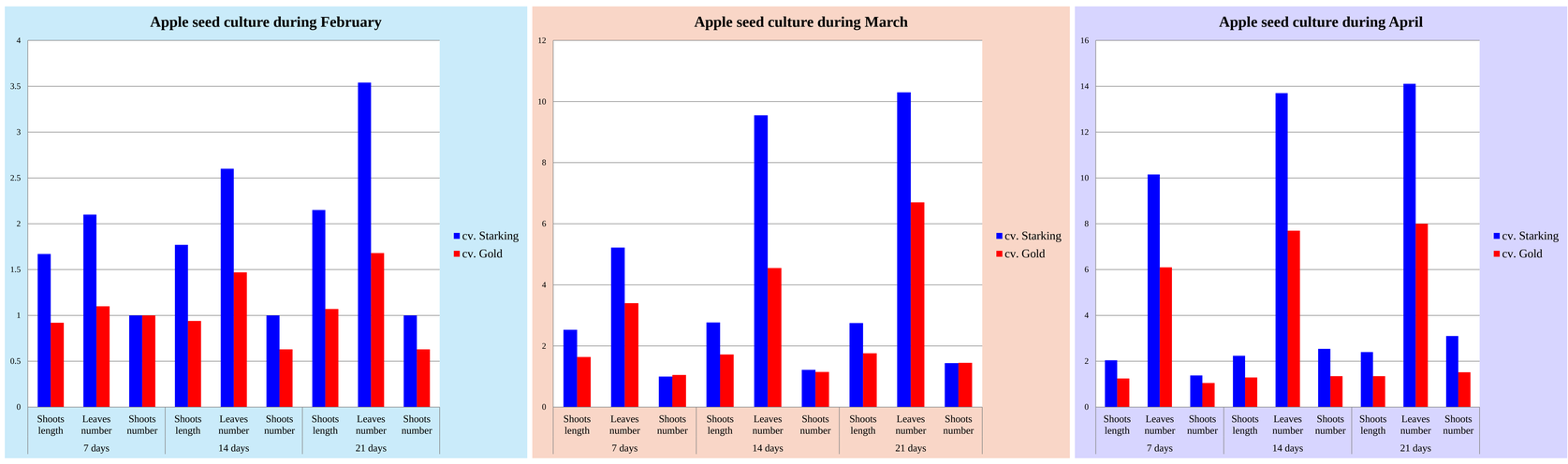
Regarding the effect of nutrient medium supplemented with several antioxidants and some methods of avoiding the phenol releasing from the explants, the type of treatment affected the response percentage during proliferation stage. The apple buds of two cultivars, cultured on MS medium combined with antioxidants (ascorbic acid and citric acid 0.1% each) presented greater survival percentage during the proliferation stage compared to the explants cultured on medium without these acids. The season in which the explants are collected is the major factor that affects the success of cultures establishment. In the other hand, the success of micropropagation is strongly depended from the use of disinfectants, antioxidants and avoiding of polyphenolics methods. Thus, the buds isolated in May, treated with additional agents (citric acid, polyvinyl pyrrolidone PVP and active charcoal) have a high survival rate (from 63% to 100%) during the first 2 weeks of proliferation stage (Graphic 1). But, was noticed that the number of survived plantlets was decreased during further periods of culture because of delayed infections symptoms or delayed polyphenolic compounds release. In this case was very difficult to establish cultures for further micropropagation.



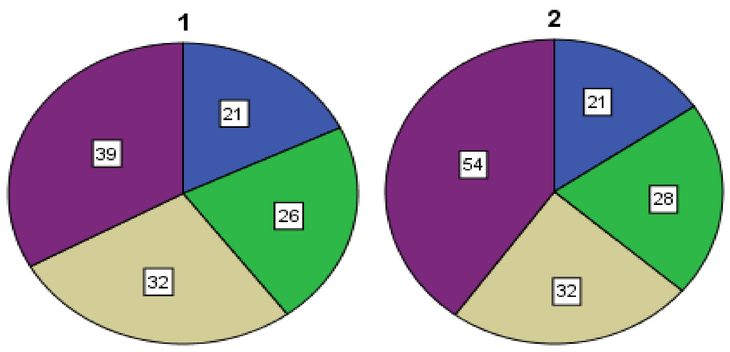
Graphic 1. Survival percentage of apple lateral buds isolated in different periods and treated by diverse methods in order to avoid polyphenolic oxidation

A parallel experiment was conducted using mature seeds as primary explants (Fig. 2). For these types of explants was followed a simple sterilization protocol and there were not added antioxidants in nutrient media. In aseptic conditions was removed the seed coat and the explants of both cultivars were inoculated in MS nutrient media. The regeneration percentage was very high (99%) and there were observed no signs of phenolics exudation onto nutrient media for both apple cultivars. Measurements of leaves number, shoots number and length were taken in different periods (January, February, March). From the results was observed that cv. Starking showed better response for all parameters measured in all periods (Graphic 2; Graphic 3; Graphic 4).

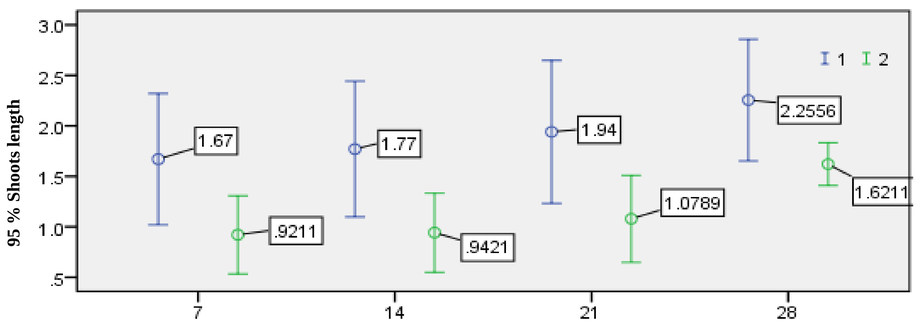
Figure 2. Plantlets development from apple mature seeds



Graphic 2. Comparison of apple cultivars performance during seed culture in different periods of in vitro propagation



Graphic 3. Comparison of leaves number for both apple cultivars (1 – cv.Starking; 2- cv. Gold) in different periods of propagation



Graphic 4. Comparison of shoots length intervals for both apple cultivars (1 – cv.Starking; 2- cv. Gold) in different periods of propagation

Conclusions

- In order to overcome the problem of media coloring by polyphenols that are released from buds, the most optimal nutrient medium is considered MS medium supplemented with MS vitamins and combined with BAP (1 mg/l^{-1}), IBA (0.1 mg l^{-1}), ascorbic acid 0.1%, citric acid 0.05%, 48 hours in darkness and twice transfers in fresh medium;
- The most optimal period for the isolation of apple buds is considered April-May period, when contamination level is lower, while regarding the avoiding polyphenolics releasing, the most appropriate period is February;
- Seed culture results an optimal method for apple micropropagation because of low contamination rates (up to 1%) and no polyphenolics releasment.
- Cv. Starking shows better performance for all paremeters evaluated, in all periods.

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