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Stabilisation of *in vitro* rhizogenesis of two apple (*Malus domestica* L.) cultivars during micropropagation

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Abstract

Rooting induction appears very difficult, especially regarding to trees species but it's necessary to stabilize a protocol for *in vitro* rhizogenesis before the acclimatization stage. These study was carry out to investigate the influence of different doses of auxinic phytohormone NAA (α-naphthaleneacetic acid) and MS nutrients on *in vitro* rhizogenesis of apple (*Malus domestica L.*) which is a fruit extremely rich in important antioxidants, flavanoids, and dietary fiber. The induction and improvement of *in vitro* rhizogenesis of microshoots of two different apple cultivars (cv. Golden Delicious and cv. Starking) was tested on three nutrient rooting media with different concentrations of NAA and macro- and micronutrients, presented in the universal medium MS: (I) - ½ MS macronutrients, MS micronutrients, MS vitamins supplemented with 0.1 mg l^{-1} NAA; (II) - ½ MS macronutrients, ½ MS micronutrients, MS vitamins with 0.1 mg l^{-1} NAA and (III) - MS macronutrients, ½ MS micronutrients, MS vitamins with 2 mg l^{-1} NAA). The first signs of roots formation were detected 10-12 days after inoculation of strongest microshoots of each cultivar of apple. There were detected significant differences in rooting percentage according to the rooting media and the highest value of rooting for both cultivars resulted in the first rooting medium (90.2%). There was not observed significant difference in rooting percentage between two studied cultivars. As the result the first medium with half concentration of MS macronutrients and 0.1 mg l^{-1} NAA is recommended.

Keywords: rhizogenesis, MS medium, NAA (1-Naphthaleneacetic acid), rooting percentage.

1. Introduction

Apples trees are the most common fruit tree type in the EU covering 450 000ha (Eurostat 2014) and in terms of quantity, with 12.7 million tones harvested in 2015 (Eurostat). The climate of Albania with warm summers and cold winters favors the cultivation of this fruit especially in northeast and southeast regions. In Albania, annual consummation of apple fruits arrives 61 553 tones. The attractiveness of fruit to consumers is determined by visual attributes that include appearance, size, uniformity, color and freshness, as well as non-visual attributes such as taste, aroma, flavor, firmness (texture), nutritional value and healthiness. Apples are the natural source of dietary mineral salts, vitamins, antioxidants, fiber, organic acids and sugars that is why there were developed many technology of breeding and preservation. Propagation of woody plants by conventional methods necessarily limits the rate of output and makes the end product expensive. Tissue culture techniques such as micropropagation provide a fast and dependable method for the production of a large quantity of uniform plantlets in a short time throughout the year [14]. The success of shoot multiplication depends not only on the genotype [9], but also on plant growth regulators (PGRs) and the interaction between these two factors. Rooting of shoots remains the most challenged point of the micropropagation process, reducing the possibilities of applying this technique on a large scale. Many woody species are difficult to root through cuttings [13] and adventitious root formation is a key step in micropropagation [5]. Several researchers have reported that consistent high frequency rooting of apple has been more difficult to achieve than shoot multiplication [12]. Root initiation is a complex morphogennic process under the control of various

endogenous and external factors including hormones, growth regulators [1], the basal culture medium concentration, the carbohydrate source, light, temperature and the presence of phenolic compounds. The aim of the present study was to investigate the influence of different doses of auxinic phytohormone NAA (α-naphthaleneacetic acid) and MS nutrients on *in vitro* rhizogenesis of apple (*Malus domestica L.*) and to establish an efficient protocol to solve this problem that affects the acclimatization stage and the efficiency of micropropagation process.

2. Material and Methods

Plant material. Cultures of *Malus domestica* Borkh. cv. Golden Delicious and cv. Starking. are established from apical and lateral buds removed from adult field-grown trees. The two apple's cultivars are more extensive population in the district of Korça (village orchards of Hoçisht, and Cangonj). Most often shoot tips and meristems are the explants of choice due to their genetic stability.

Meristem culture. Plantlets of two cultivars grown under the *in vitro* conditions, were excised carefully in pertridishs. Under a binocular dissecting microscope, the leaflets surrounding the growing point were removed. The exposed meristem tips that appeared as a shiny dome with one or two leaf primordia were gently isolated with a scalpel and cultured on WPM media [9] supplemented with 1mg.l^{-1} BAP and 0.1mg.l^{-1} NAA for 8 weeks.

Shoot multiplication. Explants of each cultivar were multiplied by the method of enhanced release of axillary buds. WPM media [9] having a combination of 1mg.l^{-1} BAP (6-benzylaminopurine) and 0.1mg.l^{-1} NAA (α-naphthaleneacetic acid), 3% sucrose and 0.6% agar was used for these stage. Plantlets of two cultivars inoculated onto aseptic conditions of laminar flow were placed then in growth chamber for 4 weeks. The experiment was repeated three times.

Rooting. Apple microshoots of two cultivars cv Golden Delicious and cv Starking (2-3cm long) were cultured on three nutrient rooting media with different concentrations of NAA and macro- and micronutrients, presented in the universal medium MS: (I) - ½ MS macronutrients, MS micronutrients, MS vitamins supplemented with 0.1 mg l^{-1} NAA; (II) - ½ MS macronutrients, ½ MS micronutrients, MS vitamins with 0.1 mg l^{-1} NAA, 1.5% (w/v) sucrose, 0.55% (w/v) agar for each media and (III) - MS macronutrients, ½ MS micronutrients, MS vitamins with 2 mg l^{-1} NAA, 2% (w/v) sucrose, 0.55% (w/v) agar. The pH of each medium was adjusted to 5.5 before autoclaving. All cultures were incubated at 23±2^{o}C. The intensity of light, consisted of incandescent lamps was 2000 lux with photoperiod of 16h light and 8h dark. The number of days required for rooting and percentage of rooted shoots was recorded during 8 weeks.

Acclimatization. Rooted plantlets were gently removed from the agar medium and cleaned under running tap water. After washing, they were potted in plastic pots containing soil substrate, covered with plastic bags to maintain high humidity and grown under growth chamber conditions with 16h photoperiod at 23±2^{o}C. The bags were gradually opened over a period of 2 to 4 weeks in the culture room to allow plantlets to acclimatize to ambient conditions.

Statistical analysis. IBM SPSS Statistics 20 was used to analyze rooting data. An analysis of variance (ANOVA) was performed to distinguish the differences between tratments ($P<0.05$).

3. Results and Discussion

Meristem culture: The aseptic dissection of the meristem is a delicate process. With the increasing number of days in the culture medium, the excised meristem dome developes bipolar axis during reorganization (Fig 1).

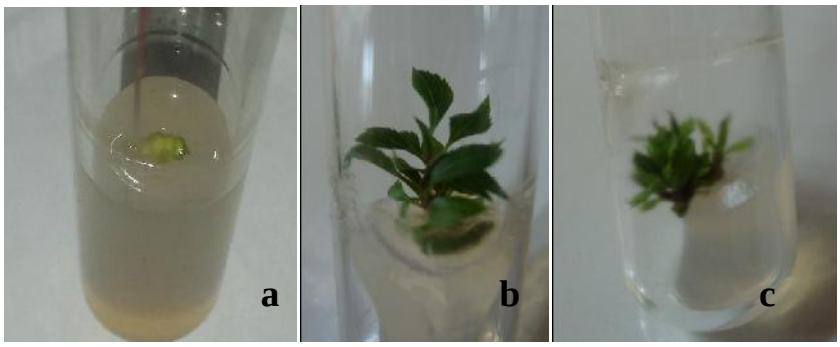

Figure 1. (a): Meristem 4 weeks after inoculation. Plantlets developed form meristem culture **(b):** cv. Starking **(c):** cv. Golden Delicious

The survival percentage of meristematic culture for both cultivars is low, about 28.5%. The meristems of cv. Golden Delicious shows a higher survival percentage (25%) than meristems of cv. Starking (3.5%) (Fig. 2).

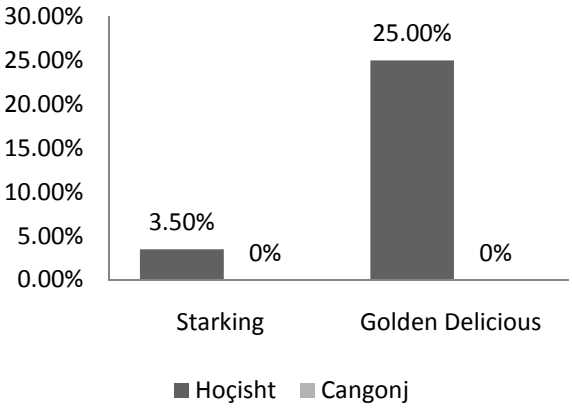

Figure 2. The survival percentage of meristematic culture

Multiplication stage: Due to contamination and phenolic exudation in nutrient media from apical and lateral buds, there are obtained only a few numbers of plants from both cultivars of Hocisht orchard, which are inoculated in nutrient media for further multiplication. During this stage, the contamination rates and the phenolic exudation are insignificant. The survival percentage is very high for all periods of culture and there is observed no difference between both cultivars for this parameter during subculture (Fig. 3a). At the same way, the few number of plantlets obtained form meristematic culture was increased during the multiplication stage. Measurements of leaves number, shoots number and length of shoots were taken during the first subculture (Fig. 3b) and were detected no significant differences between two cultivars based on these parameters ($P>0.05$).

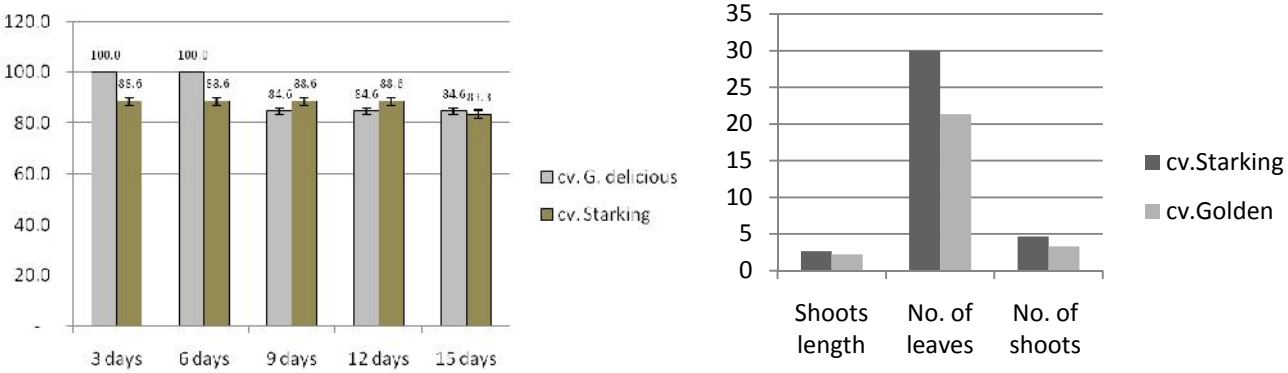

Figure 3. a) The survival percentage of apple explants during subculture stage; **b)** Comparison of apple cultivars 4 weeks after the first subculture of plantlets obtained form meristematic culture

a b

Figure 4. Plantlets development from apple explants from Hocisht orchard, ready to subculture stage: (a) – cv. Golden Delicious; (b) – cv. Starking

Rooting induction: The observations during 2 months show that plantlets react in different way among three rooting medias. When different concentrations of NAA and macro- and micronutrients were tested, the rooting percentage of both cultivars was observed in a few cases only. The data analyzed with ANOVA show that there are significant differences on rooting percentage among rooting media used ($P<0.05$). Basal portion of shoot formed callus and later root initials developed after about 10-12 days (Fig. 5). A short period of treatment of shoots in the dark to eliminate callus formation which has a negative influence on the subsequent acclimatization phase is recommended [14, 15]. In contrast, other studies show that dark have not an importance in the rooting phase of apple [3], as resulted in our experiments . The highest rooting percentage (90.2%) was observed on the first media with half MS macronutrients and 0.1mg.l^{-1} NAA and the lowest percentage (12%) on the third media with half MS micronutrients and 2mg.l^{-1}. Low concentrations of NAA for the formation of roots in apple cultivar shoots are recommended also by other authors [8]. Some studies shows that NAA concentrations influenced rooting negatively since auxin stimulates root initiation but inhibits root elongation [7]. In contrast to our studies, some authors noticed that high rooting percentage (90%) for apple root formation were obtained at 2mg.l^{-1} of NAA [11], by the other hand when effects of different inorganic salt concentrations were investigated they reported that the highest root percent (90%) were observed on half salt strength MS medium as resulted in our study. Also, other researcher have reported that MS salts (1/2 strength) yielded better results than dilution to 1/3 or ¼ strength when combined with low sucrose concentration [12]. Is proposed that reduction of salt strength improved rooting [6]. Among two cultivars studied cv Golden Delicious and cv Starking there was no detected significant differences on rooting percentage ($P>0.05$)

Figure 5. The callus and new roots formation in apple on the first rooting media

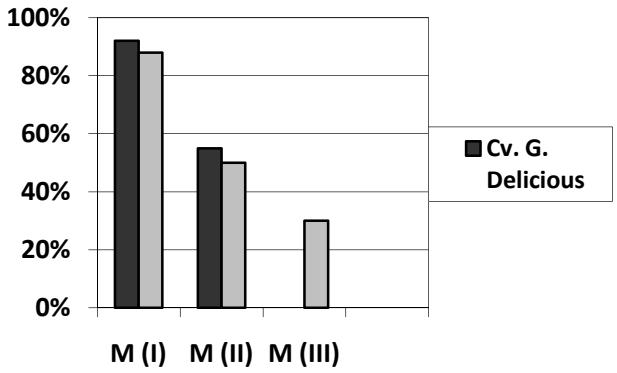

Figure 6. The percentage of rooting plantlets cultured in different rooting mediums (M) for both cultivars

Acclimatization stage: Rooted plantlets were gradually acclimatized with an average of 60% and after then they grew well and did not show morphological abnormalities (Fig 7). Other researchers reported a high percentage of apple acclimatization, about 80% [4] and 85% [11]. Indirect *in vitro* rhizogenesis through callus formation can be one of the reasons for low percentage of acclimatization in both apple cultivars as was shown in pear cultivar Bartlett [2].

Table 1. The percentage of acclimatization of two apple cultivars

Cultivars	*Acclimatization percentages (%)*
Golden Delicious	26.7
Starking	73.3
Total	60

Figure 7. Apple plants during acclimatization

4. Conclusions

The results of our study show that the survival percentage of meristematic culture is low but the number of plantlets obtained might be increased during subculture stages. The optimum media for apple rhizogenesis was the first media with half concentration of MS macronutrients and 0.1 $mg.l^{-1}$ NAA (1-Naphthaleneacetic acid). We suggest further studies to increase the survival and acclimatization percentage of apple.

5. References

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