APPLE SHOOT MULTIPLICATION AND PLANTLETS REACTION TO *IN VITRO* CULTURE

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Abstract. The present work aimed to evaluate the expression of several enzymatic systems in apple (*Malus domestica* Borkh., cvs. Florina, Romus3, Romus4, Colmar, Rebra, Goldrush, Idared) plants grown *in vitro* in comparison with the *in vivo* donor plants. *In vitro* culture was established on Murashige and Skoog (1962) basal medium supplemented with Lee and Fossard (1977) (LF) vitamins, 2 mg l^{-1} N^6-benzyladenine, 0.01 mg l^{-1} α-naphtyl-acetic acid, 30 g l^{-1} dextrose and 7 g l^{-1} agar. The highest shoot proliferation was obtained for all cultivars on medium supplemented with 1.0 mg/l N^6-benzyladenine. Our study shows that *in vivo* plants have a distinct pattern of izoesterases in comparison with *in vitro* plantlets. Several izoesterases characteristic for *in vitro* or *in vivo* plants were identified. Izoperoxidases are inducible with culture conditions, physiological condition and developmental stage. The pattern of superoxid-dismutases is less variable with the culture conditions which demonstrate that *in vitro* culture does not occur oxidative stress. According to the pattern of peroxidases, estarases and superoxid-dismutases, there are not significant differences between *in vivo* and *in vitro* plants. Valuable apple cultivars could be preserved short or medium term by *in vitro* culture without genetically changes.

Keywords: *Malus domestica*, micropropagation, peroxidase, esterase, superoxid-dimutase.

INTRODUCTION

The apple tree was perhaps the earliest tree to be cultivated, and its fruits have been improved through selection over thousands of years [31]. 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. Antioxidants as phenolic compounds (i.e., caffeic acid, ferulic acids, p-coumaric acid, protocatechuic acid) activated all apple peroxidases, so the izoperoxidases could be associated with the level of the antioxidants in apple fruit [10]. Micropropagation represents a widely known method for plant multiplication and has been extensively used for the rapid multiplication of many plant species. Tissue culture methods have been successfully applied also for the propagation of *Malus* species [19, 26]. However, it has been reported that different cultivars and rootstocks do not respond in the same way during micropropagation and *in vitro* rooting [38]. Mature woody plants are more difficult to propagate vegetatively [11] but there were developed different techniques of *in vitro* micropropagation [33]. *In vitro* mass propagation of apple rootstock has been developed using an automated bioreactor system [5]. During *in vitro* culture, plants grow under special conditions. The effects of *in vitro* culture upon an organism are often unknown and regenerated plants may be susceptible to somaclonal variation [23]. With more and more plants obtained by clonal micropropagation for greenhouse or field production the analysis of somaclonal variation or the elimination of genetic variation requires efficient screening methods. Analysis of isozymic patterns in apple regenerants showed polymorphism among regenerants and based on banding patterns, rootstocks and regenerants could be distinguished [23]. Izoenzymes are also useful for cultivar discrimination [22, 34, 39] because it is a rapid method and it is cheap in comparison with analysis of molecular markers based on DNA [40]. Izoenzymes could be used for selection of apple seedlings [42]. The content of ascorbate and the activities of peroxidases in apple fruit were considered as a marker for proper condition of fruit storage [29]. Izoperoxidases and izoesterases were used as molecular markers associated with apple resistance against pathogens [6, 8] and to different environmental conditions as cold [12, 18]. Izoperoxidases and izoesterases were used as markers for genetic variability in wild apple [30] and for genetic stability [37, 41].

The main objectives of this study was *in vitro* multiplication of shoots and the evaluation of the pattern of izoperoxidases, izoesterases and superoxid-dismutases as the major enzymes useful for cultivar discrimination and evaluation of *in vitro* plantlets belonging to seven *Malus* cultivars in comparison with the *in vivo* donor plants.

MATERIAL AND METHODS

Shoot multiplication

Initiation of *in vitro* apple (*Malus domestica* Borkh., cvs. Florina, Romus3, Romus4, Colmar, Rebra, Goldrush, Idared) cultures was made from shoots of mature plants which were cut into nodal stem segments and rinsed in tap water. Surface sterilization of initial explants was made with 70% (v/v) ethanol for 10 min followed by immersion in sodium hypochlorite (6%) for 20 min and then washed with sterile distilled water. Shoots were grown on Murashige and Skoog (MS) basal medium [24] supplemented with Lee and Fossard (LF) [20] vitamins, 2 mg l^{-1} N^6-benzyladenine (BA), 0.01 mg l^{-1} α-naphtyl-acetic acid (NAA), 30 g l^{-1} dextrose and 7 g l^{-1} agar (Sigma). The pH was adjusted to 5.7 before autoclaving. For shoot proliferation from previously established cultures the mentioned medium was used with three different concentrations of BA (0.5, 1.0 and 1.5 mg l^{-1}). The plants were grown at

24°C during a 16 h light photoperiod with a light intensity of 40 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) provided by cool white fluorescent tubes. For micropropagation nodal segments consisting of a piece of stem about 1.5 cm in length with 2-3 leaves were transferred to fresh medium as mentioned above. Subcultures were performed every four weeks.

Analysis of results

Proliferation rate and mean length of shoots were recorded after two months of culture. Explants were also visually evaluated for leaf necrosis, hyperhydricity and chlorosis. Fifteen single shoots for each cultivar were grown on different medium and the experiment was repeated twice. The results representing shoot proliferation and shoot length were expressed as the mean number ± standard deviation (S.D.).

Izoenzyme analysis

The pattern of several izoenzymes as izoperoxidases (E.C.-No. 1.11.1.7; *Per*), izoesterases (E.C.-No. 3.1.1.1; *Est*) and superoxid-dismutases (E.C. 1.15.1.1; *SOD*) was studied in leaves of apple plants prelevated from *in vivo* and from *in vitro* plantlets. Fresh leaves (100 mg) were mortared on ice with extraction buffer w/v containing 0.1 M Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$; 10 mM KCl_2; 14 mM 2-mercaptoethanol, 10-50 mg/ml solid polyvinyl-pirolidone-PVP-40). Samples were centrifuged at 10000 rpm, 10 min at 4 ºC, 15 µl supernatant were loaded in the running gel. The enzyme systems were separated on polyacrylamide gels by isoelectric focusing (IEF) [1]. Gel concentration was 5%. For gel preparation a stock solution of acrylamide/bisacrylamide mixed with ampholine A, pH=3.5-5.0/ampholine B pH=3.5-10.0 ratio 1:1, H_2O, ammonium persulphate 10% and Temed was used. Two buffers were used in cuvettes: 20 mM NaOH/10 mM H_3PO4; Running was performed at 120 V, 45 min, with a Consort device. Histochemical identification of izoenzymes was performed according to several protocols described earlier [1, 25].

RESULTS

In vitro shoot multiplication

For the success of *in vitro* culture initiation and following shoot proliferation of woody species it is important to avoid phenolic oxidation which can lead to necrosis of the tissue [19]. Shoot production increased in all cultivars in response to BA in culture medium [19]. Our results show that 1.0 mg l^{-1} BA is adequate for shoot multiplication in all cultivars. Proliferation capacity was positively influenced generating 19 shoots/inoculum for cv. Goldrush and 16.5 shoots/inoculum for cv. Colmar (Fig. 1). The lowest numbers of shoots/inoculum were obtained for cv. Romus3 (7.4) and respectively Romus4 (7.6).

After two months of cultivation the highest length of shoots was obtained for shoots grown in media with 0.5 mg/l BA regardless of the tested cultivar. The cv. Florina showed a length of 6.8 cm (Fig. 2.). The length of shoots for the other cultivars was situated between 3.2 cm for cv. Romus3 and 4.4 cm for cv. Goldrush (Fig. 2.).

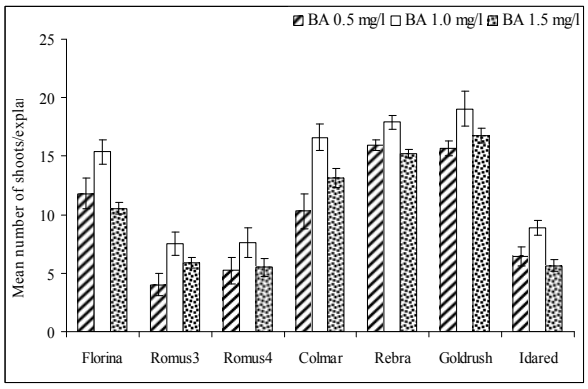

Figure 1. Mean number of shoots per inoculum as a response of BA concentration in culture media. Vertical bars represent standard deviation.

These results outlined the favorable influence of the growth regulators added. Plants did not developed symptoms of necrosis with increased subculture duration and no callus formation was observed.

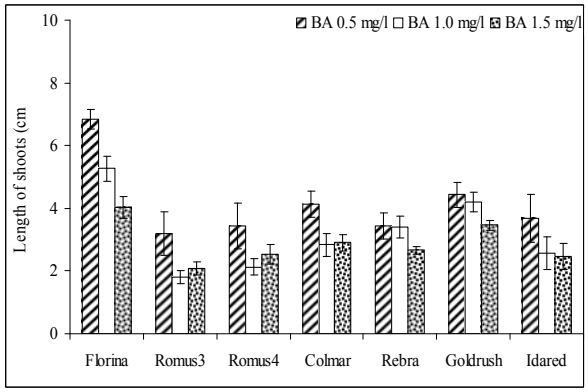

Figure 2. Length of shoots as a response of BA concentration in culture media. Vertical bars represent standard deviation.

Izoenzyme analysis

Electrophoresis on polyacrylamide gel by izoelectric focusing (IEF) allows izoenzymes separation according to their izoelectric point which makes possible the identification of izoforms with similar molecular weight according to their izoelectric point and they could be named as alkaline, neutral or acid izoenzymes. The zymogramme of isoperoxidases expressed in apple leaves is shown in Fig. 3. All the peroxidases are slightly alkaline, neutral or slightly acid, their izoelectric point being about 7.5-6 (in the middle of the running gel wich contains pH gradient). As it could be seen, each cultivar shows a specific pattern of izoperoxidases. There are some differences between *in vivo* plants (from field) and *in vitro* plantlets belonging to the same cultivar. Several izoperoxidases are common in some cultivars, some of them are distinct. Florina cv. shows 2 acid izoperoxidases in the leaves of *in vivo* plant and 4 izoperoxidases in plantlet grown *in vitro*, 2 of them are neutral and 2 of them are acid. The neutral izoperoxidases were not found in *in vivo* plants. The izoperoxidases pattern in leaves of Romus3 and Romus4 cvs. are similar to *in vivo* and *in vitro* plants. There were distinguished 4 izoperoxidases, 2 neutral and other 2 acid izoperoxidases. In case of Colmar cv. several differences were evidentiated in the pattern of

izoperoxidases in the leaves of *in vivo* plant where 3 neutral izoperoxidases were found and *in vitro* plant which exhibit 5 izoperoxidases, the first 2 being alkaline izoperoxidases and the other 3 being neutral, which are the same to the *in vivo* plant. *In vivo* plant of Rebra cv. shows only 1 neutral izoperoxidase in their leaves and *in vitro* plantlet shows 1 alkaline izoperoxidase and 2 acid izoperoxidases. In Goldrush cv. only 1 neutral izoperoxidase was distinguished in the leaves of *in vivo* plant and in plantlet grown *in vitro* there were 2 izoperoxidases, 1 alkaline and an other 1 neutral. *In vivo* plant of Idared cv. shows 1 alkaline izoeproxidase, 1 neutral izoperoxidase and 1 acid izoperoxidase, whereas *in vitro* plantlet shows only the alkaline and the acid izoperoxidases.

The izoperoxidases are very inducible enzymes, that is why there were evidentiated differences between *in vivo* and *in vitro* plants. Izoperoxidase expression depends on the culture condition, physiological and developmental stages [14, 15], oxidative stress [7, 21]. It is also known that alcaline izoperoxidases are generally involved in plant response against different stress factors, whereas acid izoperoxidases are involved in polymerization of lignin monomers and cell wall development [15].

The pattern of superoxid-dismutases (SOD) is not so variable as the pattern of izoperoxidases, and iti is not distinct for diferent cultivars (Fig. 4). Florina, Romus3 and Romus4 cvs. show in their leaves 2 izoenzymes, 1 is slightly alkaline and an other 1 acid. Both of them are present eighter in the *in vivo* plants or *in vitro* plantlets. Colmar, Rebra, Goldrush and Idared cvs. show the alkaline SOD and an other one only in the *in vivo* plants of Rebra and Goldrush cv. which is acid and is different from those expressed in Florina, Romus3 and Romus4 cvs. *In vitro* plantlets of Colmar, Rebra, Goldrush and Idared cvs. express in their leaves an other alkaline SOD wich is not expressed in the leaves on *in vivo* plants. As for the SOD is the most active enzymatic system involved in plants respons to oxidative stress [2, 3, 9, 13, 27], we conclude that *in vitro* condition did not induce metabolic changes in comparison with field plants.

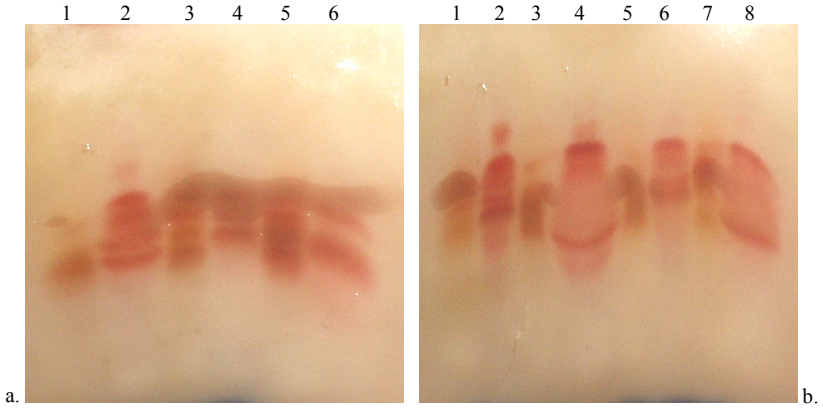

Fig. 3. Zymogramme of izoperoxidases from apple leaves prelevated from *in vivo* and *in vitro* plants (a: 1-Florina *in vivo*, 2-Florina *in vitro*, 3-Romus3 *in vivo*, 4-Romus3 *in vitro*, 5-Romus4 *in vivo*, 6-Romus4 *in vitro*; b: 1-Colmar *in vivo*, 2-Colmar *in vitro*, 3-Rebra *in vivo*, 4-Rebra *in vitro*, 5-Goldrush *in vivo*, 6-Goldrush *in vitro*, 7-Idared *in vivo*, 8-Idared *in vitro*).

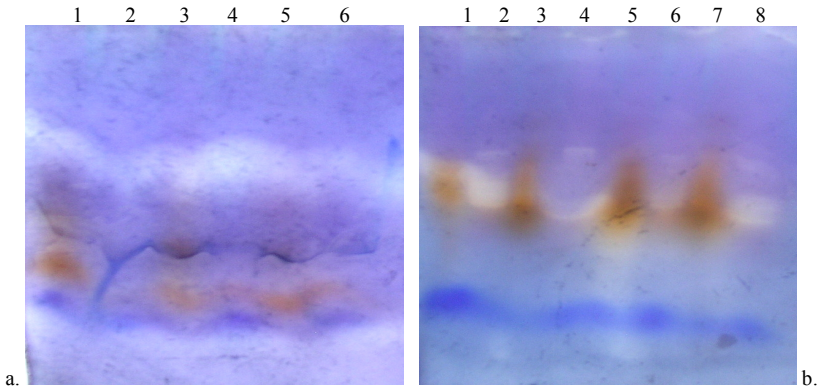

Fig. 4. Zymogramme of superoxid-dismutases from apple leaves prelevated from *in vivo* and *in vitro* plants (a: 1-Florina *in vivo*, 2-Florina *in vitro*, 3-Romus3 *in vivo*, 4-Romus3 *in vitro*, 5-Romus4 *in vivo*, 6-Romus4 *in vitro*; b: 1-Colmar *in vivo*, 2-Colmar *in vitro*, 3-Rebra *in vivo*, 4-Rebra *in vitro*, 5-Goldrush *in vivo*, 6-Goldrush *in vitro*, 7-Idared *in vivo*, 8-Idared *in vitro*).

The pattern of izoesterases shows minor differences between cultivars (Fig. 5). In alkaline region of the pH gradient there is only one izoesterase expressed only in the *in vitro* plantlets of Florina and Idared cv. In neutral region there are 3 izoesterases. The first one is expressed only in the *in vivo* plants independent of the cultivar, the second one is expressed only in the *in vitro* plants in all cultivars, and the third is expressed in all plants independent of cultivar and culture conditions. In the region slightly acid there are 2 izoesterases expressed in all plants belonging to Florina, Romus3 and Romus4 cvs. These 2 izoesterases are also expressed in the *in vivo* plants of Rebra and Goldrush cvs. The plants of Colmar cv. does not express the acid esterases and *in vitro* plantlet of Idared cv. shows only the alkaline izoesterase.

Synthetic distribution of izoenzymes in plantlets of different cultivars could be observed in Table 1. In this table could be seen the genotype of all plants analyzed, the most frequent alleles are Per-Neutral 1, SOD-Alkaline 2 and Est-Neutral 3. These izoenzyme are expressing independent of cultivar and culture conditions. The expression of Est-Neutral 1 is specific for *in vivo* plants, and expression of Est-Neutral 2 is specific for *in vitro* plantlets independent of cultivar. Several izoesterases characteristic for *in vitro* or *in vivo* plants were identified. The pattern of superoxid-dismutases is less variable with the culture conditions which demonstrate that *in vitro* culture does not occur oxidative stress. According to the pattern of peroxidases, estareases and superoxid-dismutases, there are not significant differences between *in vivo* and *in vitro* plants, plantlets could be preserved by *in vitro* culture short or medium term without genetically changes.

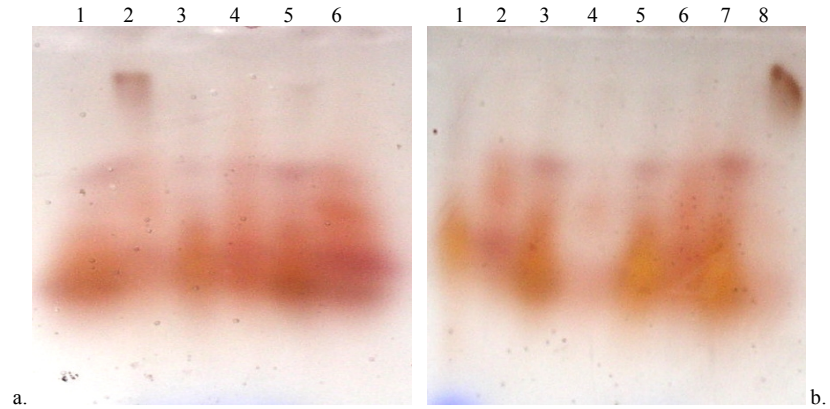

Fig. 5. Zymogramme of izoesterases from apple leaves prelevated from *in vivo* and *in vitro* plants (a: 1-Florina *in vivo*, 2-Florina *in vitro*, 3-Romus3 *in vivo*, 4-Romus3 *in vitro*, 5-Romus4 *in vivo*, 6-Romus4 *in vitro*; b: 1-Colmar *in vivo*, 2-Colmar *in vitro*, 3-Rebra *in vivo*, 4-Rebra *in vitro*, 5-Goldrush *in vivo*, 6-Goldrush *in vitro*, 7-Idared *in vivo*, 8-Idared *in vitro*).

Table 1. Expression of izoenzymes in different apple cultivars.

Enzyme coding locus	Allele	Florina		Romus3		Romus4		Colmar		Rebra		Golderush		Idared	
		Ex vitro	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*
Per-Alkaline	1	-	-	-	-	-	-	-	+	-	+	-	-	-	-
Per-Neutral	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	-	+	+	+	+	-	+	+	-	-	-	-	+	-
	3	-	+	+	+	+	+	-	+	+	-	+	+	-	-
Per-Acid	1	+	+	+	-	+	-	-	-	-	+	-	-	+	+
	2	-	-	-	-	-	-	-	-	-	+	-	-	-	-
SOD-Alkaline	1	-	-	-	-	-	-	-	+	-	+	-	+	-	+
	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SOD-Acid	1	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	+	-	+	-	-	-
Est-Alkaline	1	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Est-Neutral	1	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	2	-	+	-	+	-	+	-	+	-	+	-	+	-	+
	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Est-Acid	1	+	+	+	+	+	+	-	-	+	-	+	-	-	-
	2	+	+	+	+	+	+	-	-	+	-	+	-	-	-

DISCUSSIONS

Apple fruit is one of the most important fruits produced all over the world. Fungal diseases as fire blight infections produced by *Erwinia amylovora* and virus diseases produced by *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) causes important economic losses in apple. Solarization of entire trees under tents of clear polyethylene was used as a means to stop the progress of fire blight infections and eradicate *Erwinia amylovora* from infected tissues. Elevated temperatures obtained through solarization of soil have been shown to reduce inoculum of pathogens. High temperatures and reduced water and nutrient availability are reported to stop disease progress but growers are usually unable to control these factors [17, 32, 36]. *In vitro* culture of shoot tips associated or not with thermotherapy ensures the micropropagation of virus free plants [28]. Thus, it is very important to preserve these plantlets in gemplasm collection. In Romania there are several studies regarding short term preservation of apple cultivars by *in vitro* culture [26] but there is not a germplasm collection and the explants were not preserved long term. In order to develop a cryopreservation method it requires to obtain high number of *in vitro* plantlets to have material for cryopreservation experiment and to characterize the *in vivo* plants (donor of explants) and the *in vitro* plantlets and their reaction to *in vitro* culture. All of these

informations were obtained by analysis of some marker enzymes as peroxidases which are inducible with culture conditions, physiological condition and developmental stage, superoxid-dismutases wich are the key enzymes in oxidative stress and esterases which are not inducible in the conditions mentioned above and could be used for cultivars discrimination.

It is well known that *in vitro* regeneration of woody perennial plant species is difficult. Adventitious regeneration could result in higher shoot production for micropropagation than axillary shoot proliferation [35]. It was demonstrated that encapsulation of *in vitro* propagules could reduce the cost of micropropagated plants for commercialization [4]. The combination of synseed and traditional real seed use could find application in agriculture [4, 16]. Because the multiplication rate and growth is the major parameter for successful large-scale plant production, further investigations are required to achieve optimal propagation of these cultivars.

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