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Evaluation of morphological and chemical characteristics and micropropagation of traditionally grown domesticated apple varieties in Croatia

Određivanje morfoloških i kemijskih karakteristika te mikropropagacija tradicionalnih udomaćenih sorata jabuka u Hrvatskoj

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Abstract

Traditionally grown domesticated apple varieties are known for their adaptability to different climates and a wide range of soil types, and some for resistance to the main diseases. Their evaluation and preservation are very important, not only for restoration of traditional orchards, but also as a source of genetic material for breeding new varieties. Micropropagation of the most valuable varieties could be a way to propagate plants for restoration of traditional orchards, or as a prerequisite for *ex situ* long-term conservation through cryopreservation. Fruit samples were taken from trees 50 or more years old, of 13 traditionally grown domesticated apple varieties in Bjelovar Bilogora County in Croatia. Morphological traits of the fruits were evaluated during 3 years, and chemical characteristics (soluble solids, reducing sugars, acids and total phenol content) were evaluated during one vegetation year. Tissue culture was established from shoot apices approximately 1 mm in size isolated from buds of 1-year-old scion woods. Axillary branching was induced from two types of explants on MS medium with the addition of 2% glucose, 1% sucrose, 0.1 g*L^{-1} myo-inositol, 0.8% agar, 0.2 mg*L^{-1} gibberelic acid (GA3), 0.1 mg*L^{-1} indole-3-butyric acid (IBA) and 1.5 mg*L^{-1} 6-benzylaminopurine (BAP) in proliferation medium 1 (PM1) or 2 mg*L^{-1} zeatin (ZEA) in proliferation medium 2 (PM2). The morphological characteristics of the traditionally grown domesticated apple varieties mentioned were not different from their standard values. Average fruit weight (AFW), average fruit height (AFH) and average fruit diameter (AFD) were less than standard values, which can be explained by the age of the fruit trees. Principal component analysis of these traditionally grown domesticated apple varieties showed that fruit traits very important in today's breeding for new cultivars, like fruit weight, shape, skin color and flesh taste, stand out in explaining the variability. Despite the age of the fruit trees, the fruits still have surprisingly positive chemical characteristics, especially Canadian Reinette for which the highest concentration of total phenols was found. Of the five traditionally grown domesticated apple varieties used, tissue culture was successfully established for Jonathan and Canadian Reinette varieties. Higher multiplication efficiency was obtained for the Jonathan variety. Medium supplemented with 1.5 mg*L^{-1} BAP produced a significantly higher number of shoots/explant in comparison with those supplemented with ZEA. Morphological features of shoots produced on medium supplemented with ZEA, however, were better. Both explant types, shoots placed in the medium vertically or two-nodal stem segments placed in the medium horizontally, gave similar results for axillary branching.

Keywords: axillary shoot proliferation, cytokinins, explant types, *Malus domestica*, old domesticated varieties

Sažetak

Tradicionalno udomaćene sorte jabuka poznate su po svojoj adaptabilnosti na različite tipove tala i klimatske uvjete kao i na otpornosti prema bolestima. Njihovo poznavanje i očuvanje je vrlo bitno, ne samo radi revitalizacije tradicionalnih voćnjaka, već i zbog velikog značenja u oplemenjivanju jabuke (razvoju novih sorata). U svrhu revitalizacije tradicionalnih voćnjaka najznačajnije sorte se mogu razmnožavati mikropropagacijom, koja je i preduvjet za *ex situ* dugoročno očuvanje krioprezervacijom. S tradicionalno uzgajanih stabala jabuka, starih 50 ili više godina, uzimani su uzorci plodova jabuka u Hrvatskoj, na području Bjelovarsko-bilogorske županije. Morfološke karakteristike plodova određene su tijekom 3 godine, a kemijske karakteristike (topiva suha tvar, reducirajući šećeri, kiseline i ukupan sadržaj fenola) tijekom jedne vegetacije. Kultura tkiva je uspostavljena iz vegetacijskih vršaka veličine 1 mm izoliranih iz pupova jednogodišnjih izbojaka. Aksilarno grananje je inducirano iz dva tipa eksplantata na MS mediju uz dodatak 2% glukoze, 1% saharoze, 0,1 g*L^{-1} myo-inozitola, 0,8% agara, 0,2 mg*L^{-1} giberelinske kiseline (GA3), 0,1 mg*L^{-1} indol-3-octene kiseline (IBA) te 1,5 mg*L^{-1} 6-benzilaminopurina (BAP) u mediju 1 (PM1) ili 2 mg*L^{-1} zeatina (ZEA) u mediju 2 (PM2). Morfološke karakteristike istraživanih starih stabala tradicionalno udomaćenih sorata jabuka nisu se razlikovale od standardnih vrijednosti osim prosječne mase, visine i promjera ploda koji su bili manji od standardnih vrijednosti, što se može objasniti starošću stabala. Iz rezultata analize glavnih komponenata uočljivo je da karakteristike ploda kao što su masa i oblik ploda, boja kožice i okus mesa ploda, koje su vrlo važne u oplemenjivanju, ujedno najviše utječu na ukupnu varijabilnost. Bez obzira na starost stabala, plodovi su još uvijek imali iznenađujuće dobre kemijske karakteristike, osobito kod sorte Canadian Reinette kod koje je izmjerena najviša koncentracija ukupnih fenola. Od pet tradicionalno udomaćenih sorata jabuke uvedenih u kulturu tkiva, uspješno su mikropropagirane sorte Jonathan i Canadian Reinette. Veću stopu multiplikacije imala je sorta Jonathan. Značajno veći broj izdanaka po eksplantatu dobiven je na hranidbenom mediju s dodatkom 1,5 mg*L^{-1} BAP nego na mediju s dodanim ZEA. Međutim, morfološke karakteristike izdanaka regeneriranih na mediju s dodatkom ZEA bile su bolje. Oba tipa eksplantata, izdanci postavljeni vertikalno na hranidbeni medij ili dvonodalni segmenti stabljike postavljeni horizontalno na hranidbeni medij, dali su slične rezultate aksilarnog grananja.

Ključne riječi: aksilarno grananje, citokinini, *Malus domestica*, tip eksplantata, tradicionalno udomaćene sorte

Introduction

Among fruit species, the apple prevails; it is the most economically important fruit crop. In Croatia, the total production of apples in 2015 was 101,752 t from a production area of 5,756 ha (Croatian Bureau of Statistics, 2016). Once, apple production was much greater, but with time, it decreased on the ever-decreasing growing area.

The cultivation of fruit crops underwent transformations; old cultivation technologies were abandoned, new technologies introduced and new varieties favored. This led to the loss of old varieties, and a decrease in biodiversity, not only for less important apple varieties but also for those of agronomic importance. Many traditionally grown varieties have been abandoned, with the risk of extinction. Old varieties are a rich source of genetic material with a significant role in breeding, and some have very positive characteristics (e.g. high, regular fertility), which is very important in cultivation (Mitre et al., 2009). Apples, like other fruits, vary in chemical composition, even within the same variety, depending on maturity, production location and agricultural practices, as well as on numerous environmental factors (Lee et al., 2003; Šturm et al., 2003). However, every cultivar has its own typical chemical composition (Veberic et al., 2005). The most common phenolic compounds in apples are flavonoids, the concentration of which mostly depends on the apple variety (Yuri et al., 2014). Lee et al. (2003) reported that flavonoids such as quercetin, epicatechin and procyanidin B2, rather than vitamin C, contribute significantly to the total antioxidant activity of apples. Traditionally grown domesticated apple varieties are known for their adaptability to different climates and wide range of soil types, as well as their resistance to the main diseases. For this reason, preservation of traditionally grown domesticated apple varieties is an important task. Preservation can be done *in situ* by growing those varieties in traditional orchards. *In vitro* multiplication could be a way to propagate plants for restoration of traditional orchards consisting of traditionally grown domesticated apple varieties grown as self-rooted plants. Besides preservation of valuable germplasm, traditional orchards are hotspots for biodiversity in the countryside, supporting a wide range of wildlife (Joint Nature Conservation Committee, 2008).

Good multiplication efficiency *in vitro* is also the main prerequisite for *ex situ* long-term conservation of germplasm through cryopreservation. Micropropagation allows not only easy and efficient acquisition of cryostorage plant material but also post-cryopreservation regeneration performed with the use of an *in vitro* technique (Kwaśniewska et al., 2017). Successful micropropagation of apples using pre-existing meristems is influenced by several internal and external factors including *ex* *vitro* (e.g. genotype and physiological state) and *in vitro* conditions (e.g. media constituents and light) (Dobránszki and Teixeira da Silva, 2010). The main objectives of the current study were to (1) determine morphological traits and chemical characteristics of fruits of traditionally grown domesticated apple varieties, (2) establish tissue culture from aged trees of traditionally grown domesticated apple varieties and (3) determine the effect of variety, different cytokinins in the medium and explant type on axillary shoot proliferation.

Materials and methods

Plant material and morphological traits

Thirteen traditionally grown domesticated apple varieties (Rotter Pogatscher – RP, Gold Reinette – GR, Champagner Reinette – CR, Canadian Reinette – CA, Jonathan – JO, Ananas Reinette – AR, Adam's Pearmain – AP, Bohnapfel – BH, Belle de Boskoop – BB, Crown Prince Rudolf – PR, Reinette de Macon – RM, Steirischer Maschanzker – SM and Baumann's Reinette – BR) were analyzed in this study. The investigation area was limited to Bjelovar Bilogora County, which is located in the continental part of Croatia. In history, the region mentioned was influenced by migrations in the former Austro-Hungarian Empire as well as the Ottoman Empire, and many cultivars of apple that originate from all around both empires are still present. The average age of all trees in research was over 50 years. Trees were not cultivated nor pruned, have free canopy form and have been held under non-irrigated extensive cultural practice. The samples of 20 fruits from the same tree were collected randomly in three growing seasons (2011-2013) at full maturity stage. The full maturity stage of traditionally grown domesticated apple varieties was determined by counting number of days after full bloom and with determination of Cornel Starch - Iodine Index. All the morphological traits were characterized and evaluated using the UPOV (International Union for the Protection of New Varieties of Plants) test guideline for apples (UPOV, 2005), and the description for apples by Adamić et al. (1963), and are presented in Table 1.

Determination of chemical characteristics

Freshly squeezed and filtered juice of each fruit was used for determination of soluble solid content (SSC) using a digital refractometer (DR 201-95, A Kruss, Optronic GmbH, Hamburg Deutschland) and expressed as °Brix.

On prepared apple peel samples (chopped, homogenized and frozen at −20 °C for two weeks. The following chemical parameters were determined: reducing sugars, total acid content and total phenol content. The amount of reducing sugars expressed as invert sugar was determined by Luff-Schoorl methods (AOAC International, 1995, 2000).

Acids were measured by titration with 0.1 M NaOH and phenolphthalein as an indicator, and given in mmol*100 g^{-1} as malic acid. The total phenol content was determined by spectrophotometry, according to the method described by Singleton and Rossi (1965). The method involves the reduction of Folin–Ciocalteu reagent (Sigma Chemical, St. Louis, MO, USA) by phenolic compounds, with concomitant formation of a blue complex. In this study, 0.5 mL of the extract was mixed with 3 mL of distilled water and 0.25 mL of Folin–Ciocalteu reagent. Immediately, 0.75 mL of saturated sodium carbonate and 0.95 mL of distilled water were added. Then, the mixture was incubated for 30 min at 37 °C, and the absorbance was read at 765 nm using a UV-Vis spectrophotometer (Varian Spectrophotometer DMS 200, Palo Alto, SAD). The measurement was compared to a standard curve prepared with gallic acid solution (Sigma Chemical) and expressed in g of gallic acid equivalents (GAE)*L^{-1} of sample. Chemical characteristics were determined on samples from one vegetation year. All measurements of chemical parameters were performed in triplicate.

Micropropagation

One-year-old scion wood from five traditionally grown domesticated apple varieties (RP, GR, CR, CA and JO) was collected from the accession presented in Table 1, in early spring, prior to leaf-bud opening (green tip).

Shoot segments with few axillary buds were washed in tap water and surface-sterilized by immersion in 70% ethanol for 5 min, followed by immersion in 5% sodium hypochlorite (5% active chlorine) with the addition of 0.1% Tween 20 and 150 mg*L^{-1} of ascorbic acid for 10 minutes. Segments were rinsed four times in sterile distilled water supplemented with 150 mg*L^{-1} of ascorbic acid.

Sixteen to eighteen shoot apices of each variety, approximately 1 mm in size, were aseptically isolated under stereomicroscope, and each placed separately on the surface of 10 mL of establishment medium (EM) in 12 cm-high tubes. EM consisted of QL (Quoirin and Lepoivre, 1977) macroelements and MS (Murashige and Skoog, 1962) microelements, 3% sucrose, 0.1 g*L^{-1} myo-inositol, 0.8% agar (Difco Bacto), 1.5 mg*L^{-1} BAP, 0.1 mg*L^{-1} IBA, 0.5 mg*L^{-1} GA3 and 1 g*L^{-1} of activated charcoal. Explants were cultured on EM for 21 d at 22 °C, with a 16 h photoperiod of cool white light (40 μE*m^{-2}*s^{-1}). Surviving shoots were subcultured five times in EM (without activated charcoal after the first 3 weeks) until enough material for setting the experiment was produced.

The micropropagation experiment was performed on MS medium with the addition of 2% glucose, 1% sucrose, 0.1 g*L^{-1} myo-inositol, 0.8% agar (Difco Bacto), 0.2 mg*L^{-1} GA3, 0.1 mg*L^{-1} IBA and 1.5 mg*L^{-1} BAP (PM1) or 2 mg*L^{-1} ZEA (PM2), at pH 5.8.

Experiments were set up as a completely randomized design. Two types of explants, approximately 1 cm-long shoots placed in the medium vertically, or two-nodal stem segments placed in the medium horizontally, were used. At least 12 explants of each type of surviving variety were placed on each medium composition (PM1 and PM2). The experiment was repeated three times through successive subcultures, each lasting for 40 d. The following parameters were counted or measured: number of shoots per explant, average length of shoots and average number of nodes per shoot.

Table 1. Apple morphological traits and their state of expression according to Adamić et al. (1963) and UPOV (2005)

Trait	Graduations
AFW (g), AFH (mm), AFD (mm)	-
Fruit shape-FS	1.0 asymmetric, 2.0 roundish ovoid, 2.1. ovoid 3.0 conical, 3.1 conversely roundish conical, 3.2 elongated conical, 3.3 rounded conical 4.0 obloid, 4.1 moderately obloid, 4.2 rounded obloid, 5.0 roundish, 5.1 elongated roundish
Ground color of the skin of fully mature fruit (GC)	1 not visible, 2 whitish yellow, 3 whitish green, 4 yellow green, 5 green
Over color of the skin (OC)	1 orange red, 2 pink red, 3 red, 4 purple red, 5 brown red
Presence of stripes (PS)	1 absent or very small, 2 small, 3 medium, 4 large, 5 very large
Stem length (SL)	1 very short, 2 short, 3 medium, 4 long, 5 very long
Depth of sepal: eye basin (DS)	1 deep, 2 medium, 3 shallow, 4 without recess
Width of sepal: eye basin and openness of stamen (WSOS)	1 deep seated sepal and wide open stamen, 2 sepal without recess with open stamen, 3 medium deep of sepal and closed stamen, 4 medium deep of sepal and open stamen, 5 medium deep of sepal and wide-open stamen
Basic form of style (BFS)	1 wide style with the stamen in the lower third, 2 elongated style with the stamen in the upper third, 3 whirligig form of style with the stamen in the middle, 4 elongated whirligig form of style with the stamen at the top
Position and shape of former outer wall of ovary (PSWO)	1 flattened in the lower part of the fruit, 2 flattened in the middle part of the fruit, 3 flattened at the top part of the fruit, 4 elongated whirligig form, 5 elliptic in the central part of the fruit, 6 elongated whirligig form at the top part of the fruit, 7 heart-shaped form
Color of flesh (CF)	1 white, 2 cream, 3 yellowish, 4 greenish, 5 pinkish, 6 reddish
Taste of flesh (TF)	1 sweet, 2 sweet sour, 3 sour, 4 sour sweet, 5 tasteless, 6 bitter
Firmness of flesh (FF)	1 very soft, 2 soft, 3 medium, 4 firm, 5 very firm

Statistical analysis

Statistical analysis for morphological traits was performed using average values from 3-year measurements. Principal component analysis (PCA) was performed to summarize the various data in the principal component (PC) containing the highest possible variability of the data. The eigen values of the five PCs were compared for each trait. Data analysis was done using R (R Core Team, 2015).

For micropropagation and data of chemical properties, statistical analysis was performed using ANOVA. Mean values of chemical properties were compared by least significant difference (LSD) test at P<0.05 (SAS, 2010).

Results and discussion

Morphological traits

Results of morphological traits of fruits (Table 2) were in accordance with those obtained by Adamić et al. (1963) and Hartmann (2003). However, for traditionally grown domesticated apple varieties observed, in some fruits, a change was noticed in the fruit form where fruits inclined to asymmetry; generally, all fruits were smaller in dimensions and weight than average for the varieties (Table 2). This fact can be attributed to the age of the trees. Pirlak et al. (2003) reported similar results for some local native summer apple cultivars, with fruit weight values between 49.5 and 152.2 g.

From the results, five PCs (Table 3) with an eigenvalue greater than 1.0 were retained, and together they accounted for 83.33% of the variability. The first PC had high positive loadings for the traits average fruit weight (AFW), average fruit height (AFH), ground color of the skin (GS), basic form of style (BFS) and taste of flesh (TF). The first PC explained 24.51% of the variation, and may be interpreted as general fruit weight, ground color and a test of fruit components. The second PC showed high loadings for the traits over color of the skin (OC) and width of sepal eye basin and openness of stamen (WSOS), whereas the remaining fruit characteristics such as fruit shape (FS), position and shape of the former outer wall of the ovary (PSWO) and color of flesh (CF) had lower correlation with this component. Interpretation of this component to general fruit character should be done with caution since variable AFH had lower correlation with this component. The variation of the third PC was explained mainly by the trait OC. The traits FS, depth of sepal eye basin (DS) and PSWO had moderate correlation with these traits, with loadings of 0.52 and 0.58, respectively.

The fourth PC had high positive loadings for the traits stem length (SL) and firmness of flesh (FF). Summing up, 73.17% of variation could be explained by the first four PCs. The rest of the components varied to a lesser extent (8.83% of total variance).

Just as in this research, in most studies applying PCA, the first three or four components describe more than 70% of the variation (Iezzoini and Pritts, 1991; Ruiz and Egea, 2008).

Observing the PCA results for these traditionally grown domesticated apple varieties, noticed was that fruit traits are very important in today's breeding for new cultivars, like fruit weight, shape, skin color and flesh taste, stand out in explaining the variability. Therefore, conclusion could be made that these traditionally grown domesticated apple varieties present a good gene pool for these traits in breeding programs for new commercial apple varieties. The aforementioned apple characteristics are defined by the market that plays a major role in breeding.

Chemical characteristics

As can be seen in Table 4, SSC in traditionally grown domesticated apple varieties ranged from 14.97 °Brix (GR – Gold Reinette) to 20.27 °Brix (RM – Reinette de Macon), which are values higher than those of today's commercial varieties. The SSC of Topaz and Pinova apple varieties is 13 and 14 °Brix, respectively (Fischer and Fischer, 2002).

The reducing sugar content in the traditionally grown domesticated apple varieties evaluated ranged from 8.51% (SM – Steirischer Maschanzker) to 11.1% (RP – Rotter Pogatscher), which represents a high level of reducing sugars. A similar result with such a high reducing sugar content was shown by Campeanu et al. (2009), varying between 9.53% (Delicious and H-4/56) and 12.34% (H-3/73). The sugar content of apples differs depending on weather conditions, cultivar, culture technology, position and exposition of fruits in the crown (Mitre et al., 2009).

The acidity of the samples ranged from 2.42 (Ananas Reinette – AR) to 9.83 (Belle de Boskoop – BB), which is consistent with the results for acidity of apple varieties obtained by Babojelić Skendrović et al. (2007).

In the case of apple aroma, perception of sweetness and acidity is rather complex, and influences consumer preference as well as sweet scores obtained from sensory panels (Yuen et al., 1995). More recent studies focus directly on the sugar and acid content while older studies recognized the complexity of relationships among Brix and Brix/titratable acidity (Thiault, 1970), influence of fruit maturity and starch index, but they recognize as well that consumer acceptability and perception of sweetness is often unreliable in the case of apples (Yuen et al., 1995).

Definitively, sugars, organic acids and phenolic compounds all contribute to the aroma of apples (Mikulič Petkovšek et al., 2009), and SSC is a good indicator of the sugar content of apples, and presumably of sweetness (Hoehn et al., 2003). Titratable acidity may also be an important tool in predicting the taste of apples (Harker et al., 2002).

The total phenol content of traditionally grown domesticated apple varieties was in the range from 0.64 (Champagner Reinette – CR) to 0.95 g GAE*L^{-1} (Canadian Reinette – CA), which is a relatively high concentration. Begić-Akagić et al. (2011) showed the total phenol content (TPC) of three commercial apple cultivars (Topaz, Pinova, Pink Lady) and three autochthonous apple cultivars (Ruzmarinka, Ljepocvjetka, Paradija) from Bosnia and Herzegovina. Paradija, an autochthonous apple, had the highest phenol content (1.003 g GAE*L^{-1}), while Topaz, a commercial apple cultivar, had the lowest content (0.596 g GAE*L^{-1}). Koutsos et al. (2017) showed that the high phenol concentration in the Canadian Reinette variety compared to other varieties increased the bifidobacteria population, *Faecalibacterium prausnitzii*, butyrate levels and polyphenol microbial metabolites in in vitro analysis using a batch culture colonic model. These data suggest that apples, particularly Canadian Reinette, can induce substantial changes in microbiota composition and metabolic activity in vitro, which could be associated with potential benefits to human health.

Table 2. Average data values for 3 years' research (2011-2013) on traditionally grown domesticated apple varieties

Variable	RP	GR	CR	CA	JO	AR	AP	BH	BO	PR	RM	SM	BR
AFW	106.87	109.2	132.67	107.6	101.09	113.1	106.87	83.88	131.21	95.05	106.39	110.76	131.89
AFH	54.68	55.06	54.97	54.75	53.4	51.32	62.37	52.04	55.53	48.26	50.51	50.93	54.11
AFD	62.79	64.28	70.26	63.3	62.72	72.08	62.28	59.49	71.36	63.38	62.15	63.39	70.92
FS	4.1	3.2	4	4.2	1	5.1	3	3.3	4.1	4	4.1	9	4.1
GC	5	4	3	3	3.2	5	3	5	3	5	5	4	2
OC	4	3	2	1	3	1	3	2	3	3	1	3	3
PS	4	3	1	1	3	3	1	3	1	3	1	1	4
SL	2	3	3	2	4	1	1	3	3	2	3	4	3
DS	1	1	4	3	3	4	1	2	1	1	3	1	1
WSOS	1	1	2	4	3	4	3	5	3	1	4	1	3
BFS	1	2	4	1	3	2	4	1	4	1	4	4	3
PSWO	3	6	4	3	6	2	4	6	3	2	7	2	4
CF	1	3	1	3	2	2	1	4	3	1	1	1	2
TF	4	1	2	2	2	2	1	3	1	4	3	4	3
FF	4	4	3	4	4	2	1	4	4	4	4	4	4

Table 3. Eigenvalues, proportion of total variability, and correlation between original variables and the first five PCs of traditionally grown domesticated apple varieties

Variable	PC1	PC2	PC3	PC4	PC5
AFW	0.71	0.47	-0.05	0.27	0.36
AFH	0.68	0.01	0.46	-0.44	-0.2
AFD	0.59	0.29	-0.27	0.12	0.64
FS	-0.11	0.56	-0.58	0.22	-0.08
GC	-0.7	-0.2	-0.4	-0.19	-0.18
OC	-0.2	0.66	0.63	-0.25	-0.01
PS	-0.53	0.02	0.29	-0.29	0.56
SL	-0.18	0.17	0.38	0.86	-0.1
DS	0.26	-0.55	-0.53	0.25	0.06
WSOS	0.23	-0.81	-0.17	0.12	0.08
BFS	0.67	0.3	0.01	0.41	-0.44
PSWO	-0.03	-0.58	0.51	0.34	-0.31
CF	-0.04	-0.58	0.35	0.15	0.47
TF	-0.77	0.36	-0.31	0.12	-0.06
FF	-0.59	0.09	0.28	0.63	0.25
Eigenvalue	3.68	2.98	2.27	2.03	1.52
% Variance	24.51	19.9	15.17	13.59	10.17
% Cumulative	24.51	44.41	59.58	73.17	83.33

Culture establishment

Ten days after culture establishment, explant contamination ranged from 0-25% (Figure 1). This percentage of contamination is satisfactory considering the fact that the apple trees used for explant preparation in this study were older than 50 years, and were grown under extensive cultural practice (neither treated with fungicide nor trained). It can be supposed therefore that their phytosanitary status was bad. Three weeks after culture initiation, few explants of each variety were alive (Figure 1), but only explants of two varieties (Jonathan and Canadian Reinette) continued to grow.

Explants of the other three varieties produced rosettes, but finally did not continue to grow.

Explants were lost due to browning, or their growth was hampered. Browning of explants as a consequence of phenol oxidation is a well-documented problem during culture establishment (Hu and Wang, 1983; Laimer da Câmara Machado et al., 1991) of species with a high amount of phenols, and apples are in this category. Moreover, non-pruned apple trees can have an even higher phenol content, as was found for non-pruned olive plants in comparison with rejuvenated severely pruned plants (Basheer-Salimia, 2005). Ascorbic acid, which prevents phenol oxidation, used during surface-sterilization, and activated charcoal in EM did not prevent to a great extent of explant losses in this study. Maturation and aging in woody species imply a lack of morphogenic competence and rooting ability (Greenwood, 1987; Poethig, 1990). The study of aging in plants is very complex, and there is no consensus on general concepts related to this topic (Munné-Bosch, 2007). Because shoot meristem cells divide many times throughout plant development, they are exposed to environmental mutagens as well as the mutagenic effects of mitosis. This could be the reason for decreased vigor of aged perennials.

Table 4. Chemical characteristics of apple pulp of traditionally grown domesticated apple varieties

Apple varieties	Soluble solids (°BRIX)	Reducing sugars (%)	Total acid content (mmol*100 g^{-1})	Total phenols content (g GAE*L^{-1})
RP	18.65^{abcd}	11.1^{a}	6.58^{bc}	-
GR	14.97^{f}	9.31^{bcd}	4.95^{cd}	0.77^{b}
CR	16.58^{def}	8.92^{cd}	7.93^{b}	0.64^{c}
CA	18.28^{bcd}	9.61^{bcd}	4.05^{de}	0.95^{a}
JO	16.68^{def}	10.09^{bc}	5.93^{c}	0.71^{bc}
AR	16.99^{cdef}	9.02^{cd}	9.83^{a}	-
AP	16.1^{ef}	10.13^{bc}	2.44^{e}	-
BH	-	-	-	-
BB	18.86^{abc}	9.21^{cd}	2.42^{e}	-
PR	18.24^{bcde}	10.55^{bc}	7.08^{bc}	-
RM	20.27^{a}	9.32^{bcd}	4.65^{cde}	-
SM	17.05^{bcde}	8.51^{d}	4.91^{cd}	-
BR	19.53^{ab}	10.95^{b}	9.08^{b}	0.79^{b}

^{abcdef}Values within each column followed by the same letter are not significantly different at P<0.05 according to LSD test.

However, it would be a mistake to picture aging as directly related to the accumulation of somatic mutations in meristems. Many mutations of this sort are beneficial, and may be important sources of adaptive fitness (Thomas, 2002). The other change related to aging of perennials is a reduced level of certain cytokinins (Valdés et al., 2003). Low endogenous levels of plant growth regulators of excised shoot apices could be directly connected to interrupted growth of explants.

Axillary shoot proliferation

Variety and culture medium were the main factors that significantly affected shoot production (P=0.04 and P=0.007, respectively). The effect of explant type or any interactions among factors was not statistically significant. The mean number of shoots for the variety Jonathan (1.9) was significantly better than that for Canada Reinette (1.4) across two medium constitutions and explant types (Table 5, A). Apple variety Jonathan has already been established or propagated *in vitro* (Laimer da Câmara Machado et al., 1991; Zimmerman, 1991; Noiton et al., 1992). Yepes and Aldwinckle (1994), using medium with BAP, GA3 and IBA, produced a slightly higher number of shoots per explant (2.5) for cv. Jonathan in comparison to results of this research.

For the variety Canadian Reinette, there is no available report about micropropagation. Average length of shoots and number of nodes/shoot were similar for the two micropropagated varieties. BAP alone, or in combination with other plant growth regulators, is the most frequently used aromatic cytokinin for apple micropropagation as reviewed by Dobránszki and Teixeira da Silva (2010).

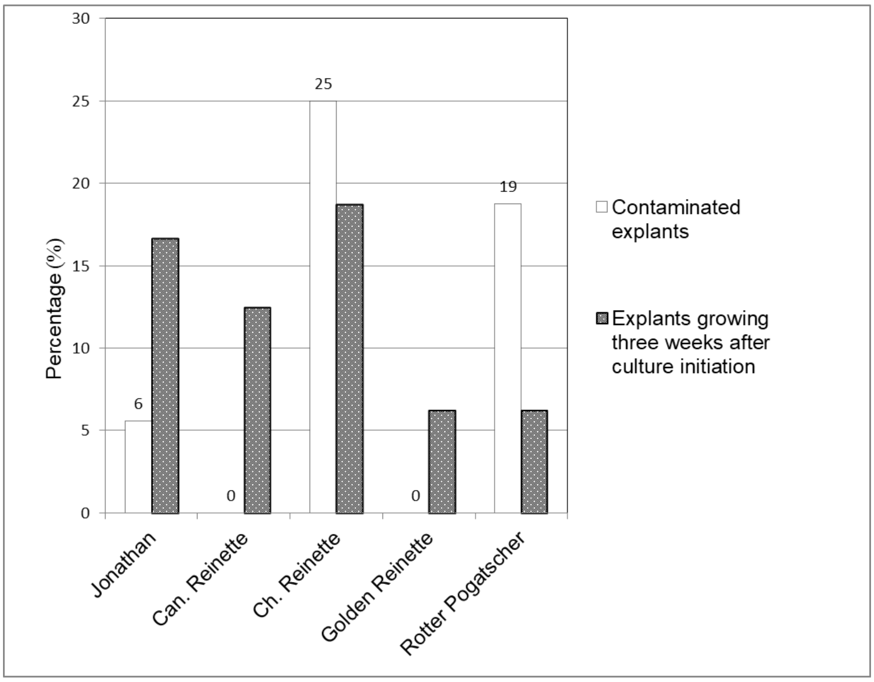

Figure 1. Percentage of contaminated and growing explants

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Its utilization, however, has several drawbacks such as shoot-tip necrosis, inhibition of rooting and problematic acclimatization of plants in the greenhouse (Szüčová et al. 2009). ZEA, a naturally occurring cytokinin, is rarely used, probably due to its cost. Moreover, ZEA has one major disadvantage when compared with aromatic cytokinins — it is susceptible to fast oxidative degradation (Plíhal et al., 2013). The average number of shoots/explant in this experiment was significantly higher on medium containing BAP (PM1) (2.0) than on medium containing ZEA (PM2) (1.4) (Table 5, B). Average length of the shoots was similar on the two media; however, shoots growing on medium with ZEA were apparently morphologically normal, with bigger leaves than shoots produced on medium with BAP (Figure 2). Briand and Hicks (1989) previously found the same morphological features of apple shoots produced on these two cytokinins. Shoots multiplied on medium supplemented with BAP had a higher number of nodes/shoot but were slightly stunted, which might be a side effect of BAP (Magyar-Tábory et al., 2010).

ZEA has been widely accepted as the only cytokinin capable of inducing satisfactory growth in cultured olive explants. ZEA replacement by single synthetic cytokinins, such as BAP or kinetin, has not been very successful as they do not allow good proliferation rates, and usually they induce explant hyperhydricity (Peixe et al., 2007). However, the authors reported that the best way to improve multiplication rates was a combination of BAP and coconut water which is known as a natural substance with high levels of ZEA in its composition. It is possible that with combined supplementation of BAP and coconut water for the traditionally grown domesticated apple varieties micropropagated here, the proliferation rate would also be satisfactory, and morphological features of shoots improved.

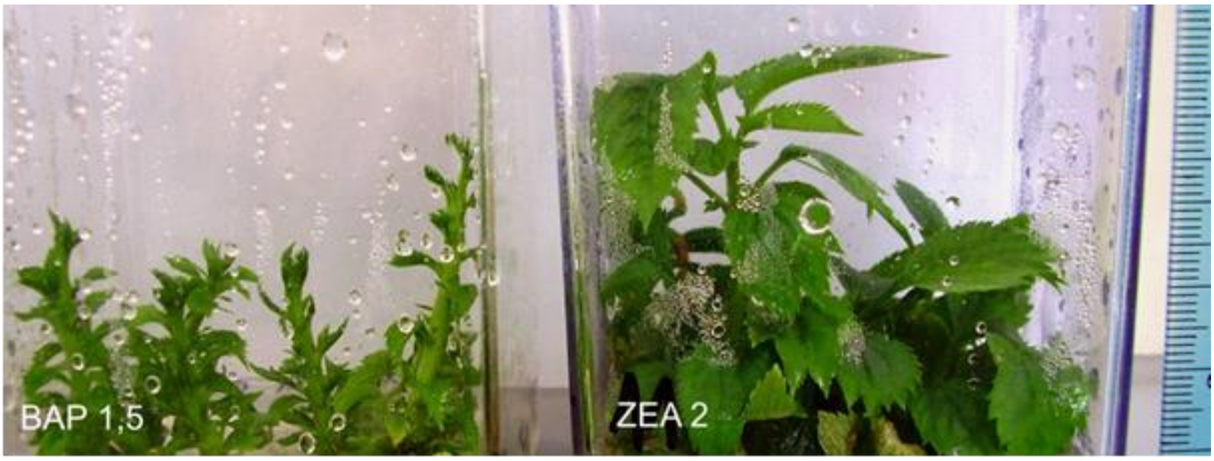

Figure 2. Morphological differences among shoots multiplied on medium with BAP and ZEA

Explant type had no significant influence on shoot number/explant; these results are consistent with those obtained by Kereša et al. (2012) for apple cv. Topaz micropropagation. Average length of the shoots was higher when shoots placed vertically were used as explants (Table 5, C).

Table 5. Efficiency of axillary shoot proliferation as affected by variety, plant growth regulators in medium and explant type

	Number of shoots/explant	Average length of the shoots (mm)	Number of nodes/shoot
A			
Variety			
JO	1.9[a]	13[a]	3.2[a]
CA	1.4[b]	11[a]	4[a]
B			
Medium			
PM1- BAP 1.5	2[a]	12.8[a]	3.6[a]
PM2- ZEA 2.0	1.4[b]	12.4[a]	2.7[b]
C			
Explant type			
Shoot-vertically	2[a]	15[a]	4.4[a]
Stem segment-horizontally	1.6[a]	11.5[b]	2.7[b]

[ab]Values within the column of each table followed by the same letter are not significantly different at $P<0.05$.

Conclusions

According to results of this research, it can be concluded that investigated traditionally grown domesticated apple varieties are valuable sources of desirable genetic characteristics including important morphological and nutritional characteristics of the fruits. The morphological characteristics of the traditionally grown domesticated apple varieties mentioned were not different from their standard values. Values of average fruit weight (AFW), average fruit height (AFH) and average fruit diameter (AFD) were less than standard. Principal component analysis of these traditionally grown domesticated apple varieties showed that fruit traits very important in today's breeding for new cultivars, like fruit weight, shape, skin color and flesh taste, stand out in explaining the variability. The fruits still have surprisingly positive chemical characteristics, especially Canadian Reinette for which the highest concentration of total phenols was measured.

Of five traditionally grown domesticated apple varieties used in this study, tissue culture was successfully established for two: Jonathan and Canadian Reinette. After establishment, shoots were efficiently multiplied by axillary branching. Greater multiplication efficiency was obtained for variety Jonathan. Medium supplemented with 1.5 mg*L^{-1} BAP produced a significantly higher number of shoots/explant in comparison with medium supplemented with ZEA. However, morphological features of shoots produced on medium supplemented with ZEA were better. Combined use of BAP and coconut water, which is known for high levels of ZEA but is cheaper, should be tested in future micropropagation trials with these two varieties. Both explant types, shoots placed in the medium vertically or two-nodal stem segments placed in the medium horizontally, gave similar results for axillary branching.

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