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IN VITRO PROPAGATION OF WILD BULGARIAN SMALL BERRY FRUITS (BILBERRY, LINGONBERRY, RASPBERRY AND STRAWBERRY)

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Abstract

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Recently, an increased interest in the identification of valuable possibilities for preserving the antioxidant properties of wild berry fruits rich in bioactive compounds can be noticed.

The interest in small berry cultivation is growing because of the high value of the fruit in global food markets. Tissue culture provides an efficient propagation method for the selected berry genotypes both for the breeding and cultivation purposes.

Four wild Bulgarian species - strawberry (*Fragaria vesca* L., Rosaceae), raspberry (*Rubus idaeus* L., Rosaceae), bilberry (*Vaccinium myrtillus* L., Ericaceae) and lingonberry (*Vaccinium vitis-idaea* L.) were evaluated in terms of their regeneration capacity when propagated *in vitro* by auxiliary organogenesis.

The highest average number of shoots (12.6) was accounted in wild strawberry, followed by wild raspberry (6.8) on MS medium supplemented with IBA, BAP and GA. The multiplication indices for bilberry and lingonberry were respectively 7 and 4.6 shoots per explant, cultivated on WPM + vitamin C with addition of growth regulators 6-[4-hydroxy-3-methylbut-2-enylamino] purine (zeatin) and N6-[2-isopenteny1] adenine (2iP). The rooting of wild strawberry was successful on MS medium supplemented with IBA, BAP and GA, while for raspberry the addition of only IBA. The rooting potential of bilberry and lingonberry varied between 1.4% to 33.3% (medium R_{10} and R_{12}).

Folin-Cio+calteu, DPPH and FRAP spectrophotometric assays were used for the assessment of the total phenolic content and the antioxidative properties of methanolic fruit extracts obtained from *ex vitro* and *in vivo* species. The antioxidant capacity determined by FRAP method was presented the values between 18.73-35.22 mM TE/g DW and in the case of DPPH assay the obtained values were situated in the range 63.75-95.65 mM TE/g DW. TPC from analyzed samples was situated in the range 28.95-53.16 mg GAE/g DW.

Key words: strawberry, raspberry, bilberry, lingonberry, *in vitro* methods, Folin-Ciocalteu, DPPH, FRAP, phenolic content, antioxidative properties

Introduction

Biotechnology has become one of the most promising branches of science in recent years. Plant biotechnology is a rapidly developing field of research and its present status cannot be evaluated without appreciation of the many possibilities and the potential of organ, tissue and cell suspension cultures. Plant tissue cultures (PTC) as the technique have many advantages and constantly growing applications. PTC can be grown in the absence of artifacts attributable to bacteria, fungi and viruses; preservation of valuable germplasm as a tool for the plant breeder.

Lately, *in vitro* propagation technology of vegetative propagated and recalcitrant plants has become a powerful industry to satisfy the needs of agricultural sector. PTC is a technique which has great potential as a means of vegetative

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propagating economically important species. However, a tissue culture system is also very often a "model" system which allows investigating physiological, biochemical, genetic and structural problems related to plants.

Historical, the works of Boxus (1974) and Anderson (1975) were the foundation for commercial micropropagation of berry crops. The commercial use of technology is directed to specific genotypes which are unique for each country. The determination of plant species as unique, depends on quality, wide-spread, investigation and areal of application. Protocols for micropropagation of berry crops have been develop in many laboratories (Turk et al., 1994). Various culture conditions, nutrient media and growth regulators have been investigated for berry crop micropropagation on semi-solid medium (Graham, 2005; Skirvin et al., 2005; Debnath, 2007).

Generally, the group of small fruits including varieties and wild species (bilberry, lingonberry, raspberry and strawberry) is one of the richest of biological active and health promoting substances. They are commercially important berry genera worldwide and good sources of natural antioxidants including vitamins, phenols, flavonoids and endogenous metabolites (Häkkinen et al., 1999; Puupponen-Pimiä et al., 2002; Hung et al., 2004; Puupponen-Pimiä et al., 2005; Dincheva I. et al., 2013). The rapid developments of technology for biology research have a relationship with the development of analytical methods and new generation of scientific infrastructure (Escarpa et al., 2001; Harnly et al., 2006; Côté et al., 2010; Vazquez-Cruz et al., 2012).

One of the potential benefits of wild berries is the ability to be included in breeding programs as donors for creation of new variety with resistance to economically important diseases but also for their use in future metabolic engineering (Debergh and Read, 1991). Another benefit is relation with a more important goal is to preservation of wild biodiversity.

The objective of this study was to develop effective technology for propagation of wild berry sp. by *in vitro* techniques, adaptation and successfully recultivation of wild small fruits (strawberry raspberry, bilberry and lingonberry) to their natural habitats.

Materials and Methods

Plant material

Strawberry (*Fragaria vesca* L., Rosaceae), raspberry (*Rubus idaeus* L., Rosaceae), bilberry (*Vaccinium myrtillus* L., Ericaceae) and lingonberry (*Vaccinium vitis-idaea* L.) were collected in 2012 from The Balkan Mountains (GPS: 42°46'44.12''N; 24°37'50.88''E); northeast slope and altitude 1460 m, Beklemeto site) in Bulgaria. Species were identified by Dr. Ilian Badjakov and voucher specimens 07546, 107547, 107548, 107549 were deposited in the herbarium collections of Sofia University "St. Kliment Ohridski" (SO), Bulgaria.

Chemicals

Gallic acid and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and sodium acetate were obtained from Fluka Chemie GmbH (Buchs, Switzerland). 2,4,6-Tripyridyl-s-triazine (TPTZ), $FeCl_3$ x $6H_2O$, $FeSO_4$ x $7H_2O$ and sodium carbonate were obtained from Merck KGAa (Darmstadt, Germany). Cyanidin 3-O-glucoside was purchased from ChromaDex. 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Fluka) and water was of Milli-Q quality > 18.2 MΩ-cm (Elga's PURELAB, UK). All solvents were of HPLC grade (EM Science, Gibbstown, NJ). $HgCl_2$, Indole-3-butryc acid (IBA), Indole-3-acetic acid (IAA), Gibberellic acid (GA_3), N6-(2-isopentyl) adenine (2ip), MS, WPM, sucrose, glucose, agar (Duchefa Biochemie, NL).

Establishment of aseptic culture

Sterilization method

Initially all separated auxiliary buds were washed with tap water and placed under constant agitation for 15 min with addition of 1-2 drops Tween 20. The procedure of surface sterilization of explants is specific for every one wild sp. following next steps:

- Strawberry - 96% ethanol for 30 sec and 0.1% $HgCl_2$ for 3 min
- Raspberry - 70% ethanol for 60 sec and 0.1% $HgCl_2$ for 5 min
- Bilberry and lingonberry - 70% ethanol for 30 sec and 0.1% $HgCl_2$ for 3 min
- Respectively for all explants - triple rinsing with sterile water.

Induction media for *in vitro* cultures

Proliferation media

Strawberry - MS medium (Murashige and Skoog, 1962) containing: IBA - 0.1 mg/L, BAP - 0.5 mg/L, GA_3 - 0.1 mg/L, Sucrose - 30 g/L or Glucose - 40 g/L, agar - 7.5 g/L and pH 5.6.

Raspberry - MS medium containing: IBA - 0.1 mg/L + BAP - 0.3 mg/L + GA_3 - 0.1 mg/L Sucrose - 20 g/L, agar - 7.5 g/L and pH 5.6

Bilberry and lingonberry - WPM - Woody Plant Medium + vitamine C (McCown and Lloyd, 1981) containing: zeatin

- 3 mg/L + 2ip - 2 mg/L. Sucrose - 20 g/L, agar - 6 g/L and pH 4.2.

Rooting media

- Strawberry - MS medium with addition of: IBA - 1 mg/L + BAP - 0.5 mg/L + GA - 0.1 mg/L and glucose 40 g/L, agar - 6g/L, pH-5.8.
- Raspberry - MS medium supplemented with only IBA - 0.2 mg/L and sucrose - 20 g/L, agar - 6g/L, pH-5.8.
- Bilberry and lingonberry - for root induction the following versions were tested (Table 1).

Growth conditions in vitro

Cultures were maintained in growth chamber at 22 ± 2˚C, white fluorescent light by 16/8 h photoperiod and intensity 2500 lux.

Acclimatization

In vitro rooted plantlets (3-4 cm) were transferred to soil from natural populations with addition of agro perlit in a ratio of 3:1 and subsequently acclimatized under greenhouse conditions involving regular irrigation and gradual reducing of humidity rate.

Preparation of extracts

The berries were lyophilized, grounded into powder using a tissuelyser (Qiagen, UK) and sonicated 0.5 g of powder for 15 min in 3 x 5 mL of 80% methanol containing 0.1% HCl. The mixture was centrifuged in room temperature for 10 min at 13000 rpm and the supernatant was subjected for analysis of TPC and antioxidant properties.

Antioxidant Activity (AA)

FRAP assay

Antioxidant activity was assessed using the Ferric Reducing Antioxidant Power (FRAP) assay as described previously (Benzie and Strain, 1996). Briefly, a sample containing 3 ml of freshly prepared FRAP solution (0.3 M acetate buffer (pH 3.6) containing 10 mM TPTZ and 40 mM $FeCl_3$ x $6H_2O$ and 100 µl of fruit extract was incubated at 37ºC for 4 min and the absorbance was measured at 593 nm (Ultraspec 2100 UV/Visible Spectrophotometer Amersham Bioscience). An intense blue color is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form at 593 nm. A standard solution of 1 mM L-ascorbic acid (AsA) in distilled water was prepared. The absorbance change was converted into a FRAP value, by relating the change of absorbance at 593 nm of the test sample to that of the standard solution of AsA and results were expressed as µmol AsA g^{-1} dry weight (DW).

DPPH assay

Each analyzed extract (0.15 mL) was mixed with 2.85 mL freshly prepared 0.1mM solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in methanol. The reaction was performed at 37°C in darkness and the absorptions at 517 nm were recorded after 15 min against methanol. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The antioxidant activity was expressed as mM Trolox equivalents (TE) per g dry weight (DW) by using calibration curve, build by 0.05, 0.1, 0.2, 0.3,

Table 1
Variants of growth regulators (R_3-R_{15}) included in MS basal medium for rooting of blueberries wild species

Code of nutrient media	IBA, mg/L	IAA, mg/L	NAA, mg/L	Myo-Inositol, mg/L	Casein hydrolysate, mg/L
R_3	1	-	-	-	-
R_4	-	0.2	-	-	-
R_5	-	0.5	-	-	-
R_6	-	1	-	-	-
R_7	-	-	0.2	-	-
R_8	-	-	0.5	-	-
R_9	-	-	1	-	-
R_{10}	-	1	-	250	250
R_{11}	-	-	-	500	500
R_{12}	-	1	-	250	-
R_{13}	-	1	-	-	250
R_{14}	-	1	-	500	-
R_{15}	-	1	-	-	500

0.4 and 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) dissolved in methanol.

Total Phenolic Content (TPC)

TPC in extracts was determined according to the Folin-Ciocalteu procedure (Waterhouse, 2002). Appropriately diluted extract (0.2 ml) was mixed with 1.0 ml of Folin-Ciocalteu reagent (1:10, v/v diluted in water) and incubated for 1 min before 0.8 ml sodium carbonate (7.5% w/v) was added. The mixture was incubated for 2 h at room temperature before absorbance followed by measurement at 750 nm (Ultraspec 2100 UV/ Visible Spectrophotometer Amersham Bioscience). TPC was estimated from a standard curve of Gallic Acid (GA) and were expressed as miligrams of GA equivalents (GAE) g^{-1} DW, and reported as a mean value ± standard deviation for three repetitions.

Results and Discussion

Propagation and rooting in vitro

The behavior of wild berry species under *in vitro* conditions is significantly different from that of the commercial varieties, especially for red and black blueberry. Testing the conditions applied for sterilization successful plantlet development in the wild strawberry and raspberry (88%) and for lingonberry (82.8%) was achieved. Most difficult it turned sterilization of bilberry where contamination was registries in 50.9%.

For the development of effective *in vitro* propagation protocol a large number of basal media supplemented with different kind and concentration of growth regulators were tested (data not shown). Many factors influenced the rate of propagation as species, genotype, seasonality, type of explants, medium, origin, etc. and the effect was specific for each wild-type.

For the wild strawberry was reported high multiplication index - 12.6 shoots per explant, cultivated on MS medium with addition of IBA - 1 mg/L, BAP – 0.5 mg/L, GA - 0.1 mg/L, sucrose - 20 g/L, agar - 7.5 g/L and pH 5.6.

Wild raspberry proliferated successfully (6.8 shoots per explants) on the same medium as composition but with different concentration of the growth regulators: IBA - 0.1 mg/L, BAP - 0.3 mg/L, GA_3 - 0.1 mg/L.

Bilberry and lingonberry explants were cultivated on WPM medium with vitamin C + zeatin - 3 mg/L + 2ip - 2 mg/L, sucrose – 20 g/L, agar - 6 g/L and pH 4.2), but the regeneration answer was different: lingonberry - 4.6 shoots per explant, while in bilberry -7 shoots per explant. The most important factor for the successful development of bilberry under *in vitro* conditions was to keep acid-base balance of the medium similar to the natural habitats (pH 4). Lack of control over this parameter can cause serious problems.

The different response of wild species can be explained by the genotype specificity and environmental conditions in which they grow. Moreover, the interaction between genotype and natural requirements of soil and climate conditions could be a factor for predetermination of their subsequent growth potential *in vitro*.

Rooting of wild strawberries was successful (100%) using MS medium supplemented with IBA - 1 mg/L, BAP - 0.5 mg/L and GA3 - 0.1 mg/L.

The observation of *in vitro* raspberry rooting reaction showed an adaptability and high rooting rate (100%) on MS medium, supplemented with IBA - 0.2 mg/L. The results obtained over many years with different cultivars and wild species clearly demonstrate that raspberry plants show higher rhizogenesis potential *in vitro* on a relatively simple culture medium. The quality of *in vitro* rooting was very good, in terms of number, length and vigor of developed roots (data not shown) as a prerequisite for efficient adaptation *ex vitro*.

Bilberry and lingonberry showed specific behavior in the course of *in vitro* rooting.

Bilberry was very difficult target for induction of roots. From all tested rooting media we received positive response only on four. The rooting potential of this species varied from 1.4% to 33.3%. Our results showed that the most suitable for rooting of bilberry auxin proved to be IAA in concentration 1 mg/L with addition of organic compounds such as myo-inositol (250 mg/L) and casein hydrolysate (250 mg/L) (R_{10}). The lingonberry rooted on the same medium as bilberry when the addition was only myo-inositol (250 mg/L) (R_{12}). The highest rooting index was 41.7%. Here established results demonstrate again the genotypic specifities in small berry species and based on these data we could suggest a very careful using of organic additives because they often can inhibit the process of rooting.

Acclimatization and adaptation rate

Adaptation of *in vitro* plants with the stem height of 3-4 cm and well developed roots was implemented usually in the spring within 30-40 days for strawberries and raspberries. Adaptation in bilberry and lingonberry was prolonged by 15 days more. Substrate for the adaptation was prepared from soil collected from the natural habitat of wild species mixed with agro perlite in a ratio 3:1. Application of the present protocol made possible to obtain 100% adapted plants which were successfully transferred to their natural habitats.

Table 2
TPC, DPPH and FRAP of *in vivo* en *ex vitro* pla1nts

#	Species	TPC (mg GAE/g DW)	± SD	TPC (mM TE/g DW)	± SD	DPPH (mM TE/g DW)	± SD
1	strawberry (*in vivo*)	44.22	0.67	30.69	3.89	90.58	0.54
2	strawberry (*ex vitro*)	41.25	0.37	26.85	2.86	84.33	0.44
3	raspberry (*in vivo*)	32.72	0.39	21.84	2.12	81.88	1.96
4	raspberry (*ex vitro*)	28.95	0.42	18.73	3.64	75.34	1.42
5	lingonberry (*in vivo*)	38.74	0.04	25.00	2.36	69.40	0.44
6	lingonberry (*ex vitro*)	34.27	1.03	22.92	1.21	63.72	1.12
7	bilberry (*in vivo*)	53.16	0.57	35.22	3.15	95.65	0.54
8	bilberry (*ex vitro*)	43.68	1.53	31.90	1.46	92.18	0.98

Total phenolic content and antioxidant capacity

The results of the TPC, radical scavenging activity (DPPH) and ferric reducing capacity (FRAP) of the investigated samples are summarized in Table 2, showing values expressed per g of DW.

Generally TPC varied between 28.95 (raspberry *ex vitro*) and 53.16 mg GAE per g DW (bilberry *in vivo*). The comparison of TPC between *in vivo* and *ex vitro* fruit extracts showed higher welues for *in vivo* fruit extracts. The established difference for bilberry fruits indicated values from 53.16 (*in vivo*) to 43.68 mg GAE per g DW (*ex vitro*), whereas for strawberry - from 44.22 (*in vivo*) to 41.25 mg GAE per g DW (*ex vitro*). This agrees with the report of Prior et al. (1998) and Buricova et al. (2011).

The two assays used represent different mechanisms of evaluating antioxidant capacity. While the DPPH assay measures the ability of plant extracts to scavenge free radicals, the FRAP assay quantifies the total concentration of redox-active compounds (Magalhães et al., 2008). The highest FRAP value was determined in bilberry *in vivo* (35.22 mmol TE/g DW), followed by strawberry *in vivo* (30.69 mmol TE/g DW). The lowest FRAP value was observed in raspberry *ex vitro* (18.73 mmol TE/g DW). Similar variations in FRAP have been reported by other researchers (Poiană et al. 2008, Deighton et al. 2000). The highest DPPH value was determined in bilberry *in vivo* (95.65 mmol TE/g DW). The lowest DPPH value was observed in lingonberry *ex vitro* (63.72 mmol TE/g DW). FRAP values were highly correlated with TPC (r = 0.98), whereas the correlation between DPPH and TPC was r = 0.73.

Conclusions

In our study we found that the antioxidant properties of wild small berry species are higher than *ex vitro* plants. Moreover, the same trend presents the total phenolic content of analyzed samples. Between antioxidant properties expressed by FRAP method and DPPH assay and TPC was established the linear correlations: r = 0.98 and 0.73, respectively. The higher values for antioxidant activity and total phenolic amount were founded in bilberry, followed by strawberry.

Our results demonstrate a reliable *in vitro* protocol for production a high quality plant material for research as well as natural habitats recultivation.

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