THE SUSTAINABLE IMPROVEMENT OF EUROPEAN BERRY PRODUCTION, QUALITY AND NUTRITIONAL VALUE IN CHANGING ENVIRONMENT: STRAWBERRIES, CURRANTS, BLACKBERRIES, BLUEBERRIES AND RASPBERRIES (EUBERRY)

SUBCONTRACT: WP2, SUB-TASK 2.1.1. ‘Evaluation of physiological properties, yeald parameters, organoleptic quality and chemical analyses of the fruits and disease resistance of raspberry and blackberry genotypes propagated with the standard technique and \textit{in vitro}’.

Genetic variability/stability of micropropagated and standard propagated raspberry and blackberry plants

Djurdjina Ružić, Tatjana Vujović and Radosav Cerović

Kralja Petra I/9, 32000 Čačak, Serbia
Phone: + 381/32/221-413; 221-375
Fax: +381/32/221-391
E-mail: institut-cacak@eunet.rs
www.institut-cacak.org
OBJECTIVE OF RESEARCH

To evaluate the potential of *in vitro* micropropagation in mass propagation of raspberry and blackberry, primarily aimed at obtaining of healthy, genetically stable and true-to-type planting material.

Experimental orchard:
Established: June 2010, 1.8 acres;
Cultivars: blackberry cv Čačanska Bestrna and raspberry cv Meeker;
Planting material: tissue culture plants (TC) and standard planting material (SP);
Planting distances: blackberry 1.5 x 3 m, raspberry 0.33 x 3 m (TC:SP = 5 : 5; 3 : 3, resp.)
1. FIELD EXPERIMENTS

1.1. Physiological properties

a) **Phenological investigation**: leafing onset, flower-cluster development, flowering onset, fool blooming, end of flowering, ripening onset, full ripening, end of ripening, as well as period of fruit ripening and duration of growing period (from bud swell to shedding onset).

b) **Yield parameters** including total number of canes, cane number per row meter, yield per cane (kg), yield per row meter (kg), total yield (kg ha\(^{-1}\)) will be also evaluated.

1.2. Organoleptic quality (standard parameters plus aroma/taste)

a) **Morphological properties**: fruit weight, height, width and thickness, drupelets properties (number within a fruit, height, diameter, shape factor and weight of drupelet seeds) and fruit colour will be evaluated.

b) **Chemical parameters of fresh fruit quality**: total dry matter, soluble solids content, total sugars content, reducing sugars content, sucrose content, titratable acidity, pH value, index of sweetness and total content of pectins.

c) **The sensory analysis of fresh fruits**: appearance, taste, aroma and consistency of fruits, rated on a scale of 1–20.

d) **Aromatic (volatile) compounds** of blackberry and raspberry fruits.

e) **Bioactive compounds** in blackberry and raspberry fruits.

1.3. Disease resistance

Evaluation of winter hardiness, susceptibility/resistance to *Didymella applanata*, *Leptosphaeria rubi*, *Botrytis cinerea*. 
2. EXPERIMENTS CONNECTED WITH ASSESSMENT OF PLANTS GENETIC STABILITY

a) ESTIMATION OF DNA PLOIDY LEVEL AND RELATIVE NUCLEAR DNA CONTENT BY FLOW CYTOMETRIC ANALYSIS;

b) DETERMINATION OF CHROMOSOME NUMBER USING LIGHT MICROSCOPY (CHROMOSOME COUNTING);

c) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF ISOPEROXIDASES.
A) ESTIMATION OF DNA PLOIDY LEVEL AND RELATIVE
NUCLEAR DNA CONTENT BY FLOW CYTOMETRIC ANALYSIS

Leaf samples:
- open field plants originated from *in vitro* micropropagated planting material –
tissue culture plants (12 TC plants for both blackberry and raspberry),
- open field plants originated from standard planting material – control plants
  for ploidy comparison (2 SP plants for both blackberry and raspberry);

Method for isolation of nuclei from plant cells – modified from Arumuganathan
  and Earle (1991);

Internal standard – *Vinca minor* leaf tissue (nuclear DNA content = 1.51 pg/2°C,
Gerard Geenen, personal communication). G₀/G₁ peak (2°C) of internal
  standard – around channel 500 set on a linear scale of fluorescence intensity
  (FL2-DAPI);

DNA-ratios were obtained by dividing mean of the dominant (G₀/G₁) peak of
the *Rubus* sample by mean of the G₀/G₁ peak of the internal standard.
Fig. 1. Representative flow cytometric histograms of DAPI-stained nuclei isolated from leaf tissue of blackberry cv Čačanska Bestrna plants of different origin: (a) control plant originated from standard planting material; (b–d) three different tissue culture plants originated from in vitro micropropagated material. Counts on y-axis represent number (x100) of nuclei. RN1 – G₀/G₁ peak of samples. RN2 – G₀/G₁ peak of an internal standard.
### Relative DNA ratio in leaf samples taken from open field plants of blackberry cv Čačanska Bestrna of different origin

<table>
<thead>
<tr>
<th>Origin of plant material</th>
<th>Relative DNA ratio</th>
<th>CV values of DNA peaks (%)</th>
<th>DNA ploidy level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control plant 1 (SP material)</td>
<td>0.830</td>
<td>4.65–4.75</td>
<td></td>
</tr>
<tr>
<td>Control plant 1 (SP material)</td>
<td>0.820</td>
<td>4.35–4.62</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 1</td>
<td>0.825</td>
<td>4.32–4.80</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 2</td>
<td>0.830</td>
<td>4.56–4.71</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 3</td>
<td>0.835</td>
<td>4.72–4.78</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 4</td>
<td>0.830</td>
<td>4.20–4.63</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 5</td>
<td>0.835</td>
<td>4.51–4.86</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 6</td>
<td>0.825</td>
<td>3.81–4.42</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 7</td>
<td>0.835</td>
<td>4.50–5.02</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 8</td>
<td>0.830</td>
<td>4.33–4.82</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 9</td>
<td>0.820</td>
<td>4.18–4.31</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 10</td>
<td>0.835</td>
<td>4.40–4.99</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 11</td>
<td>0.830</td>
<td>4.65–5.28</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 12</td>
<td>0.830</td>
<td>4.67–4.97</td>
<td></td>
</tr>
</tbody>
</table>

Relative DNA ratios are mean values of two independent measurements per each plant. Data were analyzed by ANOVA; ns – non significant. CV values of DNA peaks of internal standard (*Vinca minor*) ranged between 3.69% and 5.21% in all examined samples.
Fig. 2. Representative flow cytometric histograms of DAPI-stained nuclei isolated from leaf tissue of raspberry cv Meeker plants of different origin: (a) control plant originated from standard planting material; (b–d) three different tissue culture plants originated from *in vitro* micropropagated material. Counts on y-axis represent number (x100) of nuclei. RN1 – G0/G1 peak of the sample. RN2 – G0/G1 peak of the internal standard.
Relative DNA ratio in leaf samples taken from open field plants of raspberry cv Meeker of different origin

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<tr>
<th>Origin of plant material</th>
<th>Relative DNA ratio</th>
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<th>DNA ploidy level</th>
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</thead>
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<tr>
<td>Control plant 1 (SP material)</td>
<td>0.370</td>
<td>7.24–7.46</td>
<td></td>
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<td>Control plant 1 (SP material)</td>
<td>0.370</td>
<td>7.18–7.34</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 1</td>
<td>0.370</td>
<td>7.22–7.59</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 2</td>
<td>0.375</td>
<td>6.66–7.91</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 3</td>
<td>0.370</td>
<td>7.61–8.68</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 4</td>
<td>0.370</td>
<td>7.39–7.44</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 5</td>
<td>0.370</td>
<td>8.05–8.26</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 6</td>
<td>0.370</td>
<td>7.10–8.26</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 7</td>
<td>0.375</td>
<td>7.75–8.11</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 8</td>
<td>0.375</td>
<td>7.28–7.37</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 9</td>
<td>0.380</td>
<td>7.06–7.06</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 10</td>
<td>0.375</td>
<td>7.51–8.05</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 11</td>
<td>0.375</td>
<td>7.11–7.38</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plant 12</td>
<td>0.365</td>
<td>8.16–8.94</td>
<td></td>
</tr>
</tbody>
</table>

Relative DNA ratios are mean values of two independent measurements per each plant. Data were analyzed by ANOVA; ns – non significant. CV values of DNA peaks of internal standard (*Vinca minor*) ranged between 3.82% and 6.73% in all examined samples.
B) DETERMINATION OF CHROMOSOME NUMBER USING LIGHT MICROSCOPY (CHROMOSOME COUNTING)

Chromosomes were counted in five randomly chosen root/shoot apices sampled from ten plants:
- **root tip meristems** of blackberry cv Čačanska Bestrna – root tips were collected from the newly developed plants obtained by tip layering in mid- to late spring,
- **shoot tip meristems** of raspberry cv Meeker – 3 to 5 mm leaf buds taken from the open field plants in the spring;

Determination of chromosome number was carried out using the method described by Skene et al. (1988) and modified (pretreatment segment) by Ružić et al. (1991);

Metaphase chromosomes were counted in 2–4 cells from each sample using a light microscope;

Well-spread chromosomes at the metaphase stage were selected and photographed using Olympus DP70 digital camera (Olympus BX61, Optical Co. Ltd.).
Metaphase chromosomes in root tip meristematic cells of open field blackberry cv Čačanska Bestrna plants originated from *in vitro* propagated planting material (2n = 4x = 28)
Metaphase chromosomes in shoot tip meristematic cells of open field raspberry cv Meeker plants originated from *in vitro* propagated planting material ($2n = 2x = 14$)
C) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF ISOPEROXIDASES

The extraction of native proteins from leaf samples was performed according to the method described by Bošković (1998);

Protein extracts were prepared from young, recently expanded leaves of control plants from open field originated from standard planting material and tissue culture plants originated from *in vitro* micropropagated material;

PAGE of isozymes was performed on 5–12.5% density gradient polyacrylamide gels (1 and 5 h at 100 V and 300 V respectively, at 4 °C, TBE as a tank buffer);

Gels were stained for peroxidase (POX) activity using o-dianisidine.
Isoperoxidase patterns obtained by staining with O-dianisidine: (1–2) control plants originated from standard planting material; (3-12) tissue culture plants
Isoperoxidase patterns obtained by staining with O-dianisidine: (1–2) control plants originated from standard planting material; (3-12) tissue culture plants

(b) additional slow-migrating band identified when 100 μl of protein extract was loaded on gel

(a) isoperoxidase profile obtained by using 40 μl of protein extract per sample for PAGE
Conclusions

- Flow cytometry analysis proved that no significant differences in relative nuclear DNA content and DNA ploidy levels were detected among plants of different origin (TC and SP plants) in both *Rubus* cultivars;
- Chromosome counting in root/shoot tip meristems also showed normal tetraploid chromosome number ($2n = 4x = 28$) in blackberry cv Čačanska Bestrna plants and normal diploid chromosome number ($2n = 2x = 14$) in raspberry cv Meeker plants originated from TC planting material;
- In both cultivars, no qualitative differences (presence of additional bands) were detected in POX profiles of plants of different origin (TC and SP plants);
- The results obtained verified high genetic stability of tissue culture originated plants at this level of investigation.
Thank you for your attention!