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Research paper

An assessment of the genetic integrity of micropropagated raspberry and blackberry plants

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ABSTRACT

In vitro clonal propagation of small fruit species has been one of the most broadly exploited approaches in biotechnology. However, the most critical aspect of this propagation technology has been the maintenance of genetic fidelity of tissue culture-raised plants. In the present study, three techniques were deployed in the assessment of genetic fidelity of micropropagated plants (TC plants) of blackberry cultivar 'Čačanska Bestrna' and raspberry cultivar 'Meeker' in the second year after planting in the open field, in comparison to plants originating from the standard planting material (ST plants). Flow cytometry analysis deployed to estimate the DNA ploidy level and relative nuclear DNA content in TC and ST plants revealed no significant differences in nuclear DNA content between plants of different origin. Chromosome counting in root/shoot tip meristems also showed a normal tetraploid chromosome number ($2n = 4x = 28$) in TC blackberry plants and a normal diploid chromosome number ($2n = 2x = 14$) in TC raspberry plants. Polyacrylamide gel electrophoresis of peroxidase (POX) extracted from the leaf tissue revealed no differences in POX profiles between TC and ST plants either. The results obtained in this study verify the suitability of micropropagation by axillary branching for commercial exploitation in blackberry and raspberry.

1. Introduction

Red raspberries and blackberries have expanded arround the world due to many factors, including a global increase in berry consumption, notably as a fresh fruit (Clark and Finn, 2014; Kempler and Hall, 2013). Red raspberries and blackberries are also the primary berry fruit species in the Republic of Serbia (Leposavić and Cerović, 2009). However, red raspberry and blackberry plantations have been established using planting material originating from commercial plots, resulting in low yield, poorer fruit quality and short productive life of orchards. Therefore, the propagation of high-quality, true-to-type and disease-free nursery stock to start new raspberry and blackberry orchards is of utmost importance. Raspberries and blackberries are successfully propagated by conventional methods of vegetative propagation, which ensure that desirable genetic characteristics are preserved. However, noticeable interest has been expressed in utilizing tissue culture methods for propagation.

Micropropagation of small fruit species and vegetative rootstocks represents a major success in the commercial application of tissue culture *in vitro* in fruit crops. Micropropagation has several advantages over conventional methods of vegetative propagation, including multiplication of elite clones and recalcitrant species, year-round production, obtaining pathogen-free plants, and a large-scale production of plants within a short time-span from a single initial explant of genotypes (Rani and Raina, 2000). However, there is doubt about the maintenance of the genetic integrity of tissue culture-raised plants. Although a micropropagation protocol based on axillary branching is considered adequate in providing genetically uniform and true-to-type plants, there were several reports of incidence of somaclonal variation among plants originating from organized meristems (Rani and Raina, 2000). The occurrence of somaclonal variation can lead to enormous economic consequences, notably in fruit crops and woody species due

Abbreviations: DAPI, 4'-6-diamidino-2-phenylindole; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; G_0/G_1, resting phase of normal cell cycle; PAGE, polyacrylamide gel electrophoresis; POX, peroxidase; PVP-40, polyvinyl pyrrolidone; PVPP, insoluble polyvinyl polypyrollidone; ST plants, Plants originating from the standard planting material; TC plants, Micropropagated plants; TBE, tris-borate-EDTA buffer

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to their long lifespan (Leva et al., 2012). Hence, screening of micropropagated plants and identification of somaclonal variations during an early stage is essential.

Several strategies have been developed to assess the genetic integrity of tissue culture-raised plants involving morphological, physiological, biochemical, cytological and DNA-based molecular markers approaches (Karp, 1991). Every approach has its advantages and disadvantages in the assessment of the variations (Alizadeh et al., 2015), which determine their use for the different application. The choice of technique in identifying possible somaclonal variability depends on the material studied and the nature of the questions being addressed (Karp, 2000). Additionally, the use of different approaches is usually desirable for correct evaluation of this complex phenomenon (Jin et al., 2008).

However, most genetic variability studies involve analysis of tissue culture plants still growing *in vitro*, and there have been only few reports on the assessment of genetic fidelity of adapted plants in the open field (Castillo et al., 2010). In the case of *R. grabowskii* and blackberry 'Silvan' (Castillo et al., 2010), *in vitro* shoot tips displayed AFLP polymorphisms *in vitro*, and they were no longer detected when the plants were grown *ex vitro*. Similar results were also reported for micropropagated strawberry plants (Palombi et al., 2010). In their study, flow cytometric analysis revealed that the DNA variation induced by *in vitro* culture could be lost or retained after the transfer of plants to the greenhouse. These results indicate the need for genetic analysis of tissue culture-plants in the greenhouse or the field rather than *in vitro*, as variation might be transient. Furthermore, evaluations of field performance including important agronomic traits in plants originated from tissue culture is essential, since the good correlation between the extent of mutations (molecular level) and phenotypic changes might not be found (De Klerk, 1990).

In the present study, three techniques (chromosomal number counting, flow cytometric analysis and isoperoxidase analysis) were deployed to assess the genetic fidelity of micropropagated plants of blackberry 'Čačanska Bestrna' and raspberry 'Meeker' in the second year after planting in the open field, although they revealed no major differences in the most important biological traits (phenological, pomological and yield parameters) compared to the plants originating from the standard planting material.

2. Materials and methods

2.1. Plant material

Experiments were conducted with plant material taken from the orchards of raspberry cultivar 'Meeker' (*Rubus idaeus* L.) and blackberry cultivar 'Čačanska Bestrna' (*Rubus fruticosus* L.), both established using planting material propagated by standard method (ST plants) (Leposavić and Cerović, 2009) and *in vitro* micropropagation (TC plants). Standard plants of raspberry were produced from adventitious root buds, while blackberry was propagated by tip layering. Tissue culture plants of blackberry and raspberry were obtained according to micropropagation protocols previously described by Ružić and Cerović (1998) and Ružić and Lazić (2004), respectively. Raspberry shoots were multiplied on Murashige and Skoog (1962) medium (MS) containing 0.5 mg L^{-1} N6-benzyladenine and blackberry shoots were propagated on MS medium with 1 mg L^{-1} N6-benzyladenine, 0.1 mg L^{-1} indole-3-butyric acid and 0.1 mg L^{-1} gibberellic acid. Both genotypes were multiplied *in vitro* for eight subcultures, after which they were rooted *in vitro* and acclimatized. Both types of plant material were planted comparatively using the random block system, with the planting distance being 3 × 0.33 m for the raspberry and 3 × 1.5 m for the blackberry. Plots were maintained following the standard agro-technical and pomo-technical measures.

2.2. Estimation of DNA ploidy level and relative nuclear DNA content by flow cytometric analysis

Leaf samples of both genotypes collected from open field TC plants were used for flow cytometric analysis. In addition to this, ST plants from the open field were used as control plants for ploidy comparison. Flow cytometric analysis was performed by Gerard Geenen, Plant Cytometry Services (Schijndel, The Netherlands). To estimate relative DNA content, fresh leaf material was chopped in an ice-cold 4′-6-diamidino-2-phenylindole (DAPI) based nuclear extraction buffer, using a method modified from Arumuganathan and Earle (1991). Following this, the mixture was passed through a 40-μm nylon filter and analysed in the flow cytometer (Partec GMbH, Münster, Germany) using a linear scale. Together with each leaf sample, an internal standard, *Vinca minor* leaf tissue (2C = 1.43 pg; Obermayer and Greilhuber, 2006), was also included. G_0/G_1 peak (2C) of internal standard was adjusted to around channel 500 set on a linear scale of fluorescence intensity (FL2-DAPI). DNA-ratios were obtained by dividing mean of the dominant (G_0/G_1) peak of the *Rubus* sample by the mean of the G_0/G_1 peak of the internal standard.

For FCM analysis, DNA was extracted and analysed independently from leaf samples collected twice from each of twelve TC plants and two ST plants. The data presented in Tables 1 and 2 are mean values of two independent measurements per each plant. The data were analyzed by ANOVA.

2.3. Determination of chromosome number using light microscopy (chromosome counting)

Chromosome observations were carried out in root tip meristems of blackberry cultivar 'Čačanska Bestrna'. Root tips were collected from the newly developed plants, obtained by tip layering in mid-to-late spring. As for raspberry cultivar 'Meeker', chromosome counting was done in shoot tip meristems (3–5 mm leaf buds taken from the open field plants in the spring). For each genotype, chromosomes were counted in five randomly chosen root/shoot apices sampled from each of ten plants (total 50 root/shoot apices).

Determination of chromosome number was carried out using the method reported by Ružić et al. (1991). Actively growing root/shoot tips were pre-treated in the dark at 4 °C with 2 μM 8-hydroxyquinoline solution for 48 h, and subsequently fixed for 24 h in acetic alcohol (glacial acetic acid:ethyl alcohol 1:3, v/v), in the dark at 4 °C. Hydrolysis was done in 5 N HCl for 12 min at room temperature. After brief rinsing in distilled water, root/shoot tips were strained with the Schiff reagent for 4 h in the dark at room temperature. The tips were macerated with 2% pectinase for 30 min at room temperature. The terminal part of root/shoot tip was placed onto a clean slide and squashed under a cover slip in a drop of acetocarmine [0.5% (w/v) carmine in 45% (v/v) acetic acid]. 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.).

2.4. Polyacrylamide gel electrophoresis of isoperoxidases

Protein extracts were prepared from young, recently expanded leaves of ST control plants and TC plants from the open field. Extraction of native proteins from leaf samples of blackberry was performed according to the method described by Bošković (1998). The principal extraction solution consisted of 50 ml 0.1 M Tris-HCl buffer (pH 7.5) containing 1% (w/v) insoluble polyvinyl polypyrollidone (PVPP), 1% (w/v) polyvinyl pyrrolidone (PVP-40), 0.16 M ethylenediaminetetraacetic acid (EDTA), 0.32 M magnesium chloride, 0.18 M sucrose, 1% (w/v) polyethylene glycol 6000 and 0.12% (v/v) 2-mercaptoethanol. As for raspberry samples, 0.1 M sodium phosphate buffer (pH 7.0) containing 1% PVP-40 (Smila et al., 2007) was used for extraction.