OPTIMIZATION OF THE PROTOCOL FOR *IN VITRO* PROPAGATION OF AUTOCHTHONOUS PLUM GENOTYPE 'METLAŠ'

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Abstract

Fast and cost-effective clonal propagation of planting material is possible to achieve by application of tissue culture *in vitro*. In this way, problems associated with traditional propagation, such as rapid spreading of diseases, lack of initial material caused by dependence on seasonal growth or low propagation coefficient can be overcome. This paper deals with optimization of micropropagation of autochthonous plum 'Metlaš' (*Prunus domestica* L.) originated from Guberevci (Municipality Knić, Serbia). 'Metlaš' is very often considered to be the same genotype as 'Okruglica' (syn. 'Dragačica'), but they can be clearly distinguished according to plant habit, stone and fruit characteristics. To optimize multiplication stage, the influence of benzyladenine (BA at 0.5, 1.0, 1.5 and 2.0 mg l^{-1}) and thidiazuron (TDZ at 0.25, 0.5, 1.0 and 1.5 mg l^{-1}) on the multiplication capacity (multiplication index, length of axial and lateral shoots) was examined. Rooting ability of shoots (rooting rate, number and length of roots, and height of rooted plants) was monitored on half strength Murashige and Skoog (MS) medium containing 1.0 mg l^{-1} indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA), each combined with 0.1 mg l^{-1} gibberellic acid (GA_3). TDZ applied at 0.25 mg l^{-1} gave the highest multiplication index (9.1), while the longest axial (14.1 mm) and axillary shoots (8.6 mm) were obtained on medium with 1.5 mg l^{-1} BA. Although both auxins proved to be efficient in rhizogenesis (rooting rates being 85.7% and 95.2%), higher values of all rooting parameters were observed in the presence of IBA. Rooted shoots were successfully acclimatized.

Keywords: *Prunus domestica* L., *In vitro, Multiplication, Rooting, Acclimatization*.

Introduction

Plum is a very important fruit species native throughout the Northern Hemisphere but mostly in the temperate zone. According to the production, plum is ranked as the fourth most important cultivated fruit tree crop in temperate climate (after apple, pear and peach), accounted for 7% of total word production of temperate fruits (FAOSTAT, 2019). Today, the most globally cultivated plum species are the hexaploid ($2n = 6x = 48$) European plums (*Prunus domestica* L.) and the diploid ($2n = 2x = 16$) Japanese plums (*Prunus salicina* Lindl.) (Sottile, 2022). Contrary to Japanese plums which are widely grown for fresh consumption, European plums are commercially grown worldwide for a number of uses including fresh consumption, cooking, baking, drying, canning, distilling (brandy production), as well as other types of processing. However, cultivated European plums show very limited intra-specific genetic variability (Zhebentyayeva et al., 2019), due to modernization of agriculture, loss of local ecotypes, inbreeding and/or use of a limited number of founders in breeding processes (Sottile et al, 2022). While other breeding programs face narrowing of genetic diversity of plums, Serbia as well as other regions of former Yugoslavia is rich in old plum cultivars, primitive forms and

autochthonous biotypes (landraces) (Milošević et al., 2010; Vukojević et al., 2012) suitable for selection and breeding of both cultivars and *Prunus* rootstocks (Paunović and Paunović, 1994). Still, this rich plum germplasm is directly treated due to continuous introduction of improved newly-bread cultivars as well as to the climate change and increased rate of infection with Sharka virus (Plum pox virus – PPV) which affect many local cultivars and biotypes of plums (Botu et al., 2012). Considering the importance of these valuable plum genetic resources there is an urgent need to develop new concepts of its sustainable conservation, management and utilization. Beside that, there has been an increased interest in establishing new commercial orchards of these autochthonous cultivars as they display higher adaptability to local agroecological conditions and are suitable for low-input farming. Beside being used for production of high quality brandies (Popović et al., 2015), fruits of these genotypes have high nutritional value and antioxidant activity (Tomić et al., 2019) and could be suitable for fresh use as well (Milošević and Milošević, 2012).

In vitro approaches are useful tools for rapid clonal propagation of true-to-type, disease-free and uniform plants (Vujović et al., 2020) as well as for medium- to long-term conservation of vegetatively propagated plants such as fruit tree species (Engelmann, 2004). Micropropagation has found the widest practical application of all *in vitro* techniques in fruit growing, so it has become a standard method of propagation for many fruit species, especially for vegetative rootstocks, berry fruit species and other fruit tree species that are grown on their own roots such as autochthonous plum species. Successful *in vitro* clonal propagation is determined by many factors such as genotype, mineral composition of nutrient medium (Ružić et al., 2003), type and concentration of carbon sources (Ružić et al., 2008; Yaseen et al., 2012), light conditions and plant growth regulators (PGRs) (Ružić and Vujović, 2008).

The aim of this paper was to develop efficient protocol for micropropagation of virus-free autochthonous plum 'Metlaš' (*Prunus domestica* L.) through: i) optimization of multiplication stage by using two cytokinins – N^6-benzyladenine (BA) and thidiazuron (TDZ) applied at different concentrations, ii) evaluation of rooting ability of *in vitro* shoots on medium containing different auxins – indole-3-butyric acid (IBA) or 1-aphthaleneacetic acid (NAA), and iii) monitoring of acclimatization ability of both *in vitro* rooted and unrooted shoots under the 'mist' system in greenhouse.

Material and Methods

Field grown virus-free clone of autochthonous plum cultivar 'Metlaš' (*Prunus domestica* L.) originated from Guberevci (Municipality Knić, Serbia) was used as a source of initial material for establishment of aseptic culture. Aseptic culture of this genotype was established according to the procedure previously described by Vujović et al. (2021). Upon establishment of aseptic culture, uniform single shoots were multiplied on Murashige and Skoog (1962) medium (MS) of constant PGR composition: 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} gibberellic acids (GA_3).

To optimize multiplication, the influence of type and concentration of cytikinins (BA and TDZ) on the multiplication capacity and shoot quality was examined in the fifth subculture. Cytokinins were applied at following concentrations: i) BA at 0.5, 1.0, 1.5 and 2.0 mg l^{-1}; ii) TDZ at 0.25, 0.5, 1.0 and 1.5 mg l^{-1}. All media contained 30 g l^{-1} sucrose and 7 g l^{-1} agar. The pH value was adjusted to 5.7 before autoclaving at 121°C, 150 kPa for 20 min. Shoots were subcultured twice at a 28 day-interval on the medium of the same PGR composition, and therefore multiplication parameters were determined in the second subculture. The following parameters were monitored:

multiplication index (the number of newly formed axillary shoots >5 mm per initial shoot tip), length of axial shoots and length of lateral shoots. Some specific issues, such as leaf color, leaf and callus size, leaf roll, incidence of chlorosis, or necrosis along with occurrence of fasciation and hyperhydricity were also monitored.

Rooting was performed on the MS medium with mineral salts reduced to ½-strength and organic complex unchanged. Rooting treatments included two PGR combinations: i) 1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3, and ii) 1 mg l^{-1} NAA and 0.1 mg l^{-1} GA_3. The percentage of rooted plants was determined after 28 days along with the number and length of roots, and height of rooted plants.

Each treatment in multiplication and rooting stages included 42 uniform shoots (three replicates of two culture vessels with seven shoots each). Shoot cultures were grown in 100 ml culture vessels containing 50 ml of multiplication or rooting medium, at 23 ± 1°C and 16-h photoperiod (light intensity, 41 μmol m^{-2} s^{-1}).

Both rooted and unrooted shoots were removed from culture vessels, washed carefully with water to remove adhering medium, transferred to plastic pots containing sterile soil substrate (Klassmann Steckmed – mixture of white sod peat, white peat and perlite) and acclimatized on a 'mist' bench in a greenhouse for two weeks (Mist system type 'Electronic leaf', MC Company, Belgrade).

All data were analyzed by ANOVA, followed by Duncan's Multiple Range Test ($P < 0.05$) for means separation. Data presented in the form of percentage were subjected to arcsine transformation.

Results and Discussion

Determination of the most optimal types and concentrations of PGRs is one of the most important aspects in plant tissue culture especially in proliferation stage. It is well known that cytokinins play multiple roles in the plant development such as promotion of cell division and cell expansion, plant protein synthesis stimulation and the activities of some enzymes (Arab et al., 2014). A wide range of cytokinin types and concentrations are effective for *in vitro* culture even though the requirements among species are different. Some investigators have reported that benzyladenine (BA) and thidiazuron (TDZ) are the two cytokinins most commonly used in stone fruit micropropagation (Ružić and Vujović, 2008). BA is frequently applied in *Prunus* rootstock micropropagation (Vujović et al., 2018), while TDZ has also been reported to be appropriate for *in vitro* proliferation of some *Prunus* spp. (Arab et al., 2014).

Monitoring of multiplication capacity of autochthonous plum 'Metlaš' revealed that TDZ, although applied at lower concentrations, is more efficient for micropropagation of this genotype comparing to BA (Table 1). Namely, multiplication index of shoots grown on media with TDZ ranged between 5.9 and 9.0 which was significantly higher than multiplication indexes obtained on media with BA (2.5–5.8). The highest multiplication index was achieved with lowest TDZ concentration (0.25 mg l^{-1}) (Fig. 1a). The increase in TDZ concentration has led to a gradual and significant decline in multiplication capacity of shoots. In contrast, increase in BA concentration to 1.5 mg l^{-1} and above caused significant increase in the multiplication index (Fig. 1b). Although all cytokinins stimulate cell division, axillary bud formation and shoot multiplication, it is well known that the effect of different cytokinins is highly genotype dependent (Dobránszki and Teixeira da Silva, 2010). Similarly to our results, some investigators reported that TDZ at low concentration is more effective than purine adenine derivatives in *Prunus* micropropagation (Espinosa et al., 2006; Canli and Tian, 2008). However, Tang et al. (2002) and Ružić and Vujović (2008) reported that BAP is more

efficient than TDZ in *P. avium* and *P. cerasus*, although high concentrations of cytokinins of adenine type are often necessary for growth and differentiation in *Prunus* spp. (Arab et al., 2014) which has been confirmed in our study.

According to Huetteman and Preece (1993), TDZ may inhibit shoot elongation and cause shortening of internodes. In our research axial and lateral shoots grown on media containing TDZ were significantly shorter compared to those cultivated on media supplemented with BA at concentration of 1.5 mg l^{-1} and above. Fasciation or hyperhydricity, phenomena that are often associated with TDZ application in tissue culture (Kadota and Niimi, 2003) were not noticed even on shoots grown on medium with the highest TDZ concentration.

The rooting stage in micropropagation is very important as it directly affects greenhouse survival and acclimatization success of *in vitro* plants. Rooting difficulties occur in micropropagation of both fruit-bearing and ornamental species belonging to *Prunus* genus (Wiszniewska et al., 2016). Despite the attempts directed towards higher effectiveness of the rhizogenesis, European plums usually exhibit poor rooting ability of *in vitro* induced shoots, which could be the major drawback in commercial micropropagation. According to Tian et al. (2007), use of 1-naphthaleneacetic acid (NAA) at higher concentrations instead of indole-3-butyric acid (IBA) can increase rooting efficiency of *P. domestica L.* Vujović et al. (2020) also reported that shoots of autochthonous plum 'Crvena Ranka' cultured on the medium supplemented with NAA displayed higher rooting ability (60%) in comparison with those grown on the medium containing IBA at the same concentration (20%). Contrary to those results, 'Metlaš' exhibited a much better rooting performance (Tab. 2; Fig. 1c-d), rooting rate being between 85.7% (medium with NAA) and 95.2% (medium with IBA). Also, plantlets rooted on medium with IBA had significantly longer roots compared to those rooted on medium with NAA.

Following *in vitro* rooting shoots were successfully acclimatized (90,0%), while percentage of acclimatization of unrooted shoots was markedly lower (15,0%).

Table 1. Multiplication parameters of autochthonous plum genotype 'Metlaš' on MS medium (Murashige and Skoog, 1962) of different plant growth regulator (PGR) composition.

PGR composition of medium (mg l^{-1})	Multiplication index	Length of axial shoot (mm)	Length of lateral shoots (mm)
[a]BA 0.5	2,7 f	12,3 b	6,2 bc
BA 1.0	2,5 f	11,7 b	5,7 c
BA 1.5	3,2 e	14,1 a	8,6 a
BA 2.0	5,8 d	14,0 a	6,4 b
[b]TDZ 0.25	9,0 a	12,1 b	6,7 b
TDZ 0.5	7,9 b	12,7 b	6,5 b
TDZ 1.0	6,2 c	11,9 b	6,8 b
TDZ 1.5	5,9 d	11,9 b	6,5 b

Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); [a]BA – N^6-benzyladenine; [b]TDZ – thidiazuron.

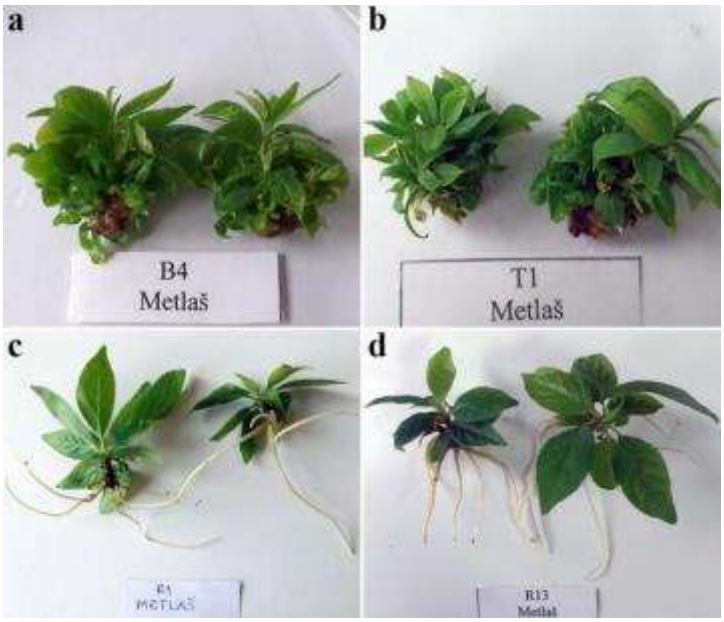

Figure 1. Shoots of autochthonous plum 'Metlaš' in the multiplication stage on MS medium with 2.0 mg l^{-1} BA (a) and 0.25 mg l^{-1} TDZ (b), and in the rooting stage on half-strength MS medium containing 0.1 mg l^{-1} GA_3 in combination with 1.0 mg l^{-1} IBA (c) or 1.0 mg l^{-1} NAA (d).

Table 2. Rooting parameters of autochthonous plum genotype 'Metlaš' on half-strength MS medium (Murashige and Skoog, 1962) of different plant growth regulator (PGR) composition.

PGR composition of medium (mg l^{-1})	Rooting rate (%)	Number of roots	Root length (mm)	Rooted shoots length (mm)
[a]IBA 1.0 + [b]GA_3 0.1	95.2 a	3.5	5.2 a	12.9
[c]NAA 1.0 + GA_3 0.1	85.7 b	3.1	3.0 b	12.5

Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); [a]IBA – indole-3-butyric acid; [b]GA_3 – gibberellic acid; [c]NAA – 1-aphthaleneacetic acid.

Conclusions

In this paper, we presented an optimized protocol for successful *in vitro* propagation of indigenous plum genotype 'Metlaš'. Analysis of the effect of cytokinin type and concentration on multiplication phase indicates that TDZ, although applied at lower concentrations, is more efficient in micropropagation of this genotype than BA. As regards of rhizogenesis, 'Metlaš' displayed high rooting ability with both IBA and NAA. Nevertheless, considering rooting percentage and root length, IBA could be recommended as more effective. The results obtained can find practical application in commercial laboratories for clonal propagation of planting material of genotype 'Metlaš' for establishment of new commercial orchards.

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