OPTIMIZING THE MICROPROPAGATION PROTOCOL FOR *Rosa canina* L. ELITE GENOTYPE PROPAGATION IN THE BELGRADE AREA

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Abstract: *Rosa canina* L. (dog rose) is an important ornamental, edible and medicinal plant. It has been used as a rootstock for ornamental roses, grown in plantations for fruit harvesting and it is suitable for revegetation of abandoned mine lands. The propagation of native genotypes that are well adapted to local conditions can provide planting material for both revegetation and plantation purpose. Micropropagation is the most suitable method for a rapid vegatative propagation of selected wild genotypes, but an increased presence of pathogens as well as higher contamination rate during culture establishment were expected. An occurrence of a specific Fe-chlorosis during *in vitro* propagation of roses is also possible. Therefore, the optimal period and disinfection protocol for establishing sterile *in vitro* culture of selected genotypes of dog rose was investigated, as well as an effect of increasing the FeEDTA concentration in the MS medium during multiplication phase. The obtained results showed that the optimal time for taking initial explants corresponds to optimal time for taking green cuttings in traditional vegetative propagation by softwood cuttings, and the best results were achieved using shoots collected in the first week of May, when the flowers were open. The iron chelate concentration in the medium affected the mean number of shoots, and doubling of its concentration resulted in a considerably higher number of shoots per explant.

Key words: dog rose, *in vitro* propagation, iron chelate, disinfection protocol, FeEDTA

INTRODUCTION

Rosa canina L. (Rosaceae) is often used as a rootstock for ornamental roses, especially cultivars 'Inermis', 'Pfänders', 'Schmids Ideal' and 'Laxa', considered as easily adaptable to different types of soil (K a r a s e k, 1976; M r a t i n i ć, K o j i ć, 1998; D e b e n e r, G u d i n, 2003).

Recent studies showed that *R. canina* can be used for the revegetation of abandoned mine lands. Thus, M a k i n e c i *et al.* (2011) investigated the plant species composition of naturally revegetated areas of abandoned open coal mines, and *R. canina* proved as one of the species that successfully adapted to unfavorable conditions in the researched area. Similar results were reported by M i l d e r *et al.* (2013) during the investigation of colonization patterns of woody species on reclaimed

coal wastes in northern Spain. Interestingly, despite the fact that *R. canina* can grow successfully on soils containing heavy metals, the concentrations of heavy metals (Cd, As, Pb, Ni, etc.) were low in fruits, and also considerably lower in fruits than in leaves (Turan, Erscisli, 2007; Tóth *et al.*, 2008; Vural, 2015).

Dog rose is also an important edible and medicinal plant. Its fruits are rich in vitamin C and phenolic compounds, it has been used for its antioxidant, antibacterial and antidiabetic properties (Mratinić, Kojić, 1998; Tucakov, 1984; Selahvarzian *et al.*, 2018). Dog rose can also be used for the treatment of cardiovascular diseases through decreasing blood pressure and LDL (low-density lipoprotein) without having any side effect (Selahvarzian *et al.*, 2018). Dog rose hips proved efficient against osteoarthritis and rheumatoid arthritis, especially with regard to its anti-inflammatory properties and their capacity to reduce pain (Selahvarzian *et al.*, 2018; Christensen *et al.*, 2008). *In vitro* studies proved that dog rose preparations are effective against certain kinds of cancer by reducing the proliferation of cancer cells (Selahvarzian *et al.*, 2018).

Hybrid varieties of *R. canina* are grown in plantations in Serbia (Dragović, 2017), but varieties from wild flora can also be grown on plantations producing high quality fruits (Šebek, Pavlova, 2019; Roman, Holonec, 2012). Most roses are sensitive to iron deficiency and show signs of specific Fe-chlorosis on alkaline soils (Bird, 2014).

Thus, the propagation of native genotypes that are well adapted to local conditions can provide planting material for both revegetation and plantation purpose. *Rosa canina* can be propagated by seed, but vegetative propagation is a better method for the propagation of selected wild genotypes in order to preserve desirable characteristics. Besides, *R. canina* seeds are characterized by complex physical and physiological dormancy and germination percentage is often low (Pawłowski *et al.*, 2020; Hoşafçı *et al.*, 2005; Alp *et al.*, 2010; Iakovoglou, Radoglou, 2015). Dog rose can vegetatively be propagated by cuttings (Kazankaya *et al.*, 2005; Izadi *et al.*, 2012), but micropropagation is more suitable for a fast production of uniform plants. Recently, many studies regarding micropropagation of *R.*

canina were published (Pahnekolayi *et al.*, 2014, 2015; Moallem *et al.*, 2012; Shirdel *et al.*, 2012; Pawłowska, 2011; Vântu, 2011; Fascella et al., 2017). However, different explant types (buds, leaves, shoots), culture mediums (MS or VS) (Murashige, Skoog, 1962; Van Der Salm *et al.*, 1994), as well as different sterilization methods and plant growth regulators were used in these papers. Besides, researchers propagated local genotypes of *R. canina*, but having large native distribution range, genetic variation of *R. canina* populations is high (Jürgens *et al.*, 2007) and there is a possibility that optimal conditions for the propagation of different genotypes will also be different.

One of the important steps for micropropagation is the establishment of a sterile *in vitro* culture.

Using young plants grown in a greenhouse or seedlings in the initial phase, the expected contamination rate is usually very low. However, during the propagation of selected wild genotypes, an increased presence of pathogens is expected as well as a higher contamination rate, and disinfection should be performed carefully. For this reason, we decided to investigate the optimal period and a disinfection protocol for establishing a sterile *in vitro* culture of selected elite wild genotypes.

In addition, Van Der Salm *et al.* (1994) showed that the occurrence of chlorosis during *in vitro* propagation of roses is not related to medium pH, but that it depends on the chelate type, and they showed that FeEDDHA is more photostable than FeEDTA which resulted in a higher availability of iron. For this reason, some researches used both the MS and VS media for the propagation of *R. canina* (Pahnekolayi *et al.*, 2014, 2015) and better results were obtained using the VS medium. However, good results of micropropagation of *R. canina* were reported on the MS medium (Moallem *et al.*, 2012; Shirdel *et al.*, 2012; Pawłowska, 2011; Vântu, 2011; Fascella *et al.*, 2017). Furthermore, the use of the MS medium has been recommended in Springer Protocols for the *Rosa* spp. micropropagation (Pati *et al.*, 2010).

Van Der Salm *et al.* (1994) also showed that the addition of a light absorbing dye to the medium containing FeEDTA also resulted in green

Fig. 1 Shoots with flowers on May 7

Fig. 2 Single-node cutting after disinfection

shoots with a higher chlorophyll content. The question arose whether increasing the FeEDTA concentration in medium could be also favourable for shoots development, and we decided to evaluate shoots response to the standard and doubled concentration of FeEDTA in the MS medium.

MATERIAL AND METHODS

Initial phase

The initial material was collected from a chosen healthy and well developed *R. canina* shrub growing in the Belgrade area during 2018. The shoots were collected on March 21, April 18 and May 7 in order to evaluate an influence of plant phenophase on the culture establishment. The shoots collected in March were woody, developed during the year before. The shoots collected in April were green, with leaves and small closed flower buds. The shoots collected in May had open flowers (Fig. 1). For this reason, the initial phase was different depending on the time of shoots collection. In all cases, the collected shoots were cut into single-node cuttings, washed using a mild liquid detergent (5% (v/v)) for 5-8 min and rinsed under running water for 15 minutes before disinfection.

Culture establishment on March 21

The cuttings were treated with 70% ethanol for 30 s or 60 s, followed by a treatment with 2% NaOCl for 25 min or 3.5% NaOCl for 15 min. The NaClO solution contained 1-2 drops of Tween-20. After disinfection, the explants were rinsed 5 times with sterile distilled water and buds were excised. The outer bud scales were removed and the buds were placed on the MS medium supplemented with 1 mg/L BAP (6-Benzylaminopurine) or 2 mg/L BAP, and 0.5 mg/L NAA (1-Naphthaleneacetic acid). The medium contained 3% sucrose, 0.7% agar, and its pH value was adjusted to 5.8 before autoclaving. The state of explants was recorded after 7 days.

Culture establishment on April 18

The leaves were cut off using scissors before cutting shoots. The cuttings were disinfected using 2% NaOCl for 15 minutes and rinsed 5 times with sterile distilled water. The upper and bottom parts of the cuttings were cut removing about 2-5 mm of the exposed parts of the cut surfaces; the remaining part of leaf stalks were completely removed and the obtained single-node cuttings were used as initial explants (Fig. 2). They were placed on the MS medium which was the same as used in the previous experiment, but supplemented only with 1 mg/L BAP and 0.5 mg/L NAA, while the variety with 2 mg/L BAP and 0.5 mg/L NAA was omitted. The state of explants was recorded after 3 weeks (contaminated, necrotic, callused, proliferated and unchanged). The number of shoots and length of the longest shoot were recorded for each proliferated cutting.

Culture establishment on May 7

The disinfection procedure and medium composition were same as in the experiment conducted on April 18. The state of explants, as well as the number and length of proliferated shoots were also recorded after 3 weeks.

Multiplication phase

The shoots obtained from the culture establishment on May 7 were used for the multiplication phase. The shoots were placed on the MS medium supplemented with 3% sucrose, 0.7% agar, 2 mg/L BAP and 0.5 mg/L NAA. In order to evaluate the effect of different concentrations of iron chelate on shoots development, the medium contained a standard concentration of $FeSO_4$ x $7H_2O$ (27.8 mg/L) and Na_2EDTA x $2H_2O$ (37.2 mg/L) or a doubled concentration of $FeSO_4$ x $7H_2O$ (55.6 mg/L) and Na_2EDTA x $2H_2O$ (74.4 mg/L), while concentrations of the remaining medium components were unchanged. The state of explants and number and length of shoots were recorded after 4 weeks.

Culture conditions and statistical analysis

In all experiments plant cultures were grown under a 16/8 h (day/night) photoperiod and a photon flux rate of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 25 ± 2ºC in the light of fluorescent white pipes. A total of 5 explants were placed in glass vessels containing 25 mL of the culture medium. Twenty-five explants were used per treatment with three replications. The obtained data were analyzed using the Version 5.0 STATGRAPHICS software (STSC Inc. and Statistical Graphics Corporation, 1994-2000, USA). The significance of differences among the treatments was determined by the analysis of variance (ANOVA), and the means were compared using the least significant difference (LSD) multiple range test (at $p < 0.05$).

RESULTS AND DISCUSSION

Initial phase

Culture establishment on March 21

All explants were contaminated or necrotic and none of them proliferated (Table 1). Since the concentration of BAP did not influence the disinfection of explants, and there were no significant differences among the explants grown on the medium supplemented with 1 mg/L BAP and 2 mg/L BAP (Table 1) the explants were grouped according to the disinfection treatment and the obtained results are shown in Table 2.

The percent of necrotic explants was very high ranging from 54.9% for explants treated with ethanol for 30 s followed by 2% NaOCl for 25 minutes to 82.9% for explants treated ethanol for 60 s and 3.5% NaOCl for 15 minutes (Table 2). It indicates that a higher concentration of NaOCl and longer exposure to ethanol caused tissue necrosis.

Table 1 State of explants cultured on March 21 on media with different BAP concentrations

Disinfection procedure	BAP (mg/L)	Contaminated (%)	Necrotic (%)
70% ethanol for 60 s, + 2% NaOCl for 25 minutes	1	29.1[abc]	70.9[abcd]
	2	50.0[c]	50.0[a]
70% ethanol for 30 s, + 2% NaOCl for 25 minutes	1	40.4[bc]	59.6[ab]
	2	48.6[c]	51.4[a]
70% ethanol for 60 s, + 3.5% NaOCl for 15 minutes	1	21.5[ab]	78.5[bc]
	2	12.7[a]	87.3[c]
70% ethanol for 30 s, + 3.5% NaOCl for 15 minutes	1	44.5[c]	55.5[a]
	2	16.7[ab]	83.3[bc]

Note: Values followed by different letters are significantly different at the P < 0.05 level according to the LSD test

Table 2 State of explants cultured from March 21

Disinfection procedure	Contaminated (%)	Necrotic (%)
70% ethanol for 60 s, + 2% NaOCl for 25 minutes	39.1[b]	60.9[a]
70% ethanol for 30 s, + 2% NaOCl for 25 minutes	45.1[b]	54.9[a]
70% ethanol for 60 s, + 3.5% NaOCl for 15 minutes	17.1[a]	82.9[b]
70% ethanol for 30 s, + 3.5% NaOCl for 15 minutes	36.2[b]	63.8[a]

Note: Values followed by different letters are significantly different at the P < 0.05 level according to the LSD test

Table 3 State of explants cultured on April 18

Necrotic (%)	Proliferated (%)	Unchanged (%)	Callused (%)	No. of shoots	Shoot length (mm)
23.4 ± 18.1	34.0 ± 8.1	25.3 ± 13.3	17.3 ± 12.8	2.9 ± 1.2	11.4 ± 4.3

X̄ ± SD*

SD – standard deviation, X̄ - mean value

Some authors state that closed winter buds are suitable for culture initiation because they can be exposed to higher concentrations of disinfection agents, especially ethanol, because in addition to disinfection, it also plays a role in dissolving the wax and resin of the protective bud scales. (Ewald, 2007a, b; Mihaljević *et al.*, 2013; Bajaj, 1996; Grbić, 2004). However, there are reports that winter buds of adult trees and shrubs are highly contaminated and thus not suitable for culture initiation. For that reason, shoots with winter buds can be collected before opening and brought in the laboratory for proliferation. Thus, young, newly developed green shoots can be used for culture initiation (Vieitez *et al.*, 2007; Skočajić *et al.*, 2017).

There are reports of using similar procedure of bud disinfection during *R. canina* sterile cul-

Fig. 3 A callused single-node cutting

ture establishment. For example Pahnekolayi *et al.* (2015) and Khosravi *et al.* (2007) used 70% ethanol for 30 s, followed by 2.5% NaOCl for 25 minutes. Similarly, Moallem *et al.* (2012) treated buds with 70% ethanol for 40 s, followed by 2.5% NaOCl for 10 minutes. However, these authors did not report the success of disinfection procedure used for culture initiation in their researches.

Culture establishment on April 18

Despite omitting ethanol pretreatment because it can damage young shoots, disinfection was successful and contaminated explants were not recorded (Table 3). However, 23.4% of explants were necrotic, probably because the concentration of NaClO was too high for young plant tissue.

The percentage of proliferated buds was relatively low, reaching only 34%, while a large number of explants remained unchanged (25.3%) or only developed callus (17.3%) (Fig. 3).

Culture establishment on May 7

The disinfection procedure was the same as on April 18, but the obtained results were considerably better (Table 4). The mean number of shoots per explant was higher (3.4) compared to the number of shoots (2.9) developed in the culture established in April. There were no necrotic explants and the proliferation rate was very high (94.6%) (Fig. 4).

Table 4 State of explants cultured on May 7

Necrotic (%)	Proliferated (%)	Unchanged (%)	Callused (%)	No. of shoots	Shoot length (mm)
0.0 ± 0.0	94.6 ± 4.1	2.1 ± 1.1	4.3 ± 3.5	3.4 ± 1.4	13.6 ± 5.1

$\bar{X} \pm SD*$

SD – standard deviation, $\bar{X}$ - mean value

The results obtained in April indicated that the proliferation rate was probably influenced by phytohormones and there was a possibility that adding some other plant growth regulators or using some other concentrations of BAP and NAA could increase the proliferation rate or the average number of shoots per explant. However, using the same procedure and the same initial medium, proliferation rate was significantly higher, thus indicating that time of culture initiation and physiological state of mother plant and level of endogenous hormones play an important role in culture establishment. Moreover, Pahnekolayi et al. (2014) cultured *R. canina* nodal cuttings with 3 axillary buds on the MS and VS media supplemented with various concentrations of BAP, NAA and GA_3 (Gibberellic acid), but the obtained proliferation rate was low, under 40% on all media used, regardless of PGRs. However, they established *in vitro* culture in February.

The time of taking softwood cuttings is very important in the vegetative propagation of woody species and it influences rooting (Grbić, 2004; Marković *et al.*, 2014; Hartmann *et al.*, 2010). Our results show that the time of taking nodal cuttings for *in vitro* culture initiation can also influence bud proliferation and the success of culture establishment.

Fig. 4 A proliferated bud on a single-node cutting

The multiplication phase

The number and length of shoots were recorded four weeks after placing the explants on the MS media containing standard and doubled concentration of chelated iron (Table 5). The concentration of iron strongly influenced the development of callus, and 50% of explants were callused on a medium with a standard iron concentration, while there were no callused explants on the medium with a doubled iron concentration (Table 5). Iron chelate concentration also significantly influenced the mean number of shoots per explant and more shoots developed on media with a doubled iron chelate concentration. However, although the shoots were also longer on a medium with a doubled iron chelate concentration, the obtained difference was not statistically significant. On the contrary, during micropropagation of *R. canina* using shoots with 3 axillary buds as explants Pahnekolayi et al. (2015) did not record a significant difference in the mean number of shoots grown on the MS or VS media, but the shoots were significantly longer on the VS medium.

In a standard plant tissue culture the medium MS precipitation of iron is common because it is ineffectively chelated (Dalton *et al.*, 1983) or exposed to photo- and thermo- degradation (Sokolov *et al.*, 2015). The concentration of Fe in a standard MS is 0.1 mmol/L. If precipitation occurs, the explants are potentially exposed to iron deficiency, which can be due to an increased *in vitro* callus proliferation and less formation of shoots in our experiments. The doubled iron concentration (0,2 mmol/L) blocks callus formation and increases the number of shoots, which indicates that iron has a significant role in *in vitro* morphogenesis of *R. canina.* A similar influence of iron on shoots formation was also observed in the *in vitro* cultures of magnolia and cherry plum (Sokolov *et al.*, 2015). There are different iron chelate forms,

Table 5 State of explants cultured on media with different concentrations of iron chelate

Concentration of iron chelate in MS	Callused (%)	Mean No. of shoots	Mean length of shoots (mm)
Standard	50.0[a]	2.6[a]	13.1[a]
Doubled	0.0[b]	3.4[b]	14.6[a]

Note: Values followed by different letters are significantly different at the P < 0.05 level according to the LSD test

such as Fe-III-EDTA and Fe-III-EDDHA which can be used for further testing and improving the protocols.

Pawłowska (2011) cultured *R. canina* shoots on MS media containing only BAP or a combination of BAP and GA$_3$. The mean number of shoots was lower on the medium with only BAP and the best results (2.5) were achieved with a low BAP concentration (0.2 mg/L), while higher concentrations produced a lower number of shoots per explant. However, a combination of BAP and GA$_3$ gave better results, and the highest number of shoots (4.1) developed on a medium with 0.2 mg/L BAP and 0.6 mg/L GA$_3$. Higher concentrations of BAP combined with lower concentrations of GA$_3$ resulted in a significantly lower number of shoots per explant.

CONCLUSION

The presented research showed that the time of establishing *in vitro* culture, using shoots and axillary buds as initial explants, are important and they considerably affect the success of culture development. The optimal time for taking initial explants probably corresponds with the optimal time for taking green cuttings in the traditional vegetative propagation by softwood cuttings.

The iron chelate concentration in the medium affects the mean number of shoots, and an increase in its concentration leads to a higher number of shoots per explant. However, the effect of adding GA$_3$ to a medium with increased iron chelate concentration, as well as the effect of other iron chelates (DTPA - Diethylenetriaminepentaacetic acid) on *in vitro* development of *R. canina* should be tested.

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