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EFFECTS OF DIFFERENT CULTURE MEDIA AND PLANT GROWTH REGULATORS ON MICROPROPAGATION OF 'GISELA 5' CHERRY ROOTSTOCK

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Abstract

We evaluated the influence of various culture media and plant growth regulators (PGR) upon axillary shoot proliferation rates and shoot lengths obtained in the in vitro multiplication stage in cherry rootstock 'Gisela 5'. The treatments consisted of the use of three basal media: Driver and Kuniyuki (DKW), Murashige and Skoog (MS), Woody Plant Medium (WPM) as well as three plant growth regulators (PGR) in various concentrations and combinations: N^6-benzyladenine (BA at 0.3 and 0.5 mg/l), DL-Dihydrozeatin (DHZ at 1.0 and 2.0 mg/l) and N-benzyl-9-(tetrahydropyranyl)-adenine (BPA at 1.0 mg/l). The results show that the type of basal media and PGRs influenced the quantity as well as the quality of axillary shoot development. In the presence of BA at 0.3 and 0.5 mg/l in DKW media the proliferation rates (PR) were superior to those in MS and WPM media, both at 0.3 mg/l BA (PR = 6.66 ± 0.65) and 0.5 mg/l BA (PR = 8.93 ± 0.86) in the aforementioned DKW treatments. The DKW culture medium supplemented with 1 mg/l indole-3-butyric acid (IBA) provided the highest in vitro rooting percentage (94.74%).

Key words*: cherry, benzyladenine, dihydrozeatin, micropropagation, shoot culture.*

INTRODUCTION

'Gisela 5' (*P. cerasus* × *P. canescens*) is known as the most popular clone of the Gisela rootstocks in Germany. One of its great advantages is that in contrast to standard *P. avium* rootstocks, it significantly reduces the vigor of sweet cherry trees and they can begin to flower and fruit in the second year in the nursery and achieve full cropping in the 5th year. The propagation and marketing of this rootstock is handled by a consortium of German rootstock nurseries Deutscher Baumschulen GmbH (http://www.cdb-rootstocks.com/en/cdb---consortium.html) (Franken-Bembenek, 2005). Since classical propagation methods of this rootstock are inefficient (Bošnjak and Kereša, 2012), several micropropagation protocols have already been developed as viable alternatives for its propagation. *In vitro* cultures of 'Gisela 5' are not dependent on season, provide clean, disease and virus-free planting material (Sharma et al., 2017). Previous reports show that the most commonly used basal medium for *in vitro* propagation of 'Gisela 5' rootstocks was MS (Murashige and Skoog, 1962) (Ružić et al., 2000; Vujović et al., 2012; Clapa et al., 2013; Xu et al., 2015; Thakur et al., 2016; Sharma et al. 2017; Tariverdi et al. 2017;). Other scientific reports show that the less tested *in vitro* culture media for 'Gisela 5' were QL (Quoirin and Lepoivre medium) (Mihovilović Bošnjak and Kereša, 2012) or DKW (Driver and Kuniyuki Juglans medium) and WPM (McCown Woody Plant medium) (Fallahpour et al., 2015; Ozudogru et al., 2017). Furthermore, N^6-benzyladenine (BA) is the most frequently used cytokinin in the initiation and multiplication stage of 'Gisela 5' cherry rootstock in various concentrations and/or combinations (Buyukdemirci, 2008; Fidanci et al., 2008; Bošnjak et al., 2012; Xu et al., 2015; Thakur et al., 2016).

Other studies show that the in vitro rooting percentage ranged from 14-93.7% when 0.5, 1 or 2 mg/l indole-3-butyric acid (IBA) was added to the culture media in the in vitro rooting stage (Fallahpour et al., 2015). Furthermore, concentrations of 0.25 and 0.5 mg/l α-naphthaleneacetic acid (NAA) led to rooting percentages of 64 and 72%.

Several investigations have been carried out regarding other aspects of micropropagation of 'Gisela 5' cherry rootstock such as in vitro conservation (Ružić et al., 2015), the effects of multiple subculturing (Vujović et al., 2012), effects of gas-tight and gas-permeable culture containers and different sucrose concentrations, as well as sucrose and mannitol combinations applied in tissue culture (Ozudogru et al., 2017). Therefore, the main aim of this research was to test different culture media and plant growth regulators such as DL-Dihydrozeatin (DHZ) and N-benzyl-9-(tetrahydropyranyl)-adenine (BPA) in micropropagation processes of 'Gisela 5' cherry rootstock, which to the best of our knowledge had not be used until now neither for 'Gisela 5' tissue cultures nor for other rootstock.

MATERIALS AND METHODS

Explant preparation and tissue culture initiation

For tissue culture initiation herbaceous shoots were used obtained from the forced twigs in laboratory conditions in March, 2018. The shoots were fragmented and internodes were eliminated followed by washing the plant material in running tap water and then rinsed with deionised water. Then the shoot fragments were sterilised with ACE 20% for 20 minutes and rinsed repeatedly with deionised water (5 rinses) in laminar flow hood. The axillary and terminal buds were excised aseptically from the nodal segments and then inoculated directly onto the MS (Murashige & Skoog, 1962) culture media in glass tubes (Table 1). The media was supplemented with 0.5 mg/l BA and solidified with cu 4 g/l Plant agar. After 8 weeks, shoot regeneration percentage was calculated, which represented the number of inoculated buds with successful shoot regeneration. For *in vitro* culture stabilisation 1.5-2 cm length shoot fragments with 3-4 buds obtained in the initiation stage were inoculated on the same above mentioned culture media. Hereafter, for all the experimental treatments 720 ml glass jars were used with crew lids with an antibacterial filter to ensure gas exchange with the atmosphere. 100 ml of culture medium was dispersed/culture vessel and each vessel contained 5 nodal segments which were introduced in the culture media in slanted position. The duration of the in vitro cycles was 6 weeks.

Effect of various culture media and plant growth regulators (PGR) on in vitro multiplication

The influence of various culture media and plant growth regulators (PGR) upon axillary shoot proliferation rates and shoot lengths were also evaluated in this study. The treatments consisted of the use of three different basal media (Table 1): Driver and Kuniyuki (DKW) (Driver and Kuniyuki, 1984), Murashige and Skoog, 1962 (MS), Woody Plant Medium (WPM) (Lloyd and McCown, 1980) as well as three plant growth regulators (PGRs) in various concentrations and combinations: N^6-benzyladenine (BA at 0.3 and 0.5 mg/l), DL-Dihydrozeatin (DHZ at 1.0 and 2.0 mg/l) and N-benzyl-9-(tetrahydropyranyl)-adenine (BPA at 1.0 mg/l).

Table 1. Chemical composition of the basal media used for the micropropagation of 'Gisela 5' cherry rootstock

	Concentrations		
Composition	MS	DKW	WPM
Macro-elements	MS	DKW	WPM
Micro-elements	MS	DKW	WPM
FeNaEDTA	36.7 mg/l	44.63 mg/l	36.7 mg/l
Myo-inositol	100 mg/l	100 mg/l	100 mg/l
Vitamin B1	2 mg/l	2 mg/l	1 mg/l
Vitamin B6	1 mg/l	-	0.5 mg/l
Acid nicotinic	1 mg/l	1 mg/l	0.5 mg/l
Glycine	-	2 mg/l	-
Sugar	30 g/l	30 g/l	30 g/l
Plant Agar	4 g/l	4 g/l	4 g/l
pH = 5.8			

Rooting and acclimatization

In vitro rooting was carried out on DKW basal media without PGRs or supplemented with IBA and gelled with Plant Agar. Thus, the following treatments (variants) were established:

V1 - DKW + 4 g/l Plant agar

V2 - DKW+ 0.5 mg /l BA + 4 g/l Plant agar

V3 - DKW+ 1 mg/l BA + 4 g/l Plant agar

The young shoots (1.5 month old) were inoculated onto the culture media (15 shoots/vessel in three repetitions). After 6 weeks of culturing shoot length, number of shoots, length of roots, number of roots and the maximum length of the roots were recorded.

Ex vitro rooting and acclimatization

The unrooted shoots taken from the multiplication stage were rooted and hardened in ex vitro conditions in floating perlite, while the *in vitro* rooted shoots were hardened in floating hydroculture (Clapa et al., 2013). After 30 days the rooting and plant survival percentages were calculated based on the data recorded from 45 shoots from three repetitions (15 shoots/repetition).

Data Analysis

To analyse the data, ANOVA analysis was performed first to check the differences among the means. When the null hypothesis was rejected, Tukey's HSD test ($P \leq 0.05$) was performed to determine the means that are significantly different from each other. Values shown are means ± SE. In addition, Pearson's correlation was assessed to check the relationships between the mean length of the shoots, number of shoots and the length of the roots developed in the *ex vitro* rooting stage of the 'Gisela 5' cherry rootstock.

RESULTS AND DISCUSSIONS

For the initiation of 'Gisela 5' cherry rootstock the tissue culture were established in March, 2018, using apical buds and nodal shoot fragments as results of forcing of the annual twigs in laboratory conditions. Thus, the in vitro culture could be established earlier, and the young shoots used for initiation were free of any type of diseases. For this reason, there were no shoot infections observed in the initiation stage of the in vitro culture as shown in Figure 1.

Figure 1. *In vitro* culture initiation of 'Gisela 5' cherry rootstock

In this manner, the initiation percentage recorded was 86.25%. In contrast, other studies show that when using 'Gisela 5' plant material obtained from greenhouse can considerably increase the risk of infections and can lead to a contamination percentage of the explants of 71.7%, and an initiation success of only 28.3% as reported by Vujović et al. (2012).

The influence of the culture medium and BA concentration on the multiplication rate of 'Gisela 5' cherry rootstock

Our results show that all the treatments had different effects on the development of 'Gisela 5' cherry rootstock in terms of number of shoots/explant/vessel and shoot length recorded in the multiplication stage as presented in Table 2. It was also observed that both the basal media and BA concentrations influenced the proliferation capacity of 'Gisela 5' cherry rootstock. The highest numbers of shoots/vessel and shoots/explant were obtained on the basal media supplemented with 0.5 mg/l BA. Thus, the highest number of shoots/vessel (42.33 ± 2.59) and shoots/explant (8.93 ± 0.86) were recorded on DKW + 0.5 mg/l BA + 4 g/l Plant agar (Figure 2) followed by 39.66 ± 2.73 shoots/vessel and 7.93 ± 0.57 shoots/explant on MS + 0.5 mg/l BA + 4 g/l Plant agar. Between these two no statistically significant differences were recorded. On the WPM + 0.5 mg/l BA + 4 g/l Plant agar medium the mean number of shoots/vessel recorded was 35.66 ± 2.87 and 7.13±0.98 shoots/explant which were significantly lower than those recorded on DKW+0.5 mg/l BA: 42.33 ± 2.59 and 8.93±0.86.

Lower number of shoots were recorded on the media supplemented with 0.3 mg/l BA but also in this case basal media DKW and MS stimulated the most the development of the

shoots/vessel (33.33 ± 1.67 and 30.33 ± 0.83), but no statistically significant differences were observed between these and those obtained on WPM + 0.3 mg/l BA (Figure 5).

Our findings reveal that the highest average lengths of the shoots were obtained on DKW+0.3 mg/l BA, reaching 3.28 ± 0.31 cm, followed by those developed on DKW + 0.5 mg/l BA with an average of 3.01 ± 0.26 cm. Statistically significant differences had been determined between these two and the rest of the media used for propagation. Therefore, the shoots developed the most on the DKW basal medium. Depending on the concentration of BA in the basal medium, the longest shoots were developed on the media supplemented with 0.3 mg/l BA, on all the three basal media (Table 2).

Table 2. The influence of culture media and BA concentrations on the in vitro multiplication of 'Gisela 5' cherry rootstock

Variant/Treatment	Average number of shoots/vessel	Average number of shoots/explant	Average length of shoots
WPM+0.3 mg/l BA	22.00±1.36 ^{e*}	4.40±0.63 ^{e}	2.41±0.22 ^{b}
WPM+0.5 mg/l BA	35.66±2.87 ^{bc}	7.13±0.98^{bc}	2.32±0.19 ^{b}
MS+0.3 mg/l BA	30.33±0.83 ^{d}	6.06±0.50 ^{d}	2.46±0.19 ^{b}
MS+0.5 mg/l BA	39.66±2.73 ^{ab}	7.93±0.57 ^{ab}	2.17±0.19 ^{b}
DKW+0.3 mg/l BA	33.33±1.67 ^{cd}	6.66±0.65 ^{cd}	3.28±0.31 ^{a}
DKW+0.5 mg/l BA	42.33±2.59 ^{a}	8.93±0.86 ^{a}	3.01±0.26 ^{a}

*The values shown are means ± SE. Different lowercase letters indicate significant differences between the means among the treatments according to Tukey's HSD test (p≤ 0.05).

Figure 2. 'Gisela 5' cherry rootstock in vitro multiplication stage on DKW culture medium supplemented with 0.5 mg/l BA

Both the number of shoots/explant and length of the shoots reached much higher values than those reported by other in all types of culture media used for this experiment (D Ružić et al., 2000; Sharma et al., 2017; Thakur et al., 2016; Vujović et al., 2012).

Effect of various plant growth regulators (PGRs) on the in vitro multiplication of 'Gisela 5' cherry rootstock

Cytokinins such as DHZ and BPA, tested for the first time in 'Gisela 5' cherry rootstock tissue culture proved to be less efficient than BA in terms of proliferation rate. The combination of 1 mg/l BPA + 1 mg/l DHZ generated longer shoots than any of the BA (Figures 3 and 5).

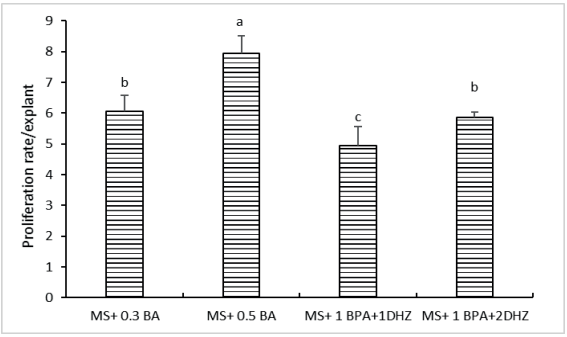

Figure 3. The influence of the Murashige and Skoog 1962 (MS) media supplemented with N6-benzyladenine (BA at 0.3 and 0.5 mg/l), DL-Dihydrozeatin (DHZ at 1.0 and 2.0 mg/l) and N-benzyl-9-(tetrahydropyranyl)-adenine (BPA at 1.0 mg/l) on in vitro the proliferation rate of 'Gisela 5' cherry rootstock. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test (p≤ 0.05)

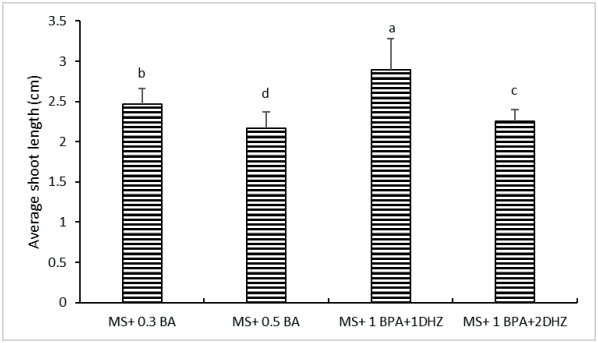

Figure 4. The influence of Murashige and Skoog 1962 (MS) supplemented with N6-benzyladenine (BA at 0.3 and 0.5 mg/l), DL-Dihydrozeatin (DHZ at 1.0 and 2.0 mg/l) and N-benzyl-9-(tetrahydropyranyl)-adenine (BPA at 1.0 mg/l) on shoot length in the *in vitro* multiplication stage of a 'Gisela 5' cherry rootstock. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

Figure 5. *In vitro* shoot regeneration of 'Gisela 5' cherry rootstock on different culture media: Murashige and Skoog (MS), Woody Plant Medium (WPM, Lloyd & McCown) and Driver and Kuniyuki (DKW) supplemented with 0.3 mg/l BA and 0.5 mg/l B.

Regarding shoot length, the combination of MS + 1 BPA + 1 DHZ generated the longest shoots (2.89 ± 0.39 cm) as compared to 2.46 ± 0.19 cm developed on MS + 0.5 mg/l BA. The average length of the shoots developed on MS supplemented with these cytokinins were much greater than those obtained on the same basal medium but supplemented with BA, Kin, TDZ and gibberellin GA3 combinations ranging between 0.75-2.25 cm (Thakur et al., 2016).

Rooting and acclimatization

Our results show that for in vitro rooting DKW basal medium proved to be the most suitable for the multiplication stage of 'Gisela 5' cherry rootstock. Based on our findings, the use of newly developed, full length shoots excised from the young plantlets obtained during the 1-1.5-months- in vitro culture is recommended due to the crucial role of the apical bud (Clapa et al., 2013). It was also observed that, shoots from the media with no PCRs added did not emerged any roots. The highest rooting percentage (94.74%), though, was recorded in shoots regenerated on DKW + 1 mg/l IBA medium, followed by 74.36% on the medium supplemented with 0.5 mg/l IBA (Figure 6). Our study provides further evidence for the effectiveness of WPM medium containing 2 mg/l IBA for rooting (93.7%) as compared to MS (53.1%) or DKW (14.0%) which showed much lower rooting percentages (Fallahpour et al., 2015). Similar results have been found by Xu et al. (2015), who used MS, 1/2 MS, 1/4 MS and 1/8 MS supplemented with 2 mg/l IBA and recorded low rooting percentages such as 77.5, 82.5, 87.5 and 77.5% as compared to WPM (Xu et al., 2015). Other low rooting percentages were also reported by Fidanci et al. (2008) when MS supplemented with 0.5 mg/l and 1 mg/l IBA generating rooting percentages of 67 and 89%, which were 8.67 and 27.74% lower than the rooting percentage obtained on DKW. The in vitro rooted shoots were acclimatised in floating hydroculture and the survival rate recorded after 30 day of hardening was 49.96%. The unrooted shoots were subjected simultaneously to ex vitro rooting and hardening in floating perlite + 1 mg/l IBA. The rooting percentage and survival rate recorded was 96.15%. The hardened shoots (plantlets) had an average length of 3.67 ± 0.23 cm, and an average number of 6.13 ± 0.64 roots/shoot, and the maximum average length of the roots recorded was 5.83 ± 0.47 cm.

These results show a good positive correlation between the measured parameters (Figure 7). Namely, the longest the shoots were the longest and highest the roots and their number were.

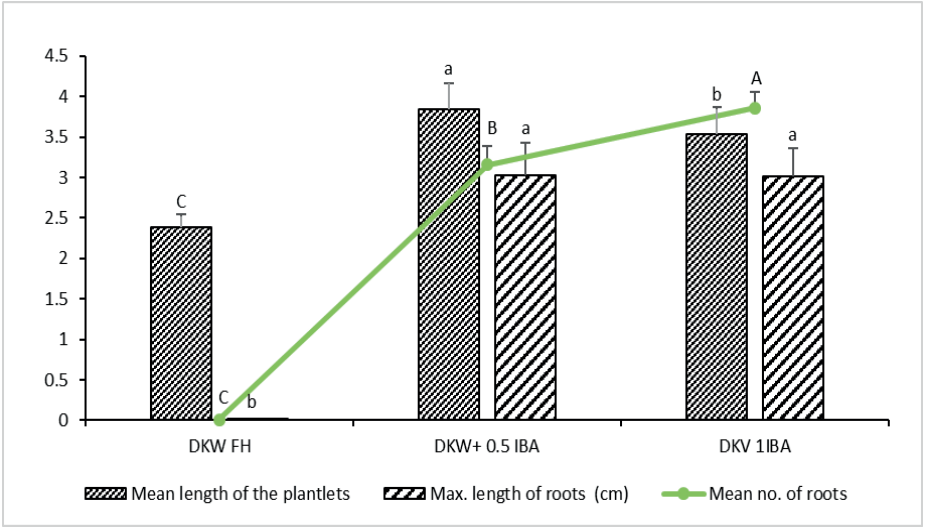

Figure 6. In vitro rooting of 'Gisela 5' on Driver and Kuniyuki (DKW) medium without PGRs and supplemented with 0.5 and 1 mg/l IB

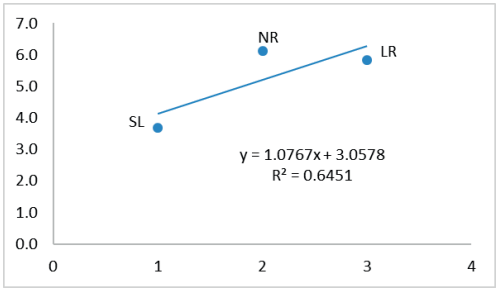

Figure 7. Correlation between the average length of shoots (SL), number of roots (NR) and the length of the roots of due to *ex vitro* rooting of 'Gisela 5' cherry rootstock

CONCLUSIONS

Our work led us to conclude that for the in vitro initiation of 'Gisela 5' cherry rootstock in spring, young, fresh and herbaceous shoots are the most suitable to be used as results of forcing the annually harvested twigs from orchard. The forcing of the harvested twigs can successfully be carried out in laboratory conditions with natural light and constant temperature. After sterilisation, shoot fragments with axillary or apical buds were the most effective to be used on MS and DKW media supplemented with 0.5 mg/l BA and gelled with Plant agar. Regarding the *in vitro* multiplication, the findings of our study indicate that MS and DKW media supplemented with 0.3-0.5 mg/l BAP and solidified with 4 g/l Plant Agar were the most adequate for this stage. The results of this research point towards the idea that 'Gisela 5' cherry rootstock can simultaneously be rooted and hardened ex vitro in floating perlite with 1 mg/l IBA to increase the economic efficiency of the production chain by eliminating the rooting stage of the regenerated plantlets. As mentioned before, in this case the rooting percentage and survival rate of the micropropagated plants can reach over 95%.

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