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Development of an in vitro micropropagation protocol for Myrobalan 29C rootstock

Murat GÜNEY*
Department of Horticulture, Faculty of Agriculture, Yozgat Bozok University, Yozgat, Turkey

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Abstract: Stone fruits are well known for their high nutritional value. Therefore, in horticulture, the micropropagation of suitable rootstocks is vital for their cultivation. The aim of the present study was to improve the micropropagation protocol of Myrobalan 29C (*Prunus cerasifera* Ehrh.) rootstock using shoot-tip culture. Murashige and Skoog (MS) basal medium containing 2 mg L^{-1} 6-benzylaminopurine (BAP) and 0.05 mg L^{-1} gibberellic acid (GA_3) resulted in the highest number (14.3) of multiple shoots. However, a greater shoot length (2.0 cm) was attained when GA_3 was excluded from the MS medium and the concentration of BAP was reduced to 1 mg L^{-1}. Root induction was best in ½MS medium containing 0.5 mg L^{-1} indole-3-butyric acid (IBA), 0.5 mg L^{-1} α-naphthaleneacetic acid, and 10 mL L^{-1} (≈13 mg L^{-1} Fe) ethylenediamine di-2-hydroxyphenyl acetate ferric with 7.0 roots per explant. On the other hand, the longest root (12.5 cm) was obtained from increased concentration to 1 mg L^{-1} of IBA. The establishment of a well-defined micropropagation protocol will lead to further biotechnological improvement of this crop.

Key words: Micropropagation, Myrobalan 29C, plant growth regulator, shoot tip culture, rooting

1. Introduction

Horticultural plants are nutritious, tasty, and healthy for humans (Sahin et al., 2002; Ozturk et al., 2009; Gündüz and Özbay, 2018). Among the horticultural fruits, consumers prefer stone fruits, like plums and apricots, for their delicious taste and higher nutritional content. However, diseases and environmental incompatibility are major issues that arise during the cultivation of these trees. In order to overcome these problems, it is necessary to produce easily propagated and adaptive rootstock of these crops. Myrobalan (*Prunus cerasifera* Ehrh.) probably originated in the Caucasus and comprises many varieties with different characteristics (Güleryüz and Ercişli, 1995). It is suitable for all soil types and adapts well to dry soils and heavy soils with low permeability. Myrobalan 29C is the selected rootstock for plum and apricot due to its resistance to root-knot nematodes (Meloidogyne). On the other hand, Myrobalan 29C is moderately sensitive to crown rot diseases, *Verticillium*, and bacterial canker. Clonal Myrobalan 29C and Myrobalan seedlings are used as standard rootstocks in most areas. The apricots grafted on Myrobalan rootstocks exhibit moderate vigor and live up to 25–30 years (Gönülşen et al., 1987; Dimitrova, 1988; Özçağıran et al., 2005). The hybrid produced in this method inherits all the properties such as adaptation ability, rapid growth, and easy reproduction from Myrobalan plum (Costa and Grandi, 1975; Nitransky, 1981; Botu, 1990). Therefore, interspecific hybrids of Myrobalan have a clear potential for breeding purposes (Arbeloa et al., 2009; Plopa et al., 2012).

Because of the short juvenile period in clonal rootstocks, they start bearing earlier than seedling rootstocks. Therefore, clonal rootstocks are more advantageous than those conventionally propagated (Arıcı, 2008). Micropropagation is a useful method for clonal propagation of rootstocks (Hossini et al., 2010). In the growth of stone fruits by tissue culture, successful results can be expected by the modification of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The diverse concentrations of plant growth regulators (PGRs) and mineral elements affect the in vitro propagation of clonal rootstock. There are limited in vitro studies on the micropropagation of Myrobalan 29C rootstocks (Arbeloa et al., 2009; Akşehirli, 2010; Movsiuw, 2011; Plopa et al., 2012; Özbek et al., 2014; Shabani et al., 2015; Nasri et al., 2019).

The aim of the present study was to mass propagate Myrobalan 29C rootstock by means of shoot-tip (ST) culture. The effects of different concentrations and combinations of MS medium, as well as PGRs, were tested on in vitro culture of Myrobalan 29C rootstock.

* Correspondence: murat.guney@yobu.edu.tr

2. Materials and methods

2.1. Plant material

Fresh shoots of Myrobalan 29C rootstock were supplied by a private company that has a certificate for producing seedlings. The explants were cut into single-node segments and surface sterilized by washing under running tap water for 15 min, followed by 70% ethanol for 2 min. Then the explants were rinsed for 20 min in 15% sodium hypochlorite solution containing 1–2 drops of Tween 20. Finally, the disinfected explants were rinsed three times in sterile distilled water for 5 min each and subsequently inoculated onto the culture medium.

2.2. Medium and culture condition

MS and ½MS basal culture media containing macro- and microelements, vitamin, ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA), and different combinations of PGRs were used for shoot induction, multiplication, and rooting of Myrobalan 29C. All of the chemicals used in the present study were obtained from Sigma-Aldrich. The pH of all the media after PGRs were added was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. After the media were dispersed into culture vessels, autoclaving was performed for 15 min at 121 °C and 15 psi pressure. All the cultures were incubated at 25 ± 2 °C under 16/8 h photoperiod with a light intensity of 3000 lux. The explants were transferred from the initiation media to the multiplication media after 2 weeks. Subcultures were done every 4–5 weeks.

2.3. Shoot proliferation and root induction

The sterilized shoots were excised further to approximately 0.5–1.0 cm consisting of the apical bud and 2–3 leaf sketches and transferred to MS medium containing 18 different combinations of BAP (1, 1.5, and 2 mg L^{-1}) and GA_3 (0, 0.025, 0.05, 0.1, 0.25, and 0.5 mg L^{-1}). All the media contained 0.2 mg L^{-1} IBA. The developed shoots were subcultured every 4 weeks. The shoots after attaining a length of 2–3 cm were inoculated into the rooting medium. Moreover, ½MS medium containing 10 mL L^{-1} Fe-EDDHA was used for rooting with different combinations of PGRs as follows: 0 mg L^{-1} IBA + 0 mg L^{-1} NAA; 0.25 mg L^{-1} IBA + 0.125 mg L^{-1} NAA; 0.25 mg L^{-1} IBA + 0.25 mg L^{-1} NAA; 0.5 mg L^{-1} IBA + 0.25 mg L^{-1} NAA; 0.5 mg L^{-1} IBA + 0.5 mg L^{-1} NAA; 1 mg L^{-1} IBA + 0.5 mg L^{-1} NAA; 1 mg L^{-1} IBA + 1 mg L^{-1} NAA. The caps of glass jars containing rooted plantlets were opened gradually after 2 weeks to ensure their adaptation to the external environment. Afterward, the plantlets were completely taken out of the jars and the number of roots was counted and then they were transferred to the soil (Figure).

2.4. Statistical analysis

The experiments were set up in a completely randomized block design with three replicates and a minimum of five explants per replication. Analysis of variance was done using the software JMP and means were compared using the least significant difference (LSD) test at 5% level of significance.

3. Results

Positive and noteworthy results for shoot multiplication and root induction were obtained from the in vitro clonal propagation of Myrobalan 29C clone rootstock. The morphology of the shoots was of high quality and no defoliation or callus-like structures were observed (Figure). The statistical analysis showed that there were significant differences among the 18 MS media containing different combinations of PGRs ($P < 0.05$).

According to the results, the average numbers of shoots per explant in 1, 1.5, and 2 mg L^{-1} BAP concentration were 9.0, 7.6, and 9.0, respectively, with no significant difference among the three BAP concentrations (Table 1). The MS medium containing 1 and 2 mg L^{-1} BAP gave the best results for the formation of multiple shoots per explant. The numbers of shoots per explant at 0, 0.025, 0.05, 0.1, 0.25, and 0.5 mg L^{-1} GA_3 were 8.0, 5.4, 11.4, 8.8, 8.1, and 9.4, respectively, which were significantly different among its concentration (Table 2). While comparing different concentrations of GA_3, the maximum shoot number per explant was obtained from 0.05 mg L^{-1} GA_3. The MS medium containing 2 mg L^{-1} BAP and 0.05 mg L^{-1} GA_3 was the best and produced the highest number of shoots per explant. The lowest shoots were recorded in 0.025 mg L^{-1} GA_3 and 1.5 mg L^{-1} BAP (Table 3).

The average length of shoots per explant in 1, 1.5, and 2 mg L^{-1} BAP concentrations was 1.1, 0.9, and 0.7 cm, respectively. BAP at 1.0 mg L^{-1} was best for higher shoot length per explant according to the statistical analysis (Table 1). Furthermore, the average lengths of shoots per explant at 0, 0.025, 0.05, 0.1, 0.25, and 0.5 mg L^{-1} concentrations of GA_3 were 1.3, 0.8, 0.9, 0.6, 0.8, and 0.8 cm, respectively (Table 2). However, the highest shoot length per explant was obtained in the MS medium containing 1.0 mg L^{-1} BAP without GA_3. The lowest shoot length (0.5 cm) was obtained from two combinations, i.e. 1.5 mg L^{-1} BAP plus 0.1 mg L^{-1} GA_3 and 2.0 mg L^{-1} BAP plus 0.05 mg L^{-1} GA_3 (Table 3).

The average percentages of explants forming shoots at 1, 1.5, and 2 mg L^{-1} concentrations of BAP were 75.5, 84.4, and 82.2, respectively (Table 1). Thus, BAP at 1.5 mg L^{-1} proved best for the highest percentage of explants forming shoots. In the case of different concentrations of GA_3, i.e. 0, 0.025, 0.05, 0.1, 0.25, and 0.5 mg L^{-1}, the average forming shoots percentages of plantlets were 82.2, 77.8, 75.5, 86.7, 77.8, and 84.4, respectively (Table 2). Comparing the different concentrations of BAP and GA_3, 0.1 mg L^{-1} GA_3 showed the maximum rate of explants forming shoots.

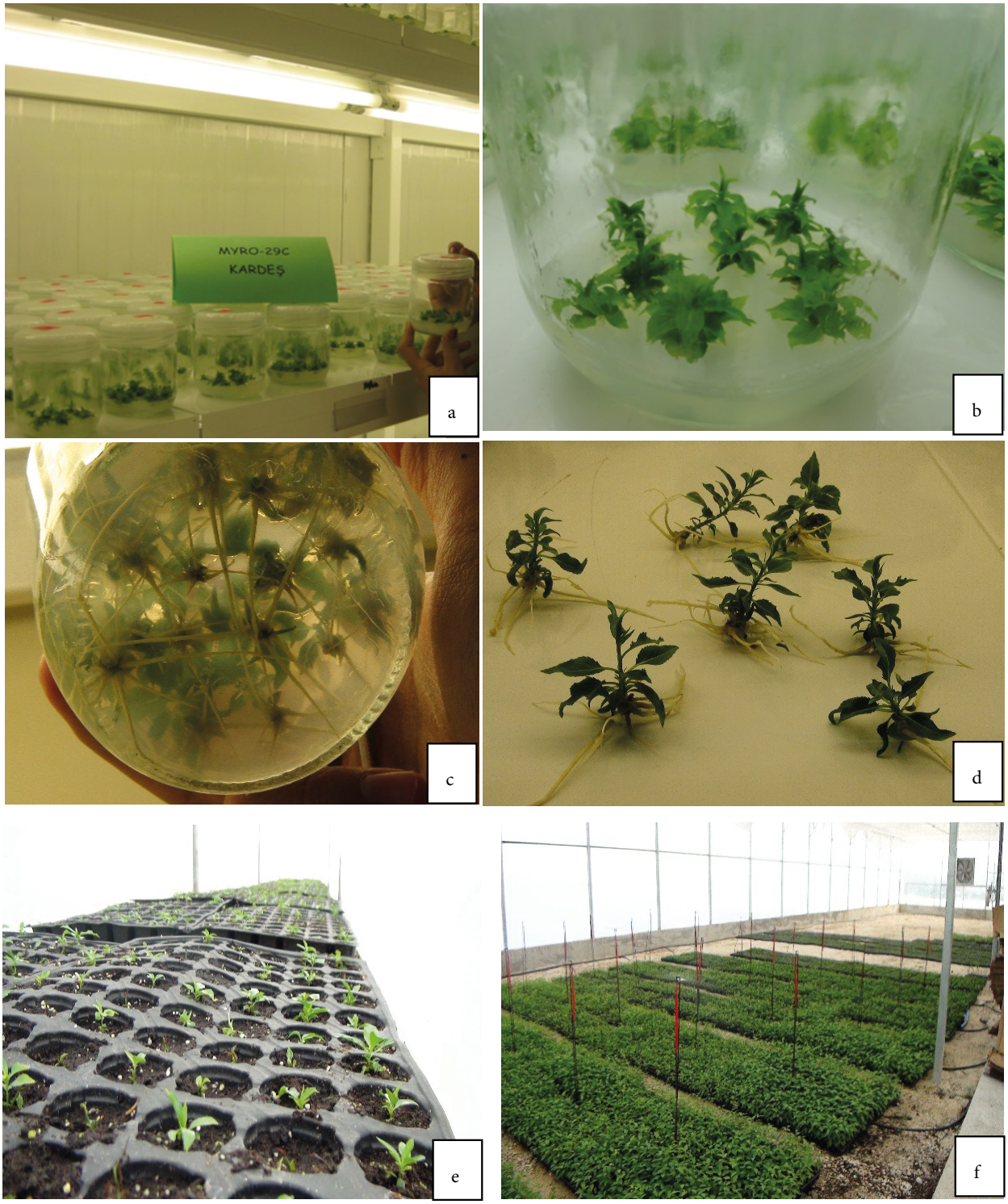

Figure. A view of micropropagation of Myrobalan 29C under in vitro conditions, a, b: shooting, c, d: rooting, e, f: explants transferred to the soil.

However, the combined effect of both the PGRs in MS media was found to be the best. A success rate of 100% was obtained when MS medium was added with 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} GA_3, and 2 mg L^{-1} BAP and 0 mg L^{-1} GA_3 as well as 2 mg L^{-1} BAP and 0.025 mg L^{-1} GA_3 (Table 3).

Table 1. The effect of BAP concentration on shooting in the in vitro propagation of Myro 29C.

BAP concentration (mg L^{-1})	Explants forming shoots (%)	Number of shoots per explant	New shoots length (cm)
1	75.5	9	1.1a
1.5	84.4	7.6	0.9ab
2	82.2	9	0.7b
LSD_{BAP}	N.S.	N.S.	0.18*

LSD. Least significant difference (LSD).
The level of significance for the differences is indicated by * $P < 0.05$ and ** $P < 0.01$.
N.S. There are no significant differences among treatments.

Table 2. The effect of GA_3 concentration on shooting in the in vitro propagation of Myro 29C.

GA_3 concentration (mg L^{-1})	Explants forming shoots (%)	Number of shoots per explant	New shoots length (cm)
0	82.2	8.0bc	1.3a
0.025	77.8	5.4c	0.8b
0.05	75.5	11.4a	0.9b
0.1	86.7	8.8ab	0.6b
0.25	77.8	8.1bc	0.8b
0.5	84.4	9.4ab	0.8b
LSD_{GA3}	N.S.	5.4*	0.09*

LSD. Least significant difference (LSD).
The level of significance for the differences is indicated by * $P < 0.05$ and ** $P < 0.01$.
N.S. There are no significant differences among treatments.

The average root length per explant in the concentrations of 0, 0.25, 0.5, and 1 mg L^{-1} IBA was 1.1, 0.9, and 0.7 cm, respectively. IBA at 1 mg L^{-1} gave the best results for root length. On the other hand, root lengths per plant in different concentrations of NAA (0, 0.125, 0.25, 0.5, and 1 mg L^{-1}) were 6.16, 9.33, 9.80, and 11.50 cm, respectively. Finally, the maximum average root length per explant was obtained from a combination of IBA at 1.0 mg L^{-1} and NAA at 0.5 mg L^{-1} (Table 4).

4. Discussion

In the present study, a stable protocol was developed for the micropropagation of Myrobalan 29C clone rootstock. It was reported earlier that the in vitro propagation of stone fruit (*Prunus*) with high BAP concentrations caused a decrease in shoot multiplication and shoot necrosis, and induced callus formation from the shoot culture (Hu and Wang, 1983; Ahmad et al., 2003). The reason for this effect is that BAP promotes cell division when added to the culture medium (George, 2008). However, the addition of auxins may directly balance the cytokinin levels (Nordstrom et al., 2004). Arıcı (2008) obtained the highest number of Myrobalan 29C rootstock shoots (5.38) in MS medium containing 1 mg L^{-1} BAP and 0.2 mg L^{-1} NAA. Shabani et al. (2015) reported that the most suitable medium for micropropagation of Myrobalan 29C rootstock was MS medium containing 2 mg L^{-1} BAP, producing 5.58 shoots per explants, and 1 mg L^{-1} NAA. Akşehirli (2010) found higher number and length of shoots as well as leaf numbers of Myrobalan 29C rootstock in medium containing 1 mg L^{-1} BAP, 0.5 mg L^{-1} IBA, and 0.25 mg L^{-1} GA_3. Recently, Nasri et al. (2019) also recorded the maximum shoot multiplication of Myrobalan 29C rootstock in culture medium including 1.0 mg L^{-1} BAP and 1 mg L^{-1} IBA. In contrast, it was reported that MS medium containing 2 mg L^{-1} BAP alone resulted in the

Table 3. The effect of PGRs combinations on shooting in the in vitro propagation of Myro 29C.

Treatments (mg L^{-1})	Explants forming shoots (%)	Number of shoots per explant	New shoots length (cm)
1 BAP + 0 GA_3	73.3a–d	6.9d–h	2.0a
1 BAP + 0.025 GA_3	46.6d	9.2b–g	1.0cd
1 BAP + 0.05 GA_3	66.7bcd	8.5b–h	1.7ab
1 BAP + 0.1 GA_3	80.0abc	11.9abc	0.7d
1 BAP + 0.25 GA_3	93.3ab	7.8c–h	0.7d
1 BAP + 0.5 GA_3	93.3ab	9.7a–f	0.7d
1.5 BAP + 0 GA_3	73.3a–d	4.1hi	1.3bc
1.5 BAP + 0.025 GA_3	86.7ab	1.7i	0.7d
1.5 BAP + 0.05 GA_3	80.0abc	11.5a–d	0.7d
1.5 BAP + 0.1 GA_3	100a	8.1c–h	0.5d
1.5 BAP + 0.25 GA_3	86.7ab	11.9abc	1.0cd
1.5 BAP + 0.5 GA_3	80.0abc	8.2c–h	1.0cd
2 BAP + 0 GA_3	100a	13.1ab	0.7d
2 BAP + 0.025 GA_3	100a	5.3f–i	0.7d
2 BAP + 0.05 GA_3	80.0abc	14.3a	0.5d
2 BAP + 0.1 GA_3	80.0abc	6.4e–i	0.7d
2 BAP + 0.25 GA_3	53.3cd	4.5ghi	0.8cd
2 BAP + 0.5 GA_3	80.0abc	10.4a–e	0.7d
$LSD_{BAP*GA3}$	31.225*	4.721**	0.617*

All media also contained 0.2 mg L^{-1} IBA. LSD. Least significant difference (LSD).
The level of significance for the differences is indicated by * $P < 0.05$ and ** $P < 0.01$. Means with the same letter within columns are not significantly different.

Table 4. The effect of PGR combinations on rooting in the in vitro propagation of Myro 29C.

IBA (mg L^{-1})	NAA (mg L^{-1})	Root length (cm)	Root number	Shoot length (cm)
0	0	6.16d	3.66c	2.08
0.25	0.125	9.33c	4.33c	2.75
0.25	0.25	9.41c	5.66b	1.66
0.5	0.25	10.16bc	6.33ab	2.00
0.5	0.5	10.50bc	7.00c	2.00
1	0.5	12.50a	5.66b	2.25
1	1	11.00b	4.33c	2.16
LSD		1.219**	1.146**	N.S.

All media also contained 10 ml L^{-1} Fe-EDDHA.
LSD. Least significant difference (LSD).
The level of significance for the differences is indicated by * $P < 0.05$ and ** $P < 0.01$.
N.S. There are no significant differences among treatments.

best shoot multiplication in *Prunus laurocerasus* L. (cherry laurel) with 6.13 new shoots and 3.26 cm formed shoot lengths (Sulusoglu and Cavusoglu, 2013). However, their results showed that with an increase in BAP concentration to 3 mg L^{-1} BAP a small callus was formed at the base of explants. When further increased to 4 mg L^{-1} BAP, the callus produced adventitious buds. Parallel to the previous studies, our research exhibited the best results from BAP (1, 1.5, and 2 mg L^{-1}), IBA (0.2 mg L^{-1}), and different concentrations of GA_3. There was no necrosis in the shoots or callus formation. The highest explants forming shoot rates in this study were obtained in medium containing 1, 1.5, and 2 mg L^{-1} BAP concentrations. Moreover, the medium containing 2 mg L^{-1} BAP and 0.05 mg L^{-1} GA_3 resulted in increased shoot multiplication. In terms of shoot length, the best medium was MS containing 1.0 mg L^{-1} BAP and 0 mg L^{-1} GA_3.

BAP stimulates shoot formation and development in stone-fruit rootstock micropropagation, whereas IBA is effective in stimulating leaf growth and stem elongation

(Francis and Sorrell, 2001; Himanen et al., 2002; De Veylder et al., 2007). Therefore, the results may prove that the auxin to cytokinin ratio is significant for optimum shoot growth as auxin may influence cell phenotype by suppressing excess cell division by cytokinin. Thus, the effect of nutrient medium and PGRs can manipulate the results of the experiment (McCown and Sellmer, 1987).

Exogenous auxins such as IBA, NAA, and IAA induce in vitro root formation (Thorpe et al., 2008). Plopa et al. (2012) reported that a higher rate of root induction could be obtained by reducing the concentration of MS culture medium to one-half. Silveira (2000) tested four different doses of IBA, IAA, and NAA (0, 0.01, 0.1, and 1.0 µM) for root induction studies in Marianna, Myrobalan, Mr.S 2/5, GF-677, and G × N22 rootstocks. It was found that IBA and IAA were effective in root induction of the mentioned *Prunus* rootstocks, while NAA was not suitable. However, Shabani et al. (2015) obtained good results in terms of root length of Myrobalan 29C rootstock (14.5 cm) when 2 mg L^{-1} NAA was added to MS medium, which was comparable to the present study. In our study, the highest root length was obtained in ½MS medium containing 1 mg L^{-1} IBA, 0.5 mg L^{-1} NAA, and 10 mL L^{-1} Fe-EDDHA. In another study of root induction in Myrobalan 29C rootstock, different doses of NAA and IBA were tested and the best result was achieved in ½MS + 2 mg L^{-1} IBA medium (Akşehirli, 2010). Although a desirable outcome was obtained in different combinations of IBA and NAA, Akşehirli (2010) also mentioned that there were some instances in which in vitro root induction did not occur in Myrobalan 29C rootstocks. It was proved that alteration in the concentration of culture media had a direct effect on the rate of root induction and number of roots per shoot in in vitro clonal propagation of Myrobalan 29C rootstock. The results of several studies have shown that nutrient media containing NAA promote growth and development of roots in *Prunus* species (Ruzic et al., 2000; Lauri et al., 2001). However, the concentration of NAA in the medium is important, since low levels of NAA induced more roots while higher NAA concentration led to callus formation (Hossini et al., 2010; Plopa et al., 2012; Vujovic et al., 2012; Shabani et al., 2015). Moreover, the addition of Fe-EDDHA to the nutrient media gives superlative results in rooting of *Prunus* rootstocks (Antonopoulou et al., 2007).

In the present research, the effect of Fe-EDDHA in root induction was also investigated in Myrobalan 29C rootstock. The maximum root formation was achieved in ½MS medium formulated with 0.5 mg L^{-1} IBA, 0.5 mg L^{-1} NAA, and 10 mL L^{-1} Fe-EDDHA. Özbek et al. (2014) also recorded higher root induction of Myrobalan 29C rootstock in MS medium containing 1.0 mg L^{-1} IBA, 0.05 mg L^{-1} GA_3, and 100 mg L^{-1} Fe-EDDHA. Molassiotis et al. (2003) and Antonopoulou et al. (2007) further confirmed that the addition of Fe-EDDHA to culture medium stimulated rooting in GF-677 (*Prunus amygdalus* × *P. persica*) rootstock. There was no root induction in the medium devoid of Fe-EDTA.

In the present study, a competent in vitro propagation protocol was developed for Myrobalan 29C rootstock. BAP, GA_3, IBA, and NAA in optimal doses showed positive effects on multiple shoot formation, shoot growth, and rooting. There was a great reduction in time, energy, and production cost of micropropagated plantlets and enhancement in the shoots forming. The novelty of this protocol relies on its PGR combinations, which enabled increased multiplication rates in a short time. Direct regeneration reduces the possibility of somaclonal variations. There is a possibility of quick grafting at any time of the year and transplanting the micrografts into the field as and when required. Owing to its many benefits, this technology can be practical and convenient for technicians and researchers. The method has great potential for enhancement of this crop using other biotechnological advances such as genetic transformation studies.

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