

DETERMINATION OF REGENERATION EFFICIENCY IN DIFFERENT APPLE SPECIES AND VARIETIES

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Abstract

Genetic transformation technology emerged as a useful tool to shorten the breeding time in plants and also allowed the improvement of the plants by only intended character without changing their other characters. To carry out gene transfer studies, the regeneration efficiency of plants must be known. In this study, *in vitro* regeneration efficiency was investigated in 9 genotypes in 3 different apple species; *Malus domestica*, *Malus sieversii* and *Malus niedzwetzkyana*. After explants shoot in the media, callus culture was applied to sterile leaves. Genotypes' shoot formation ratio was ranged from 8.33% to 75%. The genotype with the highest shoot was 'genotype 8', species of *M. domestica*, with a rate of 75%. Sterile leaves were taken from shoots for callus culture. The regeneration potential of genotypes ranged from 0% to 90%. While callus formation was not observed in 'genotype 4' and 'genotype 5' (*M. domestica*), 90% callus formation was observed in 'genotype 3' (*M. domestica*). With this study, it has been determined that there is a serious variation in the regeneration efficiency of different apple species and genotypes.

Keywords: Apple, Callus culture, Regeneration efficiency

1. INTRODUCTION

Conventional clonal propagation methods such as grafting and budding are used successfully by many nursery growers. While successful, these methods are labor intensive and may require large amounts of land and slow. As an alternative, successful *in vitro* clonal propagation methods are reported in apple for commercial applications in the tree fruit industry (Bommineni et al., 2001). The tissue culture of domesticated apple has an extensive and rich history spanning approximately sixty years (Dobrąnszki and Teixeira da Silva, 2010). *In vitro* propagation of apple generally includes 4 stages, as in other plants: (1) *in vitro* culture establishment from *in vivo* plants; (2) shoot multiplication and/or regeneration; (3) rooting of microshoots; (4) acclimatization of plantlets and the establishments of plant in an *in vivo* environment. The most frequently used explants in Stage 1 in apple *in vitro* propagation are shoot tips or axillary buds when propagated by axillary or terminal buds, or leaves when propagation is based on adventitious shoots (Teixeira da Silva et al., 2019). Genetic transformation in apple depends largely on specific tissue culture techniques that lead to effective regeneration. First, Liu et al., (1983) described this process with lot of explant types from *in vitro*-grown apple cultures of open-pollinated 'Golden Delicious' seedlings. Then, in addition to studies on apple cultivars, many reports of apple rootstock regeneration and genetic transformation also have been published. Several researchers have used rootstock M.26 for shoot regeneration

experiments, where *in vitro* culture of leaves can regenerate shoots at frequencies close to 100% on a medium based on MS (Murashige and Skoog, 1962) salts, LS vitamins (Linsmaier and Skoog, 1965), containing N6 -benzyladenine (BA), and 1-naphthaleneacetic acid (NAA). They resulted in transformation efficiencies ranging widely from 0.9% to 80.0% (Predieri and Malavasi 1989; Norelli et al. 1994; Holefors et al. 1998). Shoot and leaf tissues from Jork 9 rootstock have also served as explant sources. Its transformation efficiency ratio was 3.3% to 6.5% (Sedira et al., 2001). Protocols have been developed for regeneration of N545 (Dolgov et al. 1999), M.9/29 (Zhu et al. 2001), and 'Balenghaitang' (Qu et al. 2005) apple rootstocks. Thus, regeneration and transformation efficiencies vary significantly with genotype. Identification of the critical factors that affect these processes is crucial (Zhang et al., 2014).

In this study, *in vitro* regeneration efficiency was determined in 9 genotypes in 3 different apple species; *Malus domestica*, *Malus sieversii* and *Malus niedzwetzkyana*.

2. MATERIALS AND METHODS

Plant material and establishment of culture: Nodal explants were collected in autumn from 5-year old mature tree of *Malus domestica*, *Malus sieversii* and *Malus niedzwetzkyana* established in the collection garden of Erciyes University Agricultural Research Center (Turkey). The excised nodal explants ($\pm 1-2$ cm) were washed thoroughly under running tap water for 30 min and then surface-disinfested in 70% (v/v) ethanol for 30 s. Rinsed three times with sterile distilled water. Then, soaked for 8 min in a 0.1% (v/v) HgCl₂ solution. Finally explants rinsed 4 or 5 times in sterile distilled water with duration of 5 min each. four times with sterile distilled water (Dobr anski and Teixeira da Silva 2010). The sterilized single nodal explants were inoculated into test tubes with 15 ml of nutritive solution. The medium contained the mineral salts and vitamins of Murashige and Skoog (1962) supplemented with 6-benzylaminopurine (BAP: 1.0 mg.L⁻¹), indole-3-butyric acid (IBA: 0.1 mg.L⁻¹) for shooting, enriched with 30 g.L⁻¹ sucrose and gelled with agar (8 g.L⁻¹). The pH was adjusted to 5.8 with 1 M NaOH prior to autoclaving at 121°C for 20 min (Boudabous et al., 2010). All explants were cultured at 25°C under a 16-h light photoperiod and 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of light intensity from white light (Amoo et al., 2011).

Shoot multiplication: Shoot tips (1 to 3 cm) obtained from *in vitro* differentiated shoots were transferred to MS medium supplemented with BAP (2.0 mg.L⁻¹) for induction of multiple shoots. The average number of shoots induced per explants as well as the length of the shoots was recorded each 4 weeks.

Shoot regeneration from leaf explants: Subcultured shoots of 4 week old were used as source material for leaf explants. Healthy, fully expanded young leaves were collected from the sub-cultured shoots. Leaf blades were cut transversely across the midrib into three or four pieces, or were wounded by crushing the leaf tissue with forceps (Norelli et al., 1996). Explants were placed with their adaxial surface in contact with the medium (MS supplemented with 7.5 g L⁻¹ agar, 30 g L⁻¹ sucrose, 2.3 μM TDZ, and 0.9 μM NAA) and cultured for 5 wk. Culture conditions included darkness for the first 3 wk followed by 2 wk of light exposure (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). After 3 wk in the dark, a stereomicroscope was used to observe the regeneration events at the time of calli development (Zhang et al., 2014).

3. RESULTS AND DISCUSSIONS

Shoot induction: All genotypes from various *Malus* species (*M. niedzwetzkyana*, *M. sieversii*, *M. domestica*) were performed different rates of shoot development. Genotypes' shoot formation ratio

was ranged from 8.33% to 75%. The genotype with the highest shoot was 'genotype 8', species of *M. domestica*, with a rate of 75%. This species was followed by the *M. niedzwetzkyana* and *M. sieversii* species, respectively for shoot induction with %58.3 ratio. The lowest shoot development was observed in 'genotype 9' and 'genotype 2'. Leaf explants from 30-day-old *in vitro* propagated shoots were used for callus induction. Explants produced calli on media depending on hormone concentrations and combinations. Most of the leaf explants showed callus formation after 15 days, producing milky yellow-greenish callus from the edges of the explants on media containing both auxin and cytokinin (Figure 1-D). The regeneration potential of genotypes ranged from 0% to 90%. Callus formation was not observed in 'genotype 4' and 'genotype 5' (*M. domestica*) but 90% callus formation was observed in 'genotype 3' (*M. domestica*). 'genotype 8' and 'genotype 3' has achieved both high rate of shoot growth and callus formation (Table 1). This genotype can be use in genetic transformation studies in apple because of its regeneration efficiency.

Table 1. Effects of different genotypes on shoot and callus induction ratios

Genotype No	Species	Shoot Induction	Regeneration
		%	%
1	<i>M. niedzwetzkyana</i>	50	66.6
2	<i>M. sieversii</i>	8.33	66.6
3	<i>M. domestica</i>	50	90
4	<i>M. domestica</i>	16.6	-
5	<i>M. domestica</i>	58.3	33.3
6	<i>M. domestica</i>	16.6	58.3
7	<i>M. sieversii</i>	58.3	-
8	<i>M. domestica</i>	75	83.3
9	<i>M. domestica</i>	8.33	66.6



Figure 1. A- Shoot induced from the axillary bud of nodal explant. B-Multipliated shoots. C-Leaf explants from *in vitro* shoots D- Regenerated shoots on MS medium supplemented with 2.7 μ M TDZ and 0.9 μ M NAA

Zhang et al., 2020, aimed to investigate the influence of different concentrations of hormones on wild apple regeneration from leaf and stem explants to establish an optimal regeneration system in their study; An efficient *in vitro* regeneration system from different wild apple (*Malus sieversii*) explants. Leaves and stems derived from seedlings were cultured on several media supplemented with various concentrations of TDZ or BA in different combinations with NAA. The results showed that the most efficient shoot formation media (35% and 90%) were MS medium containing 4.0 mg L⁻¹ TDZ and 1.0 mg L⁻¹ NAA for leaf explants and MS medium containing 1.0 mg L⁻¹ BA without NAA for stem explant. MS medium supplemented with 0.4 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA (for shoot multiplication) and 1/2 MS+0.1 mg L⁻¹ NAA+1.5% sucrose (for rooting) were effective media. Shoot regeneration from leaf explants was the most effective when the explants were placed abaxial side down onto the medium and were subjected to a pre-treatment of 3 weeks in darkness. The genetic diversity and inheritance of BRR and NAB were evaluated using 153 *Malus* germplasm accessions and 78 hybrid trees of Jonathan × Golden Delicious. *Malus sieversii* 31, Liberty, and Smoothie exhibited significantly high BRR (98.33%, 98.33%, and 93.33%, respectively) and a large NAB without vitrification. Bud regeneration rate (BRR) and the number of adventitious buds (NAB) linearly correlated with each other but not with callus formation rate. The broad sense heritability of the regeneration rate was 92.16%. The three *Malus* accessions that had high regeneration ability, and some of their sexual descendants, might be outstanding genetic resources for future genetic transformation (Tan et al., 2017).

Zhang et al., 2014 determined apple (*Malus domestica*) rootstock G.41 is an excellent member of the Geneva series because it has traits for resistance to abiotic and biotic stresses. A simple and efficient protocol for obtaining shoots from leaf explants was established by optimizing the combinations of plant growth regulators, mode of wounding, and explant orientation on the culture medium. The best shoot multiplication index (2.58) was obtained from successful subculture medium that was the standard Murashige and Skoog (MS) medium supplemented with 7.5 g L⁻¹ agar, 3.55 μM N6 -benzyladenine, 0.16 μM indole-3-butyric acid, and 30 g L⁻¹ sucrose. Regeneration rates were highest (99%) when MS medium was supplemented with 2.7 μM thidiazuron and 0.9 μM 1-naphthaleneacetic acid, and cut-wounding explants before placing the abaxial surface in contact with the medium.

A high frequency of sprouting (85.0%) and shoot differentiation is observed in the primary cultures of nodal explants of *Malus domestica* L. cultivar Douce de Djerba on MS medium supplemented with BAP (1.0 mg.L⁻¹) plus IBA (0.1 mg.L⁻¹). *In vitro* proliferated shoots are multiplied rapidly by culture of shoot tips on MS medium with BAP (1.0 and 2.0 mg.L⁻¹) which produce the greatest multiple shoot formation. The BAP has a positive effect on the multiplication and growth, but a concentration that exceeds 4.0 mg.L⁻¹ decreases the growth (Boudabous et al. 2010).

4. CONCLUSIONS

Determination of regeneration efficiency in plants is very important because of reference to gene transfer studies. As seen in this study, the regeneration activities of different apple species and varieties are different from each other. The use of prominent genotypes in terms of regeneration efficiency to be conducted for obtaining transgenic plants will contribute positively to the studies.

5. REFERENCES

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