An In Vitro Germination Technique for Some Stone Fruit Species: the Embryo Isolated from Cotyledons Successfully Germinated without Cold Pre-treatment of Seeds

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Abstract. Seeds of temperate fruit species need a long time to germinate as a result of their requirement of stratification or cold treatment. Therefore, fast and uniform germination techniques are desirable and important for fruit tree propagation and especially for breeding studies. The effects of combinations of benzylaminopurine (BAP) and gibberellic acid (GA₃) on in vitro embryo germination of apricot, peach, and wild cherry were determined without seed cold pretreatment. The results showed that no germination was recorded in all the seeds with testa. In the seeds without testa, no germination (wild cherry) or limited germination (less than 10%) was recorded. When the embryos separated from cotyledons were cultured, successful germination was obtained for all species. In general, the addition of different combinations of BAP and GA₃ into the Murashige and Skoog (MS) medium significantly increased the germination ratios of embryos without cotyledons in all species. For wild cherry, the best treatment (66.7% germination) was MS media containing 0.5 mg·L⁻¹ BAP + 2.0 mg·L⁻¹ GA₃ or 0.5 mg·L⁻¹ BAP + 4.0 mg·L⁻¹ GA₃. For peach, the best treatment (86.7% germination) was MS medium supplemented with 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃. For apricot, the best treatment (93.3%) was MS media containing 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃, 1.0 mg·L⁻¹ BAP + 1.0 mg·L⁻¹ GA₃, or 1.0 mg·L⁻¹ BAP + 2.0 mg·L⁻¹ GA₃.

Seeds of temperate fruit species do not germinate as a result of seed dormancy even if conditions such as water, temperature, and oxygen are suitable for germination. Seed dormancy is classified as physiological, morphological, morpho-physiological, physical, and combinational dormancy (physical and physiological) (Baskin and Baskin, 1997, 2004; Finch-Savage and Leubner-Metzger, 2006). These mechanisms of dormancy are present in the seeds of all temperate fruit species, including peach, cherry, and apricot (Han et al., 2002; Martinez-Gomez and Dicenta, 2001). On the other hand, some chemicals such as abscissic acid (ABA), coumarin, and jasmonates could also impose seed dormancy (Bewley, 1997; Linkies and Leubner-Metzger, 2012). Moreover, other substances such as phenolics could prevent seed germination (Yukiko et al., 2001). The germination inhibitors exist at different concentrations in various parts of a seed, including pericarp, seedcoat, cotyledons, and embryo. The proportion of inhibitors could be decreased by removing either one or several

parts of the seeds and thus germination percentage could be increased (San and Yildirim, 2009). Generally, these germination problems in temperate fruit species are successfully overcome by cold stratification of seeds for several months during winter. However, seeds of temperate fruit species require a long time to germinate under traditional stratification or natural conditions. Therefore, fast and uniform germination techniques are desirable for fruit-growing and breeding studies (Arbeloa et al., 2009; Bridgen, 1994). For this purpose, some growth regulators such as GA₃ and cytokinin as antagonists of ABA (Nicolas et al., 1996) are used to induce the germination of seeds in some species such as Arabidopsis, caper, and hazelnut (Aygün et al., 2009; Debeaujon and Koornneef, 2000; Soyler and Khawar, 2007). Additionally, to overcome seed dormancy, in vitro germination was also successfully used in strawberry (Miller et al., 1992), *Citrus* (Hassanein and Azooz, 2003), walnut (Kaur et al., 2006), and almond (San and Yildirim, 2009). Germination of immature embryos has been successfully accomplished in some stone fruits including cherry (Hormaza, 1999), apricot (Ning et al., 2007), peach, almond, and peach × almond hybrids (Ledbetter et al., 1998). However, few studies have been performed about in vitro germination of embryos isolated

from cotyledons until now (Arbeloa et al., 2009; San and Yildirim, 2009), although there are some studies on in vitro germination of whole embryo.

The objective of the present study was to determine the effects of isolating embryo from cotyledons and the combination of BAP (0, 0.5, and 1.0 mg·L⁻¹) with GA₃ (0, 1.0, 2.0, 3.0, 4.0 mg·L⁻¹) on the in vitro germination of apricot, peach, and wild cherry without cold treatment.

Materials and Methods

Seeds from apricot (*Prunus armeniaca* L. cv. Alyanak), peach (*Prunus persica* L. cv. Redhaven), and wild cherry (synonym: bird cherry) (*Prunus avium* L.) fruits collected in the same year were used as material in the study. The maturation time of apricot cultivar Alyanak, a midseason cultivar (Uslu et al., 1996), is 15 to 25 June in the Isparta region of Turkey. The maturation time of peach cultivar Redhaven, a midseason cultivar, is 1 to 5 Aug. in the Isparta region of Turkey (Akgül et al., 2005). Wild cherry fruits were harvested from a tree in the first week of July in Isparta region of Turkey. Seeds isolated from mature fruits were dried at room temperature for 30 d before they were used. After cracking the shells by hand, the seeds were removed from the shells (Fig. 1A–B) and pre-sterilized by immersion into 3.75% (v/v) sodium hypochlorite solution containing two to three drops of Tween 20 (Merck KGaA, Darmstadt, Germany) for 25 min followed by rinsing three times for 5 min in sterile distilled water. Then, sterilized seeds were incubated in sterile distilled water for 60 h for easy removal of testa by replacing the water every ≈12 h. Turgid seeds were sterilized again as described previously (San and Yildirim, 2009). After the testa (seedcoat) was removed, seeds with/without testa were cultured in petri dishes (100 × 10 mm) on MS medium (Murashige and Skoog, 1962) containing no plant growth regulators. Three percent (w/v) sucrose (Merck KGaA) and 0.7% (w/v) agar (Oxoid; Thermo Fisher Scientific Inc.) were added to the MS medium. After pH was adjusted to 5.7 with potassium hydroxide (KOH) and hydrochloric acid (HCl), the medium was autoclaved at 121 °C for 15 min and distributed to the petri dishes under sterile conditions. Because the whole embryos (seeds) with/without testa (Fig. 1B–C) did not germinate, embryos isolated from cotyledons were cultured on MS medium containing different combination of plant growth regulators (Fig. 1D–E).

To determine the best combination of plant growth regulators for in vitro embryo germination, the embryos were carefully excised from the cotyledons under sterile conditions and were cultured on MS medium supplemented with BAP (Merck KGaA) and GA₃ (Merck KGaA) in the quantities as shown in Table 1. Three percent (w/v) sucrose (Merck KGaA) and 0.7% (w/v) agar (Oxoid; Thermo Fisher Scientific Inc.) were added into the MS medium. After the pH was adjusted to 5.7 with

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KOH and HCl, the medium was autoclaved at 121 °C for 15 min and distributed to the petri dishes under sterile conditions. Filter-sterilized GA₃ doses were added to media cooled to ≈50 °C after autoclaving.

Cultures were incubated in a growth chamber at 25 ± 1 °C under a 16-h photoperiod provided by cool white fluorescent lamps (140 to 150 μmol·m⁻²·s⁻¹) for 3 weeks in both the whole embryos with/without testa and embryos isolated from cotyledons. The germination percentages were recorded at the end of 3 weeks. Each treatment consisted of three replications and each replication contained four petri dishes with five explants per petri dish.

Data were subjected to analysis of variance using Minitab software (MINITAB Inc.). Duncan's multiple range test was applied to determine significant differences between the means ($P \leq 0.05$). An arcsin transformation was performed to stabilize the variance of percent germination values and angle values were used in the analysis of variance. The real values obtained from the research were presented in the Table 1.

Results and Discussion

Table 1 shows the germination percentages of seeds and isolated embryos obtained from the MS media containing different concentrations of BAP and GA₃ in apricot, peach, and wild cherry species. Seeds with testa did not germinate on the MS medium containing no growth regulators in all species studied (Table 1; Fig. 2A). No germination was recorded in the seeds with testa. Likewise, in the seeds without testa, either no germination (wild cherry) or limited (less than 10%) germination was shown (Table 1; Fig. 2B). The isolation of the embryo from testa did not significantly increase the germination ratios of the stated species. This might be the result of the presence of inhibitors in the cotyledons and/or testa (Martinez-Gomez and Dicenta, 2001; Mehanna and Martin, 1985; San and Yildirim, 2009). However, in all species, germination ratios significantly increased when embryos isolated from cotyledons were cultured on the MS medium containing no plant growth regulator.

These results highlighted that cotyledons may contain some germination inhibitors such as ABA. San and Yildirim (2009) reported that almond embryo with cotyledons did not germinate because of presence of inhibitors in cotyledons even if BAP and GA₃ were added to the MS medium. Although germination ratios increased with the isolation of embryo from cotyledons, results were not satisfactory in the present study possibly as a result of the presence of inhibitors in the embryo (Bewley, 1997; Linkies and Leubner-Metzger, 2012). Therefore, we cultured the embryos (without cotyledons) of apricot, peach, and wild cherry on MS medium fortified with different combinations of growth regulators. Addition of different combinations of BAP and GA₃ into the MS medium significantly increased the germination ratios of embryos of the species

Fig. 1. Germination stages. (A) An apricot seed with shell; (B) an apricot seed isolated from shell; (C) a whole embryo isolated from shell and testa; (D) the separation of cotyledons from embryo; (E) embryos without cotyledons cultured on Murashige and Skoog medium.

Table 1. The effects of some combinations of benzylaminopurine (BAP) and gibberellic acid (GA₃) on the embryo germination in apricot, peach, and wild cherry species (%).

Treatments	Apricot	Peach	Wild cherry	Avg
Seed germination				
Seed with testa on the MS medium ²	0	0	0	0
Seed without testa on the MS medium ²	5.0	10.0	0	5.0
Average	2.5	5.0	0	
Embryo germination (without cotyledons)				
MS medium (control) ²	53.3 b ^y	53.3 b	33.3 c	46.6
MS medium + 0.5 mg·L ⁻¹ BAP + 1.0 mg·L ⁻¹ GA ₃	80.0 a	53.3 b	40.0 bc	57.8
MS medium + 0.5 mg·L ⁻¹ BAP + 2.0 mg·L ⁻¹ GA ₃	80.0 a	73.3 ab	66.7 a	73.3
MS medium + 0.5 mg·L ⁻¹ BAP + 3.0 mg·L ⁻¹ GA ₃	93.3 a	86.7 a	60.0 ab	80.0
MS medium + 0.5 mg·L ⁻¹ BAP + 4.0 mg·L ⁻¹ GA ₃	86.7 a	80.0 a	66.7 a	77.8
MS medium + 1.0 mg·L ⁻¹ BAP + 1.0 mg·L ⁻¹ GA ₃	93.3 a	66.7 ab	53.3 abc	71.1
MS medium + 1.0 mg·L ⁻¹ BAP + 2.0 mg·L ⁻¹ GA ₃	93.3 a	53.3 b	46.7 abc	64.4
MS medium + 1.0 mg·L ⁻¹ BAP + 3.0 mg·L ⁻¹ GA ₃	86.7 a	40.0 b	46.7 abc	57.8
MS medium + 1.0 mg·L ⁻¹ BAP + 4.0 mg·L ⁻¹ GA ₃	73.3 ab	53.3 b	50.0 abc	58.9
Average	82.2	62.2	51.5	

²MS medium without plant growth regulator was used for the in vitro germination.

^yDifferent letters indicate significantly different means at the level of $P \leq 0.05$ in the same column.

MS = Murashige and Skoog.

Fig. 2. In vitro seed and embryo germination in some stone fruits; (A) seed with seedcoat did not germinate on Murashige and Skoog (MS) medium; (B) seed without seedcoat did not germinate on MS medium; (C) apricot embryos (without cotyledon) successfully germinated on MS medium containing 0.5 mg·L⁻¹ benzylaminopurine (BAP) + 3.0 mg·L⁻¹ gibberellic acid (GA₃); (D) peach embryos (without cotyledon) successfully germinated on MS medium containing 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃; (E) wild cherry embryos (without cotyledon) successfully germinated on MS medium containing 0.5 mg·L⁻¹ BAP + 2.0 mg·L⁻¹ GA₃.

(Table 1; Fig. 2C–E). The highest germination percentage was 93.3% on the MS media containing 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃, 1.0 mg·L⁻¹ BAP + 1.0 mg·L⁻¹ GA₃, or 1.0 mg·L⁻¹ BAP + 2.0 mg·L⁻¹ GA₃ in apricot. However, the differences between the germination percentages obtained from MS media containing all combinations of BAP and GA₃ were not statistically significant. Germination ratios of peach embryos increased to 86.7% when embryos were cultured on MS medium

supplemented with 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃. The MS media containing 0.5 mg·L⁻¹ BAP + 2.0 mg·L⁻¹ GA₃ or 0.5 mg·L⁻¹ BAP + 4.0 mg·L⁻¹ GA₃ gave the highest germination percentage in wild cherry (66.7%). The positive effects of BAP and GA₃ on the embryo germination might be the result of their antagonistic effects to certain inhibitors such as ABA (Aygun et al., 2009; Debeaujon and Koornneef, 2000; Linkies and Leubner-Metzger, 2012).

Seeds with cotyledons and/or testa of apricot, peach, and wild cherry did not germinate on the MS medium without cold treatment or stratification. However, seeds could be germinated successfully without cold treatment through isolation of embryos from cotyledons. In this way, the time required for obtaining plants from seeds could be shortened. For successful *in vitro* embryo germination, the MS medium should be fortified with 0.5 mg·L⁻¹ BAP + 3.0 mg·L⁻¹ GA₃ in apricot, peach, and wild cherry.

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