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Original Research Article

Embryo Rescue Efficiency Affected by Developmental Stages of Embryo and Medium Composition in Early-Ripening Peach (*Prunus persica*)

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Abstract - Embryos of early-ripening peaches could not achieve physiological maturation or undergo abortion before harvest. Embryo rescue is an effective strategy to rescue embryos from early-ripening peaches. Thus, the current study was carried out to determine the appropriate developmental stage and optimal medium composition for embryo rescue in early-ripening peach. Development of open-pollinated 'Yumi' fruit was investigated from 20 to 90 days after full bloom (DAFB) to explore period occurring endocarp hardening. After endocarp hardening, embryo development was observed by light microscopes. Shoot and root meristems were observed at 65 DAFB and embryo size rapidly increased at 75 DAFB. Embryos collected at 75, 80, 85, and 90 DAFB were cultured on four media based on Driver and Kuniyuki (DKW) medium. Germination rate of embryos cultured on four media gradually increased from 75 to 90 DAFB and reached 100% at 90 DAFB. Notably, M3 medium (0.5 DKW supplemented with 6-benzylaminopurine (BAP) 1.0 ㎎/L) displayed the highest germination rate at 75 and 80 DAFB stages. Growth and development of shoot and root were pronounced in plantlet cultured at 90 DAFB stage. While delayed shoot growth was evident in plantlets cultured at 75, 80, and 85 DAFB stages, this retardation could be overcome through the application of growth regulators, particularly in M3 and M4 (0.5 DKW supplemented with BAP 1.0 ㎎/L and indole-3-butyric acid 0.5 ㎎/L) media. Remarkably, roots of plantlet grown in M4 medium exhibited limited elongation. In conclusion, germination rate of embryo and growth of embryo cultured plantlet can be enhanced by collecting seeds from early-ripening 'Yumi' at the 90 DAFB stage and conducting embryo culture using the M3 medium.

Key words – Breeding, DKW medium, Immature seed, Plant growth regulator, Tissue culture

Introduction

Peach (*Prunus persica*), a prominent deciduous fruit trees, undergoes intensive harvesting during the summer season in South Korea. The peach harvest coincides frequently with rainy and typhoon seasons. Early or extra-early ripening peaches have the potential to make a substantial contribution to the orchard economy during the off-season. It has been confirmed that the fruit maturation date of peaches exhibits relatively high heritability (Dirlewanger *et al*., 2012; Hansche *et al*., 1972; Hansche, 1986; de Souza *et al*., 1998). Therefore, for the purpose of breeding early or extra-early ripening peaches, the parental selections should consist of early-ripening peaches.

The growth and development of peach fruit exhibits a double sigmoid pattern and can be divided into three stages (Chalmers and van den Ende, 1975). The initial phase, Stage 1 (S1), encompasses a period characterized by rapid growth. During S1, fruit expansion is accelerated by concurrent processes of cell division and expansion. Subsequent to S1, the fruit transitions into Stage 2 (S2), a phase marked by slower growth. In this stage, seed maturation takes place, accompanied by endocarp hardening, embryo maturation, and endosperm enlargement (Dardick *et al*., 2010). Following

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S2, the fruit enters the second rapid growth phase, Stage 3 (S3), characterized by fruit maturation. This phase manifests notable changes, including fruit skin pigmentation, flesh softening, and an increase in sweetness. Notably, the S2 period in early ripening peaches is comparatively shorter than in mid- and late- ripening peaches. Consequently, seeds of early-ripening peaches do not attain physiological maturity until the harvest period. This characteristic presents a challenge in establishing cross population through seed germination.

Embryo rescue is a technique employed to facilitate the propagation of immature embryos, utilizing tissue culture method (Shen *et al*., 2011). This method has been used to cultivate valuable genetic resources of fruit trees, such as apples (Druart, 2000), grapes (Li *et al*., 2014), and peaches (Devi *et al*., 2017), as well as various herbaceous plant species. Because the short S2 period in early-ripening peaches, embryos may not attain physiological maturity or may undergo abortion, studies have been conducted on the acquisition of hybrid seedlings through embryo rescue.

Early investigations were undertaken to examine the media components that could enhance the germination rate and embryo growth (Ledbetter *et al*., 1998; Pinto *et al*., 1994; Sinclair and Byrne, 2003). Sinclair and Byrne (2003) performed embryo rescue using Woody Plant Medium (WPM) and various carbohydrates and suggested that suitable carbohydrates differ depending on embryo size. In addition, 90% germination rate was achieved in *Prunus* species, including peach, plum (*P. salicina*), and apricot (*P. armeniaca*), using 0.5 Murashige and Skoog (MS) medium supplemented with 4.0 ㎎/L 6-benzylaminopurine (BAP) and 0.5 ㎎/L indole-3-butyric acid (IBA) (Liu *et al*., 2007). However, the embryo germination also affected by embryo developmental stage. The germination rate of low chill peach hybrid embryos was observed to increase when embryos were collected at 85 days after crossing (Devi *et al*., 2017). Moreover, according to Sallom *et al*. (2021), it was proposed that plum × apricot embryos harvested at 65 days after pollination exhibited the highest germination rate when cultured on completed MS medium supplemented with 0.5 ㎎/L BAP and 1.0 ㎎/L IBA.

Although diverse studies for embryo rescue have been conducted in peach, the medium components for peach embryos rescue varied across the studies. In addition, the establishment of an embryo rescue process for Korean peaches is imperative. Therefore, we investigated to determine the optimal developmental stage and medium components for embryo rescue, employing the early-ripening peach cultivar ‘Yumi’ (*P. persica*).

Materials and Methods

Plant materials and investigation of fruit developmental stages

The early-ripening ‘Yumi’, mid-ripening ‘Mishong’, and late-ripening ‘Sumee’ maintained in the orchard of the National Institute of Horticultural and Herbal Science, Rural Development Administration, in Wanju, Korea (35°50'2.7"N, 127°1.8'47.3"E) were used for experiment.

The fruit developmental stage of open-pollinated ‘Yumi’ was investigated by measuring the longitudinal and horizontal lengths using a digital vernier caliper (CD-15CP, Mitutoyo, Tokyo, Japan) at 5-day intervals from 20 to 90 days after full bloom (DAFB).

Microscopy

Seeds of open-pollinated ‘Yumi’ collected at 5-day intervals from 50 to 90 DAFB and seeds of open-pollinated ‘Mishong’ and ‘Sumee’ collected at 65 DAFB. The seed samples were fixed in 2.5% glutaraldehyde (v/v in a 0.1 M phosphate) at pH 7.2 in the 4% sucrose (w/v) for 24 h. After three 30 min rinses with the aforementioned buffer, they were dehydrated in an alcohol series and embedded in historesin. Semi-thin sections (3.5 ㎛) prepared by an ultra-microtome were collected on glass slides, and the Periodic Acid-Schiff (PAS) polysaccharide-specific reaction was conducted. A PAS reaction was indicated by a red color. For staining, sections were initially immersed in 1% periodic acid (w/v) for 30 min, followed by treatment with Schiff’s reagent for 40 min, and finally exposed to 5% sodium bisulfate (w/v) for 35 min. The sections were than rinsed in distilled water, dried on a warm plate, and mounted in Histomount. A negative control was performed by omitting the oxidation step with periodic acid, and observed using a light microscope (Axioscop2, Carl Zeiss, Oberkochen, Germany).

Embryo culture

Approximately 200 seeds of open-pollinated 'Yumi' collected at 75, 80, 85, and 90 DAFB and surface vacuum sterilized using a vacuum pump (G-50DA, ULVAC-KIKO, Saito, Japan) before embryo culture. The collected seeds were washed three times with tap water and subjected to vacuum sterilization with 70% ethanol for 1 min. Following this, the seeds underwent three additional washes with sterilized water. Subsequently, the seeds were vacuum sterilized with 1% NaOCl for 4 min and then transferred to a sterilized clean bench. The 1% NaOCl solution was discarded, and the seeds were rinsed three times with sterilized water.

The surface-sterilized seeds were placed on filter paper, and the seed coat was removed. For seeds collected at 75 DAFB, the seed coat was not removed, thus sterilized seeds were directly used for embryo culture. Embryos were cultured in four media based on Driver and Kuniyuki (DKW) medium (Driver and Kuniyuki, 1984) containing 2% glucose and 0.8% agarose. These four media were designated as M1 (1DKW), M2 (0.5 DKW), M3 (0.5 DKW supplemented with BAP 1.0 ㎎/L), and M4 (0.5 DKW supplemented with BAP 1.0 ㎎/L and IBA 0.5 ㎎/L). Because previous studies used MS or WPM media for peach embryo culture, we used DKW medium to investigate the effect of mineral-supplemented media on embryo culture. Moreover, the M3 and M4 media were established to confirm the effect of cytokinin and auxin on embryo germination. The *in vitro* embryos were maintained in a culture room with a 16 h light period and a photosynthetic photon flux density (PPFD) of 100 ㎛ol/㎡/s.

Germination and growth in *in vitro*

The germination of embryos was assessed one weeks after embryo culture. Due to a limited quantity of fruits obtainable from three 'Yumi' trees, 35 replicates were established for the study. Embryos with a radicle protruding over 1 ㎜ were considered germinated. The germination rate was determined by calculating the percentage of germinated embryos relative to the total number of embryos. Plantlet development was compared at 2 weeks after cultivation.

Results

Development of open-pollinated 'Yumi' fruits

The fruit size of open-pollinated 'Yumi' exhibited a progressive increase from 20 to 90 DAFB and the development of open-pollinated 'Yumi' fruits showed double sigmoid pattern with short S2 period (Fig. 1). The endocarp of open-pollinated 'Yumi' fruits was begun to harden at 55

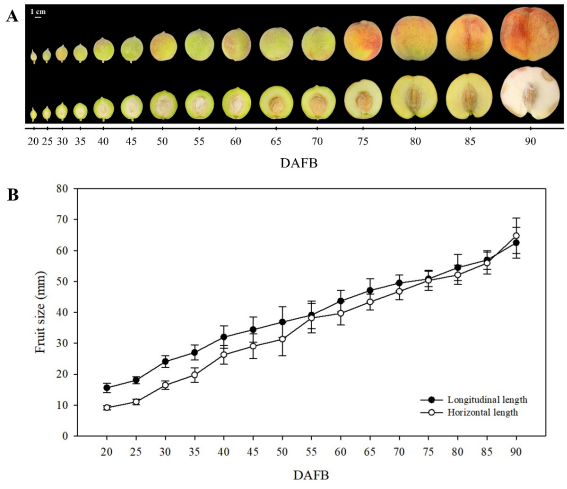

Fig. 1. Changes of fruit development of open-pollinated 'Yumi' from 20 to 90 days after full bloom (DAFB) with 5-days interval. A, Fruit appearance; B, Fruit size.

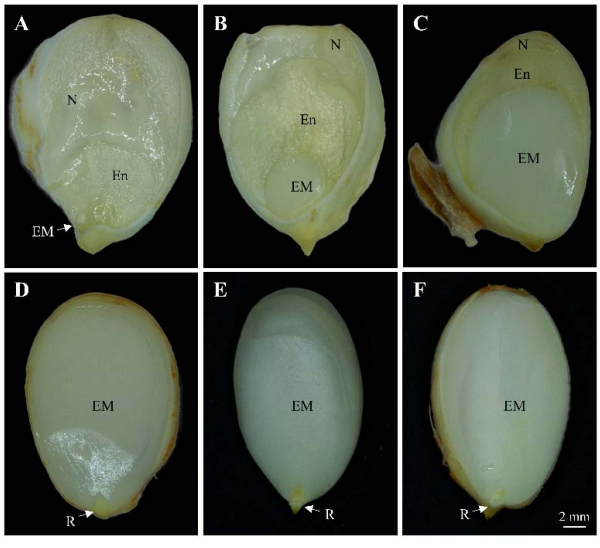

Fig. 2. Changes of embryo development in open-pollinated 'Yumi' seeds at 65 (A), 70 (B), 75 (C), 80 (D), 85 (E), and 90 days after full bloom (F). N, nucellus; En, endosperm; EM, embryo; R, radicle.

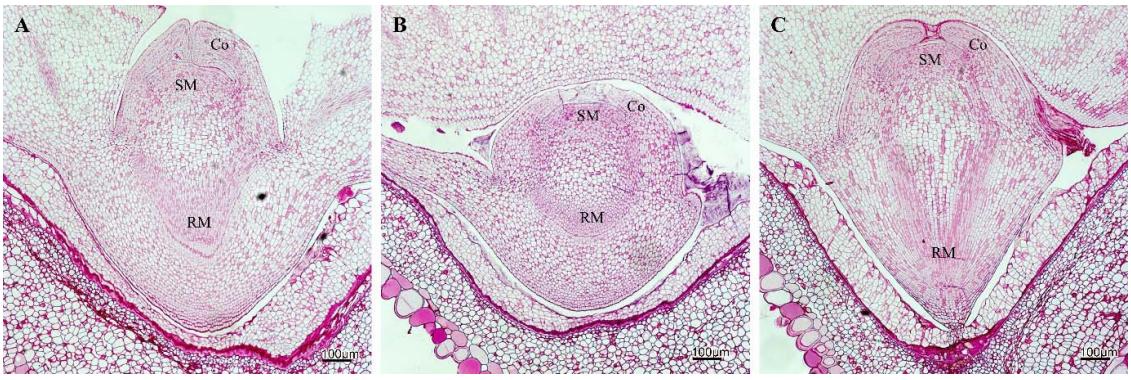

Fig. 3. Comparison of embryo development in early-, mid-, and late-ripening peaches at 65 days after full bloom. A, early-ripening peach 'Yumi'; B, mid-ripening peach 'Mishong'; C, late-ripening peach 'Sumee'. Co, cotyledon; SM, shoot meristem; RM, root meristem.

DAFB and the fruit size did not change during 55 to 60 DAFB. Flesh rapidly softened after 85 DAFB and reached physiological maturation at 90 DAFB.

Development of embryos of early-, mid-, and late-ripening peaches

Clear embryos of open-pollinated 'Yumi' developed during 50 to 60 DAFB, and clear endosperm and nucellus were also observed. At 65 DAFB, diminutive embryos were detected (Fig. 2A) and the embryos gradually enlarged. This embryo enlargement phase was accompanied by a reduction in both endosperm and nucellus, ultimately resulting in the embryos occupying the entire seed area with the exception of the seed coat from 80 to 90 DAFB.

At 65 DAFB, the embryos of open-pollinated 'Yumi' exhibited the development of cotyledon and meristems (Fig. 3A). Similarly, mid-ripening 'Mishong' and late-ripening 'Sumee' also displayed the development of cotyledon and meristems at the corresponding stage of 65 DAFB (Fig. 3B, C)

Germination rate according to the developmental stages of embryo and medium components

The germination rate of open-pollinated 'Yumi' increased followed with embryo development across four media and reached 100% at 90 DAFB (Table 1). At 85 DAFB, embryos cultured in M1, M2, and M3 showed 100% germination rate, while 85.7% embryos were germinated in M4. Although, germination rate was lower at 75 and 80 DAFB compared to 85 and 90 DAFB, embryos cultured in M3 displayed the highest germination rate among the four media.

Table 1. Germination rate of open-pollinated 'Yumi' according to the medium components and developmental stages of embryo (n=35).

Medium	Germination rate (%)			
	75 DAFB[z]	80 DAFB	85 DAFB	90 DAFB
M1	11.4	42.9	100	100
M2	11.4	68.6	100	100
M3	28.6	97.1	100	100
M4	8.6	65.7	85.7	100

[z]Days after full bloom.

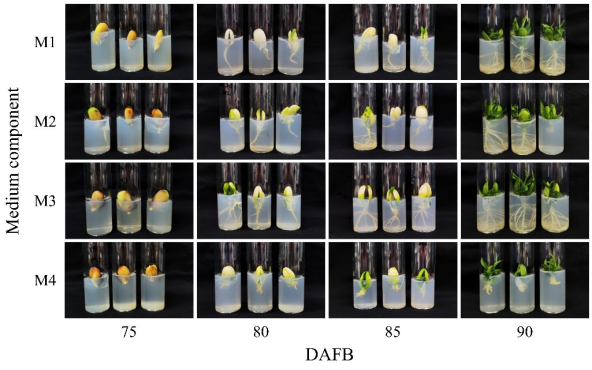

Fig. 4. Embryo rescued plantlets of open-pollinated 'Yumi' grown on four media. Plantlets from embryos collected at 75 days after full bloom (DAFB) were observed at 3 weeks after embryo culture. Plantlets from embryos collected at 80, 85, and 90 DAFB were observed at 2 weeks after embryo culture.

Two weeks after cultivation, embryos collected at 90 DAFB developed plantlets with shoot and roots (Fig. 4). The shoot and root growth of plantlets derived from embryos

collected at 75, 80, and 85 DAFB displayed a comparatively slower growth than the plantlets derived from embryos collected at 90 DAFB. Notably, in media M3 and M4, shoot growth was accelerated. In the M3, rootlet development was observed from 80 DAFB. Embryos grown in M4 manifested the development of a short main root and rootlets throughout the entire embryo developmental periods.

Discussion

Identification of the optimal period for embryo culture requires comprehensive understanding of embryo development process. The growth rate of peach embryos unaffected by the harvest time, while the physiological maturation of the embryo advances during the endocarp hardening period, corresponding to the S2 (Dardick *et al*., 2010). Thus, fruit growth stages of the early-ripening 'Yumi' were investigated to identify S2 and embryo developments were observed.

The duration of S2 is depended on cultivars, and early-ripening peaches has shorter S2 period than mid- and late-ripening peaches (Bonghi *et al*., 2011). The S2 of early-ripening 'Yumi' was determined to span from 55 to 60 DAFB (Fig. 1) and this significantly shorter than the late-ripening 'Fantasia', which manifested 32-days of S2 (Bonghi *et al*., 2011). During S2, transparent embryos were observed, and approximately 1 ㎜ size of embryos were observed after S2 (Fig. 2A). The embryos had enlarged while endosperm and nucellus areas were gradually reduced (Fig. 2B, C). This might be influenced by nutrient absorption of embryos from the endosperm (Fukuda *et al*., 2006; Van Dongen *et al*., 2003). This implies that the embryos of early-ripening peach undergo maturation until S3. The embryos occupied whole seed area at 80 DAFB and displayed non-albuminous seed form (Fig. 2D, E, F). In the embryos at 65 DAFB of early-, mid-, and late-ripening peaches, cotyledon and meristem tissues were observed. This observation suggests a uniformity in the developmental process of embryos among peach cultivars, irrespective of harvest time. Furthermore, embryo enlargement after 65 DAFB was thought to be caused by cotyledon development.

Embryo rescue was performed using seeds collected from 75 to 90 DAFB and four media. Germination started at 3 weeks after cultivation in the seeds collected at 75 DAFB stage, whereas embryos germinated within 3 to 7 days in the embryos collected at 80, 85, and 80 DAFB stages. It was thought that embryos at the 75 DAFB stage might require additional time for maturation before initiating germination. The presence of observed endosperm at 75 DAFB stage (Fig. 2C) supports this. Pérez-Jiménez *et al*. (2021) suggested that seed coat did not influence germination in embryo rescue, but the result of this study suggests that seed coat could act as a physical obstacle (Mehanna and Martin, 1985) in embryo germination of open-pollinated 'Yumi'.

The visible development of embryos was similar during 80 to 90 DAFB stages, but the germination rate increased according to the developmental stages of embryos (Table 1). Thus, the embryos of open-pollinated 'Yumi' might continue physiological maturation until fruit harvest period. Large embryos, which can be obtained at late developmental stages of embryo, showed the highest germination rate (Ramming *et al*., 2003; Sallom *et al*., 2021). Because the embryos of open-pollinated 'Yumi' were not aborted until harvest, 90 DAFB stage is suitable for collection seed for embryo rescue.

Germination rate of embryos cultured in the M3 medium maintained highest germination rate until 80 DAFB stage and showed 100% germination rate with M1 and M2 media from 85 to 90 DAFB (Table 1). Uma *et al*. (2011) suggested that the medium containing plant growth regulators could enhance the germination rate of embryos. The M4 medium contains BAP and IBA, but germination rate was lower compared to the M3 medium from 75 to 80 DAFB. This result implies that, within this experiment, the embryo developmental stage is more affected on germination than plant growth regulators.

Moreover, the *in vitro* plantlets cultivated in M4 medium have significantly short roots (Fig. 4). Generally, auxins such as IBA, indole-3-acetic acid (IAA), and 1-naphthaleneacetic acid (NAA) facilitate rooting, however, IBA has been recognized to impede adventitious root elongation in *in vitro* plantlets of *Prunus* rootstocks (Justamante *et al*., 2022). However, the IBA pulse treatment was able to overcome the suppression of root elongation caused by continuous IBA treatment (Lawson *et al*., 2023; Song *et al*., 2022). Hence, IBA in the M4 medium may have disrupted radicle protrusion

and hindered root elongation.

MS and WPM media have widely used for embryo rescue of *Prunus* species, showing germination rate up to 82~90% germination rate (Devi *et al*., 2017; Sinclair and Byrne, 2003). On the other hand, we could improve germination rate up to 100% using DKW medium, which have higher mineral contents compared to MS and WPM media. Although the M1 and M2 media showed 100% germination rate at 85 and 90 DAFB stages, the shoot growth was facilitated in M3 and M4 medium at 80 and 85 DAFB stages. Embryos of open-pollinated 'Yumi' did not abort until 90 DAFB stages, whereas several early-ripening peach and nectarine cultivars experience embryo abortion and pit splitting during harvest period. Consequently, for embryos susceptible to early abortion and pit splitting, improved germination rate and plant differentiation can be achieved by collecting the embryos before the standard harvest period and performing embryo rescue them on M3 medium.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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