Effects of Cultivar and Ethanol Disinfection on Aseptic Germination of Loquat (*Eriobotrya japonica*) Seeds

Kunkun Li, Weiqi Dong, and Yun Zhao

The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zijingang Campus, Zhejiang University, Hangzhou 310058, P. R. China

Hongxia Xu and Junwei Chen

Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, P. R. China

Changjie Xu¹

Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zijingang Campus, Zhejiang University, Hangzhou 310058, P. R. China

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Abstract. Loquat (*Eriobotrya japonica* Lindl.) is an economically important subtropical fruit tree, originating and commercially produced mainly in China and Japan. The aseptic seed germination of 13 loquat cultivars, including 9 white-fleshed cultivars and 4 red-fleshed ones, was studied, and the relationship between the germination rate and the content of endogenous ABA in seeds was explored. The germination rate and the seedling height at 80 days after sowing of white-fleshed cultivars were generally higher than that of red-fleshed ones. The ABA content in seeds was generally lower in white-fleshed cultivars, and the ABA content negatively correlated with the germination rate at 80 days after sowing. A moderate detrimental effect of 75% ethanol disinfection on aseptic germination of loquat seeds was observed, especially, for the cultivar Dahongpao, and the germination rate and seedling height were reduced by around half and one-third, respectively. The addition of plant preservative mixture (PPM) to the germination medium at a final concentration of 25 mg·L⁻¹ effectively controlled contamination when the 75% ethanol disinfection procedure was omitted.

Loquat (*Eriobotrya japonica* Lindl.), a Rosaceae fruit crop, originated mainly in China and Japan (Lin et al., 1999) and is now also cultivated in other countries such as Spain, Pakistan, Brazil, etc. Generally, loquat fruits ripen from February to June in China and can be consumed fresh or processed to other forms. Loquat fruit, leaves, and roots are rich in active compounds such as carotenoids, phenolics, and triterpenes, etc., and possess high medicinal value for treating inflammatory diseases such as cough and asthma and counteracting diabetes, cancer, and allergy (Liu et al., 2016). Moreover, loquat fruit has contrasting differences in lignification and carotenoid accumulation between cultivars (Fu et al., 2014; Xu et al., 2014). Therefore, loquat is important in both fruit production and research.

Currently, genetic transformation systems are important for plant science because they can be used for verifying gene functions, improving specific traits without affecting other traits, and shortening the breeding period (Aldwinckle and Malony, 2009). There have been some successful reports about regeneration and genetic transformation of some Rosaceae fruit trees such as apple, pear, and strawberry (Aldwinckle and Malony, 2009). However, the genetic transformation of loquat has so far not been successful; the regeneration system is still unstable, and further research on loquat regeneration and genetic transformation is necessary.

Since explants prepared from field-grown plants are susceptible to contamination and easily damaged during the process of disinfection as well as being more recalcitrant to regeneration and transformation, explants prepared from aseptically grown seedlings are usually adopted as with many other Rosaceae or non-Rosaceae woody plants (Aldwinckle and Malony, 2009; Petri and Burgos, 2005). However, in loquat, there are few studies on aseptic germination of seeds and use of seedlings for regeneration (Wu et al., 2007), and a comparison of aseptic seed germination in different cultivars is not available.

The aim of this study was to explore the differences in the aseptic seed germination between the white- and red-fleshed loquat cultivars and possible reasons for differences relating to endogenous ABA content. In addition, the effect of ethanol disinfection on aseptic seed germination and the prospect of supplying PPM, a broad-spectrum biocide/fungicide (Niedz, 1998), to the germination medium for controlling contamination were evaluated.

Materials and Methods

Plant materials. Thirteen loquat (*Eriobotrya japonica* Lindl.) cultivars, including nine white-fleshed and four red-fleshed ones, were used in this study (Table 1). The trees were cultivated at the loquat germplasm nursery of the Zhejiang Academy of Agricultural Sciences, Zhejiang province, China. Fruit were harvested at commercial maturity (different cultivars were harvested on different days), and the seeds were collected on the day of harvest and subjected to disinfection procedures and sowing. For each cultivar or each treatment, three biological replicates were used, with 15 seeds for each replicate.

Seed disinfection. As a routine disinfection scheme, the loquat seeds were washed with flowing tap water for 2 h and then were soaked in 75% (v/v) ethanol for 30 s followed by a rinse with sterile water. After that, the seeds were soaked in 2% NaClO solution for 10 min followed by rinsing five times with sterile water. Finally, the seeds were placed on a sterile filter paper to remove free surface water before sowing on the germination medium. For cultivar comparison experiments, this routine disinfection scheme was followed. Another scheme with 75% (v/v) ethanol disinfection procedure omitted was also used for the study regarding the effects of ethanol disinfection on seed germination.

Sowing and culture. The disinfected seeds were placed on the germination medium, either 1/2 MS for the study regarding the differences in germination between cultivars, or 1/2 MS supplemented with 25 mg·L⁻¹ PPM for the study regarding the effects of ethanol disinfection on seed germination. The seeds were first cultured in the dark at 25 °C for 15 d and then transferred into artificial climate chambers at 25 °C under long-day conditions (16 h light: 8 h darkness), with illumination at 250 μmol·m⁻²·s⁻¹. The germination rate and seedling height were recorded every 10 d from the beginning of sowing. Ungerminated seeds were not included in the calculation of seedling height.

Determination of ABA. The content of ABA in loquat seeds on the day of harvest was determined following an ELISA protocol (Li et al., 2011) by the Phytohormones Research Institute, China Agricultural University, China. Briefly, the frozen seeds (including seedcoat) were ground under liquid nitrogen. One gram of each sample was extracted with 10 mL cold 80% (v/v) methanol containing 1 mmol·L⁻¹ butylated hydroxytoluene as an antioxidant overnight

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¹Corresponding author. E-mail: chjxu@zju.edu.cn.

at 4 °C in the dark. The supernatant was collected after centrifugation, passed through Chromosep C18 Sep-Park Cartridge (Waters, Milford, MA) prewashed with 10 mL 100% (v/v) and 5 mL 80% (v/v) methanol, and dried under nitrogen stream. The residue was dissolved in 2 mL phosphate-buffered saline containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for ELISA analysis of ABA as described by Yang et al. (2001).

Statistical analysis. The obtained data were statistically analyzed by applying the

least significant difference at 5% for the comparison among the treatment means. Duncan's new multiple range test and regression analysis were also used.

Results and Discussion

Effects of loquat cultivars on seed germination. Seeds of loquat germinated gradually after sowing (Fig. 1). At 20 d after sowing, seeds of nine cultivars germinated, but the other four did not, as indicated by the

absence of a 20 d column in Fig. 1. At 40 d, seeds of all cultivars germinated, but the germination rate ranged in different cultivars from 10.00% ('Dahongpao') to 80.70% ('Ruantiaobaisha'). At 60 d, the germination rate of 'Ruantiaobaisha' and 'Taipingbai' was over 85.00% and similar at 80 d after sowing. However, the germination rate of other cultivars at 60 d was significantly lower than at 80 d, when the germination rate of seven cultivars was over 85.00%. In three cultivars, a rate below 70.00% was observed (Fig. 1). The profiles of changes in seedling height after sowing were generally similar to that of the germination rate, and a large difference was observed between cultivars, e.g., the average seedling height of 'Ruantiaobaisha' reached 8.63 cm at 80 d, whereas for 'Oobusa' it was only 1.87 cm (Table 2). There are some previous reports on seed germination of loquat, with germination rates ranging from 0% to 51.00% at 4–8 weeks following sowing for 'Advance' (El-Dengawy, 2005) from 54.70% to 81.90% at 20 to 40 d after sowing for 'Dahongpao' (Wu et al., 2007) and over 90.00% for 'Jiefangzhong' (Chen et al., 1998). The germination

Table 1. List of 13 loquat cultivars included in this study.

Group	Cultivar	Origin
White-fleshed	Changlvviahao	Suzhou, Jiangsu, China
	Guanyu	Suzhou, Jiangsu, China
	Ninghaibai	Ningbo, Zhejiang, China
	Qingzhong	Suzhou, Jiangsu, China
	Ruantiaobaisha	Hangzhou, Zhejiang, China
	Taipingbai	Lishui, Zhejiang, China
	Tangkebaisha	Hangzhou, Zhejiang, China
	Wuerbaisha	Hangzhou, Zhejiang, China
	Yingtiaobaisha	Hangzhou, Zhejiang, China
Red-fleshed	Algerie	Spain
	Dahongpao	Hangzhou, Zhejiang, China
	Liufenzhong	Hangzhou, Zhejiang, China
	Oobusa	Japan

Fig. 1. Germination rates of seeds from 13 loquat cultivars. The data were collected from 10 to 80 d after sowing at 10 d intervals, but only the data at 20, 40, 60, and 80 d are shown. Error bars mean SE ($n = 3$). Note that, the absence of a 20 d column indicates that no seed had germinated. A significance test was conducted and values with the same letter indicate no significant difference at $P < 0.05$.

rates obtained in this study are generally in the range of those reported.

Loquat cultivars can be divided into white- and red-fleshed types. By comparing the germination rate and seedling height at 80 d after sowing, it was concluded that seeds of white-fleshed loquat had generally stronger germination ability than red-fleshed ones. The average germination rate of white-fleshed cultivars at 20, 40, 60, and 80 d was 2.65, 1.36, 1.41, and 1.17 times of red-fleshed cultivars, respectively, and the seedling height of white-fleshed ones at 80 d was on average 1.67 times for red-fleshed ones (Fig. 1; Table 2). The white-fleshed cultivar Ruantiaobaisha had the highest germination rate as well as seedling height among 13 cultivars analyzed, whereas the red-fleshed cultivars Dahongpao and Oobusa had the lowest germination rate and seedling height, respectively (Fig. 1; Table 2). It is worthy of further study, using more cultivars, on whether the difference in seed germination ability between white- and red-fleshed cultivars is a general rule.

The relationship between ABA content and the germination rate. The process of seed germination was complex and affected by endogenous factors including plant hormones such as ABA, gibberellin (Finch-Savage and Leubner-Metzger, 2006; Guan and Scandalios, 2002; Kucera et al., 2005), and ethylene (Beaudoin et al., 2000; Pandey et al., 2006), and hardness of seedcoat (Chen et al., 1998), as well as environmental conditions such as temperature and humidity, etc. (Chen and Jones, 2004). Among these factors, the inhibitory effects of ABA on seed germination have been reported for various plants (Kanjana et al., 2016; Linkies and Leubner-Metzger, 2012; Nambara et al., 2010; Wang et al., 2013). However, the role of ABA in loquat seed germination has not been previously reported. Therefore, in this study, the content of ABA in loquat seeds on the day of harvest was determined, and the correlation between ABA content and the germination rate was analyzed.

As shown in Fig. 2, the ABA content in loquat seeds on the day of harvest ranged greatly between cultivars from 42.76 ng·g⁻¹ FW ('Ninghaibai') to 81.61 ng·g⁻¹ FW ('Oobusa'). The content is lower than those reported for some other Rosaceae plant seeds, e.g., 0.55 pmol·mg⁻¹ (equals to 145.20 ng·g⁻¹ FW) for rose (*Rosa hybrida*) (Bosco et al., 2015) and 354.65 ng·g⁻¹ FW for 'Cuiguan' sand pear (*Pyrus pyrifolia*) (Wang et al., 2013). The relatively low content of ABA in loquat seeds might be related to the lack of obvious dormancy in contrast to the obvious seed dormancy in rose and sand pear (Pipino et al., 2013; Wang et al., 2013). Although the ABA content in the seed was not high enough to result in seed dormancy, it did affect the seed germination rate. A significantly negative correlation was observed between the content of ABA in seeds on the day of harvest and the germination rate at 20, 40, 60, or 80 d after sowing, with the highest regression coefficient obtained when the germination

Table 2. Seedling height for 13 loquat cultivars at 80 d after sowing.

Group	Cultivar	Seedling ht at 80 d (cm) ^a
White-fleshed	Changlvviahao	5.57 ± 0.35 bc
	Guanyu	5.23 ± 0.23 bc
	Ninghaibai	5.17 ± 0.55 bc
	Qingzhong	6.00 ± 1.05 b
	Ruantiaobaisha	8.63 ± 0.49 a
	Taipingbai	6.07 ± 0.55 b
	Tangkebaisha	5.53 ± 0.65 bc
	Wuerbaisha	5.60 ± 0.78 bc
	Yingtiaobaisha	6.60 ± 1.05 ab
Red-fleshed	Algerie	3.53 ± 1.33 cd
	Dahongpao	3.47 ± 1.13 cd
	Liufenzhong	5.63 ± 1.09 bc
	Oobusa	1.87 ± 0.67 d

^aThe data are means ± SE (n = 3), and the different letters indicate significant differences at P < 0.05.

Fig. 2. The correlation between the content of ABA in seeds and the germination rate at 80 d after sowing of 13 loquat cultivars.

rate at 80 d was taken (Fig. 2). This data, in combination with a previous report regarding the promotive effect of GA₃ treatment on seed germination of loquat (El-Dengawy, 2005), suggested that ABA can be a predominant endogenous factor affecting seed germination. A generally higher content of ABA in seeds of red-fleshed cultivars can account for a relatively lower rate of germination as compared with white-fleshed cultivars (Fig. 2). On the other hand, although the correlation between ABA content in seeds on the day of harvest and seed germination rate at 80 d after sowing was significant (P = 0.007), the data points did not closely distribute alongside the linear regression line (Fig. 2), indicating that other factors also play roles in regulation of seed germination and this is worthy of further research.

Effect of ethanol disinfection on aseptic germination of loquat seeds. During the process of determining the germination rates of various loquat cultivars, we realized that the aseptic germination rate obtained in this study was somewhat lower than the germination

rate of seeds sown in soil, although we did not carry out detailed control trials. It is considered unlikely that the germination conditions on the medium can be less favorable than those in soil. Therefore, we suspected that the disinfection procedures for aseptic germination might have adverse effects on seed germination. Ethanol disinfection was considered in particular because previous studies showed that ethanol had adverse effects on seed germination of two other species (Li et al., 2013; Yang et al., 2014). Therefore, a "no ethanol" disinfection process was tested, by omitting ethanol disinfection and compared with the control, using otherwise routine procedures. To control contaminations, PPM was added to 1/2 MS germination medium, both for the control and the "no ethanol" treatment. PPM is a relatively new, broad-spectrum biocide used in plant tissue culture and can effectively prevent microbial contamination of plant cell and tissue culture without affecting the seed germination and callus regeneration as reported for various plants such as

Fig. 3. The effect of ethanol disinfection on germination of loquat seeds. The “ethanol” scheme following routine procedures includes 75% ethanol disinfection, whereas the “no ethanol” scheme excludes the 75% ethanol disinfection procedures. For both schemes, plant preservative mixture was added to the germination medium for restricting contamination. Error bars mean SE ($n = 3$). Asterisks indicate significant differences, $P < 0.05$ and $P < 0.01$ are designated by * and **, respectively.

cauliflower, chrysanthemum, and petunia, etc. (George and Tripepi, 2001; Miyazaki et al., 2010; Niedz, 1998; Rihan et al., 2012).

As shown in Fig. 3, ethanol disinfection had adverse effects on the seed germination of two loquat cultivars tested. For ‘Dahongpao’, the ethanol-disinfected seeds rarely germinated at 30 d after sowing while about half of the seeds of “no ethanol” treatment had germinated at this time. At 80 d, the germination rate and seedling height of ethanol-disinfected ‘Dahongpao’ seeds were about half and one-third, respectively, of those disinfected without ethanol. The adverse effect of ethanol disinfection was not so serious on ‘Yingtiaobaisha’, but still the germination was about one-third lower at 60 d, when all seeds not disinfected with ethanol had germinated. These data showed that ethanol inhibited loquat seed germination and the extent varied between cultivars. Whether there is an interaction between ethanol disinfection and endogenous factors such as ABA needs to be investigated further.

No contamination was encountered for the “no ethanol” treatment with PPM. Another treatment, without ethanol disinfection and without PPM in the medium, was also tested. However, the seeds began to be contaminated from 15 d after sowing. Therefore, no data on germination rate and seedling height were recorded. These data suggested that PPM is required and can effectively restrict contamination when ethanol disinfection is omitted.

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