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HORTICULTURAL STUDIES

RESEARCH PAPER

Effects of Different Embryo Development Stages and GA_3 Doses on Germination in Clementine Mandarin × Carrizo Citrange Immature Embryos

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Abstract

Citrus rootstock breeding is important to improve their resistance to diseases, pests and adverse environmental conditions. The majority of citrus species have nuceller embryony. Although Clementine mandarin is monoembryonic, in some cases (intercrossing etc.) abortive seed formation can be seen frequently. In this study, Clementine mandarin × Carrizo citrange were crossed. In the study, interspecies hybridization (*Citrus* × *Poncirus*) was used. Embryo rescue technique was used to prevent the loss of hybrid individuals due to abortive seed formation. The seeds were gathered from the crossed plants 80, 100, and 120 days after pollination. The immature embryos were removed from the seeds and these embryos were transferred to control, 0.5, and 1.0 mg l^{-1} GA_3 containing medium to germination. The highest germination rate with 100% was observed from 1.0 mg l^{-1} GA_3 containing media harvested 120 days after pollination. Additionally, the highest rate of trifoliate seedlings was obtained from embryos gathered after 120 days of the pollination.

1. Introduction

The citrus, which has major importance in the world and Türkiye, is propagated by vegetative and generative methods. However, in particular diseases and because of some soil and climatic conditions rootstock uses is essential in citrus. Therefore, almost all types of citrus are grafted on rootstocks which are grown from seeds and the rootstock has a significant effect on some properties of grafted cultivars. The Mediterranean basin, with world citrus production by 22%, is under threat because of sour orange rootstock uses (Pestana et al., 2005). If CitrusTristeza virus and its pest vector *Toxoptera citricida* is spread, the uses of sour orange rootstock, tolerant to salinity and calcareous soil, will be limited in the Mediterranean region. Therefore, new rootstocks urgently are required as alternatives to the sour orange (Ollitrault et al., 2006).

Monoembryonic diploid varieties are effective when used as the female parent in crosses (Xie et al., 2019). However, the presence of a few monoembryonic parents causes problems in intraspecies or interspecies hybridizations. Although this problem is partially reduced by breeding studies, there is a need to obtain new individuals (Spiegel-Roy and Goldschmidt, 1996). With some exceptions, hybrid breeding is the most used method in rootstock breeding, which includes the same methods as variety breeding, which is difficult, costly and needs in long duration (Barrett, 1985; Cheng and Roose, 1995). Citrus rootstock breeding has focused on crossing a selected male or female parent with a trifoliate, which is still important as a genetic resource recently (Castle, 2010).

Although embryos are produced from such inter species and genus hybridizations in citrus, seed development is halted because the normal embryo

to endosperm ratio is not achieved. In such crosses, different types of seeds are obtained and multiple small embryos are also observed exclusively in partially developed seed (abortive seeds). Embryo rescue is necessary to obtain these genotypes from abortive seeds. In addition, Monoembryonic female parents are widely used in interploid hybridizations in citrus, and then genotypes are obtained by embryo rescue technique (Oiyama and Kobayashi, 1990; Oiyama et al., 1991; Shen et al., 2011).

One of the major problems in citrus breeding is competition between zygotic and nucellar embryos (Soost and Roose, 1996). Generally, to determine hybrid embryo some additional experiments require such as cytological, flow cytometry, isoenzyme analysis or molecular analysis (Tusa et al., 2002). This negative situation is eliminated by *in vitro* embryo rescue techniques for developing embryos. The success of embryo rescue depends on the ingredients of medium and embryo developing stages (Jaskani et al., 2005). The germination capacity of citrus embryos can be affected by the embryo's genetic structure and embryo developing stage (Viloria et al., 2005). Embryos of some citrus species have developed more easily than others in culture, and sometimes there are differences between varieties (Collins and Grosser, 1984; Rangan, 1984; Jia, 1993).

Various studies reported that the addition of 0.01 mg l^{-1} GA_3 (Riberio et al., 2000; Chagas et al., 2005); 0.1 mg l^{-1} GA_3 (Pasqual et al., 1990; Jumin and Nito, 1996; Singh et al., 2020); 1.0 mg l^{-1} GA_3 (Ollitrault et al., 2007; Zhang et al., 2013; Kurt and Ülger, 2014); 1.5 mg l^{-1} GA_3 (Perez-Tornero and Porras, 2008; Soni et al., 2019) and 2.0 mg l^{-1} GA_3 (Gmitter et al., 1990; Turgutoğlu et al., 2015) in growing media for embryos developing of citrus is to be appropriated.

Rangan et al. (1969) studied nuceller embryos developing in common sour orange. They indicated that nuceller embryos developing were not seen in growing seeds in 120 days after anthesis. It was determined depending on the examined species and varieties of citrus that 50 days (Wang et al., 1999); 80 days (Tan et al., 2007); 85 days (Xie et al., 2019); 95 days (Singh et al., 2020); 100 days (Tusa et al., 1996; Deng et al., 1996), 105 days (Scarano et al., 2005; Ferrante et al., 2010), 118 days (Chagas et al., 2005); 120 days (Perez-Tornero et al., 2011; Kurt and Ülger, 2019); 130-140 days (Soni et al., 2019) and 135-150 days (Perez-Tornero and Porras, 2008) after pollination was found to be suitable for embryo rescue.

The objective of the study to determine the effect of different embryo development stages (80, 100 and 120 days after pollination-DAP) and GA_3 concentrations (control, 0.5, and 1.0 mg l^{-1} GA_3) in the culture medium of Clementine mandarin (*Citrus clementina* Hort. ex. Tanaka) × Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] hybrid seeds.

2. Materials and Methods

2.1. Plant materials

Clementine mandarin and Carrizo citrange in the Citrus Genetic Resources Collection located in Batı Akdeniz Agricultural Research Institute were used as plant materials. The study was conducted in 2012. Carrizo citrange was used as the male parent and Clementine mandarin was used as the female parent in the crossing combinations.

2.2. In vitro experiments

Murashige and Tucker (1969) medium was used as a basic culture medium and 50 g l^{-1} sucrose, 25 mg adenine sulfate, and 500 mg l^{-1} malt extract were put in medium. Then, control (0), 0.5, and 1.0 mg l^{-1} GA_3 were supplemented to the prepared medium and medium pH was adjusted to 5.7 and 8.0 g l^{-1} agar was added. After sterilization, the prepared medium was distributed in petri dishes as 40 ml medium containing.

The fruits were taken 80, 100 and 120 days after crossing, were washed with water and detergent, and the fruits were soaked in 70% ethyl alcohol for 5 min and 20% sodium hypochlorite for 30 min to make surface sterilization (Ollitrault et al., 2007). Then, the fruits were cut horizontal. The seeds were removed from the fruit by forceps and immature embryos were taken from the microphyl parts of the seeds by cutting with a surgical blade under binocular (Figure 1a). Two embryos were placed into each petri dishes containing a culture medium. And then, the petri dishes were incubated at 25ºC under 1000 lux light intensity and 16 h photoperiod in a growth chamber. Germinated embryos were counted and the germination rate of embryos was calculated (Figure 1b).

Germinated embryos were sub-cultured Murashige and Skoog (1962) medium containing 0.02 mg l^{-1} NAA and 20 mg l^{-1} sucrose in culture tubes for seedling growing (Perez-Tornero and Porras, 2008). Then the plantlets in culture tubes were incubated at 25ºC under 1000 lux light intensity and 16 h photoperiod in a growth chamber (Figure 2).

The developing plants in the sub-culture were transferred to plastic pots (Figure 3). The plastic pots were put in a chamber with 25-26ºC temperature and 80-85% humidity for two weeks.

Trifoliate seedlings in sub-culture were counted and the rate of trifoliate was calculated. Trifoliate is controlled by two dominant genes in citrus and this feature is shown in the hybrids of zygotic dominant. This feature was taken into consideration when the trifoliate rate was determined. Heterozygous and recessive zygote seedlings are not taken into consideration as they have no trifoliate features.

The developing plants in the sub-culture were measured at 15 days intervals to observe growing

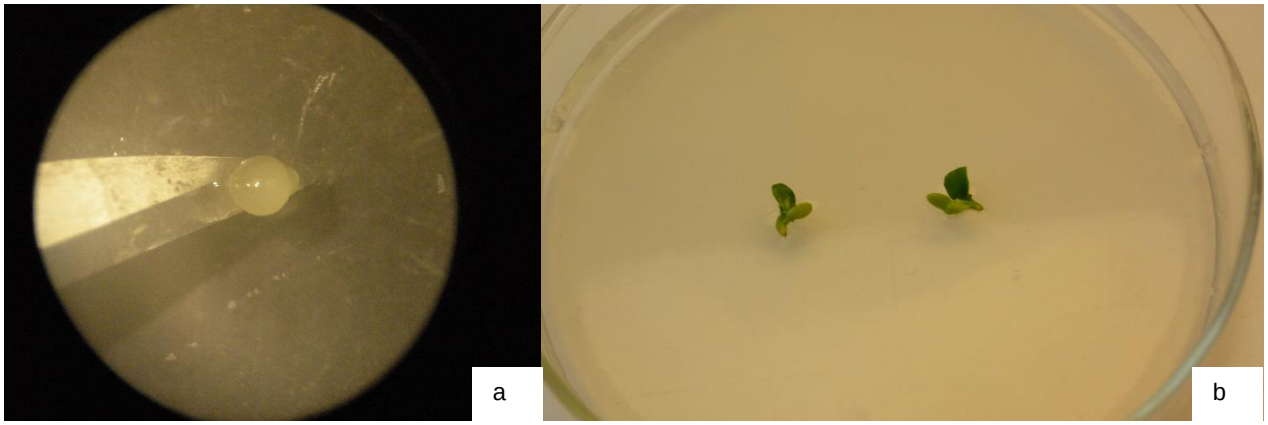

Figure 1. Immature embryo under binocular (a) and germinated embryos (b).

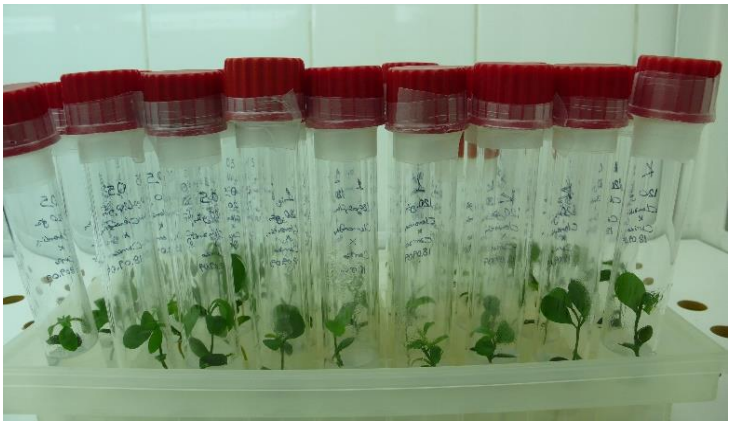

Figure 2. Developed plantlets in the culture tubes.

Figure 3. Hybrid bifoliate and trifoliate seedlings transplanted into plastic pots.

seedlings. As a result of the measurements, the plant height development was evaluated.

2.3. Experimental design and data analysis

The experiment was conducted as random plots with 10 replications and each replication have two embryos. Data were subjected to analysis of variance with mean separation by Least significant difference (LSD) test. Square root transformation was made to data before the compare the percentage values with variance analysis.

3. Results and Discussion

3.1. Germination of embryos

Embryo development stages, GA_3 concentrations in the medium and their interactions were significant to the germination rate of *Clementine mandarin* × *Carrizo citrange* hybrid embryos ($p \leq 0.05$). The highest germination rates in embryos were obtained 120 days later taken after pollination on all embryo stages. The germination rate of embryos that were taken 120 days after

pollination and germinated 1.0 mg l^{-1} GA_3 containing media was found as 100%. The lowest germination rate in embryos was obtained 80 days later taken after pollination with 20% and 22%. (Table 1).

3.2. The rate of trifoliate in plantlets

Trifoliate is controlled by two dominant genes in citrus and this feature is shown in the hybrids of zygotic dominant. The highest trifoliate rate was observed in 120th days with 74% and it was followed by 100th days later taken after pollination with 18%. The lowest rate was found in the 80th day with 8%. (Figure 4). In the study, this feature was taken into consideration when the trifoliate rate was determined. Heterozygous and recessive zygote seedlings are not taken into consideration since they have no trifoliate features.

3.3. The growth of the seedling's height

According to Figure 5, the growth of seedlings height occurred as a linear increase at all embryo development stages and all GA_3 doses. It observed that the growth of seedlings' height taken from 120 days after pollination was higher than other DAP at all GA_3 doses.

Table 1. The effect of embryo development stages and GA_3 concentration on germination rate.

Days after pollination (DAP)	GA_3 doses (mg l^{-1})			Average of days after pollination
	Control	0.5	1.0	
80th DAP	20.00 g *	22.00 g	30.00 f	24.00
100th DAP	15.00 h	42.50 d	35.00 e	30.83
120th DAP	82.50 c	92.50 b	100.00 a	91.67
Average of GA_3 doses	39.17	52.33	55.00	

*Different letters indicate significant differences (P<0.05) according to the Least Significant Difference test (LSD: 3.2346).

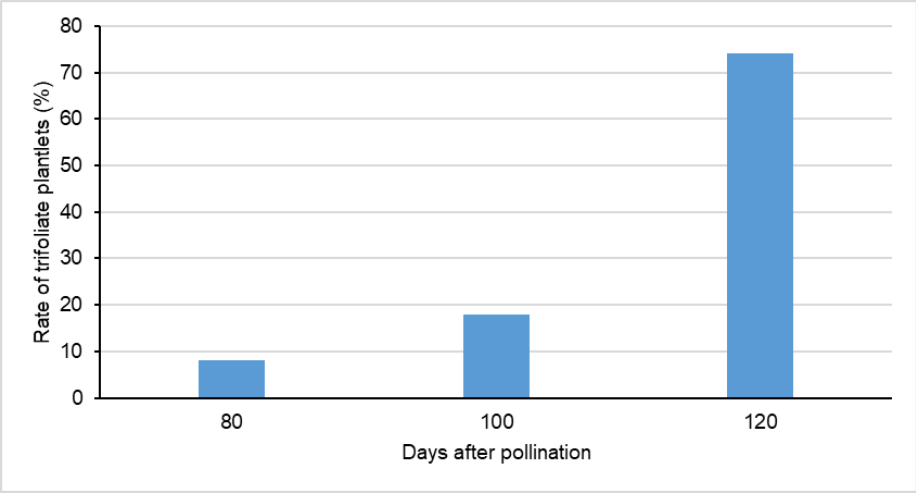

Figure 4. The effect of embryo development stages on the rate of trifoliate plantlets.

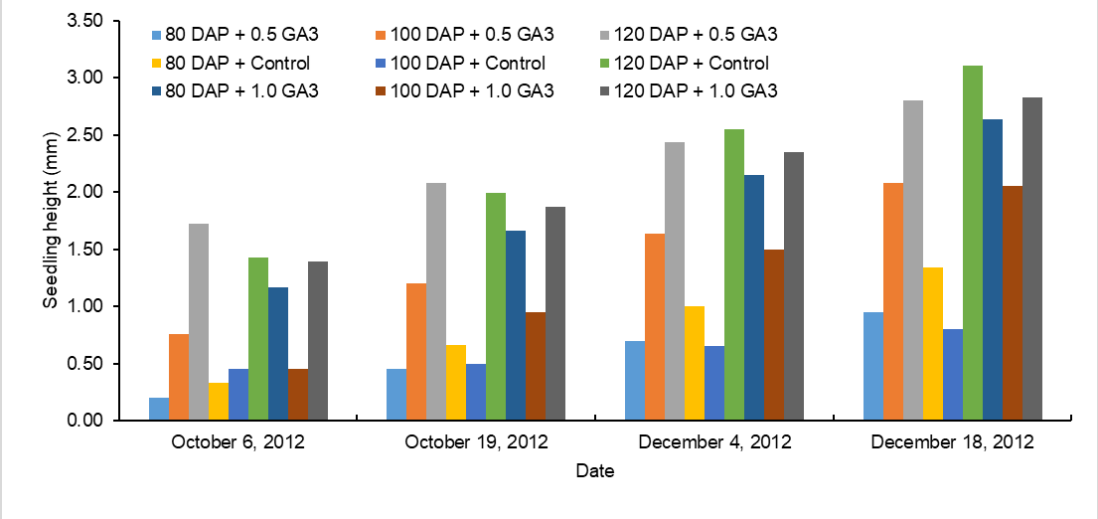

Figure 5. The effect of embryo development stages and GA_3 concentration on seedling height.

According to the experiment results, it was postulated that there was a relation between embryo development stages and embryo germination since the highest germination results were obtained 120 days after pollination in all GA_3 doses. In accordance with the results, Carimi et al. (1998), Viloria et al. (2005), Perez-Tornero et al. (2011) and Kurt and Ülger (2014) had good embryo germination from 120 DAP has taken embryos and they were indicated that genetic and embryo developing stages were effected the germination capacity of embryos. On the other hand, there were some results that good embryo germinations were obtained in 50 days (Chen and Wang 1986; Wang et al., 1999), 80 days (Tan et al., 2007), 85 days (Xie et al., 2019), 95 days (Singh et al., 2020), 100 days (Deng et al., 1996; Tusa et al., 1996), 105 days (Ferrante et al., 2010) and DAP has taken embryos after pollination. This may be due to the growing location and cultivars used.

Since the best results were obtained from 1.0 mg l^{-1} GA_3 containing medium 120 days after pollination has taken embryos, it showed that this dose and embryo development stage was appropriated for germination of embryos. Similarly, Button and Kochba (1977), Kunitake et al. (1991), Carimi et al. (1998), Das et al. (2000), Wakana et al. (2004), Jaskani et al. (2005), Ollitrault et al. (2007) and Zhang et al. (2013) studied in different citrus species and cultivars and they indicated that adding of 1 mg l^{-1} GA_3 to the medium was given good results in the germination of embryos in citrus. Some experiments reported that 0.01 mg l^{-1} GA_3 (Ribeiro et al., 2000; Chagas et al., 2003), 0.1 mg l^{-1} GA_3 (Pasqual et al., 1990; Jumin and Nito 1996; Singh et al., 2020) and 2.0 mg l^{-1} GA_3 (Gmitter et al., 1990; Turgutoğlu et al., 2015) appropriated for embryo germination of citrus.

4. Conclusion

In this study was found that 1.0 mg l^{-1} GA_3 dose and 120 DAP development stage appropriated for immature embryo rescue. In addition, it was determined that the best embryo rescue time was 120 days after pollination since germination and trifoliate seedling rate were higher than others. Immature embryo culture, which is a preferred and valuable method in parallel with the advances in classical and genetic breeding studies in citrus, creates an important potential in the development of superior new varieties and shortening the breeding period.

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