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Optimizing embryo stage and GA_3 doses in Common mandarin x Carrizo citrange crosses on embryo rescue technique

Yerli mandarin x Carrizo sitranjı melezlerinde, embriyo kurtarma tekniğinde embriyo gelişim aşaması ve GA_3 dozlarının optimizasyonu

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ABSTRACT

Many difficulties are exist in citrus breeding due to incompatibility, long juvenility, sterility and nucellar embryo. Zygotic embryo in the seeds of polyembryonic citrus cultivar is degenerated by nucellar embryos during embryo development. Accordingly, in vitro embryo culture is a useful tool in citrus breeding, since it assures embryo germination and development. In this study, Common mandarin x Carrizo citrange were crossed in order to produce new rootstock genotypes. Immature embryos were taken from fruit after 80, 100 and 120 pollination days to determine the suitable embryo rescue stage. Then, the embryos were germinated in Murashige and Tucker (MT) culture medium including 0, 0.5 and 1 mg l^{-1} GA_3. According to the results, high germination rates were materialized as 100% and on 95% to taken embryos from 120 days after pollination (DAP) in supplemented with 1 mg l^{-1} GA_3 and 0.5 mg l^{-1} GA_3 in MT culture medium respectively. The embryos taken from 80 days after pollination did not germinate on MT medium without GA_3 (control) and including with 0.5 mg l^{-1}. Generally, the existence of GA_3 on medium increased the ratio of germination compared to the control. The highest trifoliate ratio was determined as 33.33% at the embryos taken from 100 and 120 days after pollination and medium including 0.5 and 1 mg l^{-1} GA_3 consecutively. The results have revealed that the optimal time for embryo rescue at citrus is 120 DAP and including GA_3 in the medium increased embryo germination.

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ÖZ

Turunçgil ıslahında; tür ve çeşitler arasında uyuşmazlık, uzun gençlik kısırlığı, gametik kısırlık ve polyembriyoni gibi nedenlere bağlı olarak birçok engel bulunmaktadır. Poliembriyonik turunçgillerin tohumlarındaki zigotik embriyo gelişim sırasında nüseller embriyolar tarafından dejenere edilmektedir. Buna göre, zigotik embriyoların çimlenmesini ve gelişimini sağlaması nedeniyle embriyo kültürü ıslah çalışmalarında yararlı bir araçtır. Bu çalışmada, yeni anaç bireylerinin elde edilmesi amacıyla Yerli mandarin x Carrizo sitranjı melezlenmiştir. Embriyo kurtarma için uygun embriyo gelişim aşamasının belirlenmesi amacıyla olgunlaşmamış embriyolar tozlanmadan 80, 100 ve 120 gün sonra alınmıştır. Daha sonra, alınan embriyolar 0, 0.5 ve 1 mg l^{-1} GA_3 içeren Murashige ve Tucker (MT) besi ortamında çimlendirilmiştir. Sonuçlar; en yüksek çimlenme oranlarının tozlanmadan 120 gün sonra alınan embriyolarda 1 mg l^{-1} GA_3 içeren ortamda %100 ile ve 0.5 mg l^{-1} GA_3 içeren ortamda %95 olduğunu göstermiştir. Tozlanmadan 80 gün sonra alınan embriyoların 0 mg l^{-1} GA_3 (kontrol) ve 0.5 mg l^{-1} GA_3 içeren ortamlarda çimlenme olmamıştır. Genel olarak, ortama GA_3 ilavesi kontrole göre çimlenme oranını artırmaktadır. En fazla üç yapraklı bitki oranı %33.33 ile tozlanmadan 100 ve 120 gün sonra alınan ve sırasıyla 0.5 ve 1 mg l^{-1} GA_3 içeren ortamlardan elde edilmiştir. Sonuçlar, turunçgillerde embriyo kurtarma için en uygun zamanın tozlanmadan sonraki 120 gün olduğunu ve ortamda GA_3 bulunmasının çimlenmeyi artırdığını göstermiştir.

1. Introduction

Citrus aurantium L. that mainly in good compatibility with main citrus types, productive and resistant against calcerous soil, is commonly used as rootstocks in Mediterranean basin where 22% of world citrus production. However, there is an urgency to develop alternative rootstocks resistant to tristeza (CTV) and well adapted to the constraints of the area because of sour orange rootstock is very sensitive against tristeza virus (Ollitrault et al. 1998) and it has sometimes incompatibilities with some lemon cultivars (Tuzcu 1978; Tuzcu et al. 1999).

Although nucellar embryony is genetically valuable in production of homogeneous seedling, it is a big obstacle for breeding of citrus. There is both nucellar embryos and zygotic embryo in polyembryonic citrus seeds. The total number of embryos per seed varies greatly within a tree, as well as among cultivars and there is little consistency within species having nucellar embryony (Ray 2002).

Cultivars that produce nucellar seedlings also occasionally produce zygotic seedlings, but typically monoembryonic cultivars have not been shown to produce nucellar seedlings (Soost and Cameron 1975).

One of the important problems in breeding of citrus, the zygotic embryo in polyembryonic citrus species must compete both nutrients and space with embryos developed from nucellar tissue (Soost and Roose 1996). This negative situation can be eliminated with using embryo rescue technique. Embryo rescue is a very beneficial method for triploid hybrid citrus plants can be produced by interploid crosses followed by *in vitro* embryo rescue techniques to overcome the problem of embryo abortion and recovery of zygotic triploids from polyembryonic seeds (Yi and Deng 1998; Jaskani et al. 2005; Viloria et al. 2005). The success of embryo rescue depends on the embryo development stage is excised and the composion of the medium used for germination (Jaskani et al. 2005). Genetic structure of embryo and embryo development stage may affect germination capacity of embryos (Viloria et al. 2005). The addition GA_3 to culture medium positively contributed to the development of embryos (Button and Kochba 1977; Kunitake et al. 1991; Carimi et al. 1998; Das et al. 2000; Wakana et al. 2004; Jaskani et al. 2005). However, GA_3 concentrations in culture medium may give different results depending on the species or variety. Some authors observed that supplementing 0.01 mg l^{-1} GA_3 to the medium (Ribeiro et al. 2000; Chagas et al. 2003), supplementing 0.1 mg l^{-1} GA_3 to the medium (Pasqual et al. 1990; Jumin and Nito 1996) and supplementing 2 mg l^{-1} GA_3 (Gmitter et al. 1990) favored the growth and development of embryos.

The optimal time for embryo rescue vary significantly according to the species and cultivars. Some researcher found that 50 DAP (Chen and Wang 1986; Wang et al. 1999), 80 DAP (Tan et al. 2007), 100 DAP (Wang et al. 1981; Deng et al. 1996; Tusa et al. 1996) 105 DAP (Scarano et al. 2005) and 118 DAP (Chagas et al. 2005) for the optimal time of embryo rescue. Rangan et al. (1969) declared that nucellar embryos had not yet been found in the developing seeds after 120 days pollination.

Trifoliate is controlled by two dominant gene in citrus and this features is shown in the hybrids of zygotic dominant. Generally, to determine hybrid embryo some additional experiments require such as cytological, flow cytometry, isoenzyme analysis or molecular analysis (Tusa et al. 2002).

In this study, Common mandarin (*Citrus deliciosa* Ten.) and Carrizo citrange [*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] have been hybridized in order to find new rootstock genotypes. Immature embryos taken 80, 100 and 120 DAP were cultured in MT culture medium supplemented with GA_3 concentrations in order to rescue the zygotic embryos.

2. Materials and Methods

Common mandarin (female) and Carrizo citrange (male) at the "Citrus Genetic Resources Collection" of Bati Akdeniz Agricultural Research Institute were used as parents. The research area is 7.62 km away from sea and its altitude is 9 meters. Its coordinates are 36° 55' 32.40" N and 35° 00' 35.75" E. Soil has salt less-alkali reaction and calcic. Soil structure is sandy-clay- loam as general in soil structure of Antalya region.

Unopened or partially opened flower buds of Carrizo citrange as paternal parent was collected before anthesis and petals and styles were removed with forceps in the laboratory. The flowers were placed at 25°C to promote anther dehiscence. The flowers of Common mandarin as maternal parent were emasculated prior to opening and emasculated flowers were pollinated by touching the dehisced anthers of the pollen parent gently to the stigma with brush and paper or linen bags were replaced after pollination and were removed approximately one month later. Then fruits were covered by net and marked with labels. Cultural practices like irrigation, fertilizing, weed control, disease and pesticides control were applied on time and according to the technique to the trees.

Modified Murashige and Tucker (1969) medium was used as basic culture medium for embryo germination and 50 g l^{-1} sucrose, 25 mg adenine sulfate, 500 mg l^{-1} malt extract were put in medium. Then, 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 then 8 g l^{-1} agar was added. After sterilization, the prepared medium was distributed 40 ml volume per petri dish.

For embryo culture, hybrid fruits were harvested at 80, 100 and 120 days after cross-pollination. Fruits were washed with water and detergent later, surface-sterilized by immersion for 5 min in 70% ethyl alcohol and 30 min in 20% sodium hypochlorite (Ollitrault et al. 2007). Then, the fruits were cut horizontal with the care of not damaging seeds. The seeds were removed from the fruit by forceps and immature embryos were taken from the microphyl parts of seeds by cutting with a surgical blade under binocular. Two embryos were placed into each petri dish containing modified MT culture medium

Later, the embryos were incubated at 25°C and 90% humidity, in 16 h photoperiod with 1000 lux light intensity in growth room. The embryos which have cotyledon leaves were counted and embryo germination ratio (%) was determined by rating the germinated embryo number to total embryo number.

Germinated embryos were sub-cultured Murashige and Skoog (1962) medium containing 0.02 mg l^{-1} NAA and 20 g l^{-1} sucrose in culture tubes to provide seedling growing (Perez-Tornero and Porras 2008). Later, the seedlings were incubated at 25°C and 90% humidity, in 16 h photoperiod with 1000 lux light intensity in growth room.

Trifoliate seedlings in sub-culture were counted and trifoliate ratio (%) was determined. Developed plants were transferred into 12x8 cm plastic pots. The seedlings were taken to growth rooms at 25-26°C temperature and 80-85% humidity in order to increase the survival rates. Later, the plants were

transferred to greenhouses for acclimatization to *in vivo* conditions.

The experiment was conducted as random plots with 10 replications and each replications have two embryo. Data were subjected to analysis of variance with mean separation by Duncan's multiple range test. Before comparing the percentage ratio with variance analyses, square root transformation was made to the data.

3. Results

3.1. Germination of embryos

Embryo development stages (day after pollination), GA_3 concentrations in the medium and their interactions were significant on germination rate of Common mandarin x Carrizo citrange hybrid embryos ($p \leq 0.05$).

High germination rates were materialized as 100% and on 95% to taken embryos from 120 DAP in supplemented with 1 mg l^{-1} GA_3 and 0.5 mg l^{-1} GA_3 in MT culture medium respectively. The embryos taken from 80 days after pollination did not germinate on MT medium without GA_3 (control) and including with 0.5 mg l^{-1}. The including of 1 mg l^{-1} GA_3 in medium was increased the ratio of germination of taken embryos at 80, 100 and 120 DAP compared to the control. However, medium supplemented with 0.5 mg l^{-1} GA_3 did not affect the ratios of germination of taken embryos at 80 DAP. The ratios of germination of taken embryos at 100 DAP decreased while the ratios of germination of taken embryos at 120 DAP increased at medium supplemented with 0.5 mg l^{-1} GA_3. The germination ratio of the embryos increased with the progress of the embryo development stage (Table 1).

3.2. Trifoliate plant ratio

The highest trifoliate ratio was determined as 33.33% at the embryos taken from 100 and 120 DAP and medium including 0.5 and 1 mg l^{-1} GA_3 consecutively. Because of the fact that embryos taken from 80 days after pollination did not germinate on MT medium without GA_3 (control) and including with 0.5 mg l^{-1}, trifoliate rate could not determine at these applications. The embryo development stage and the including of GA_3 to the culture medium did not affect the trifoliate rate (Table 2).

4. Discussion and Conclusions

As the highest germination ratio was obtained from embryos taken from 80, 100 and 120 days after pollination at culture medium supplemented with 1 mg l^{-1} GA_3, this study shown that the suitable dose as 1 mg l^{-1} GA_3 for immature embryo germination. Our results are in agreement with those of other authors; it have been accepted adding 1 mg l^{-1} GA_3 to the culture medium for different citrus types and species (Button and Kochba 1977; Kunitake et al. 1991; Carimi et al. 1998; Das et al. 2000; Wakana et al. 2004; Jaskani et al. 2005).

On the other hand, there were some results showing that high embryo germinations were obtained in adding 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) and 2 mg l^{-1} GA_3 (Gmitter et al. 1990) in culture medium. These situations may vary due to the embryo development stage, species and cultivars used. There is not an ideal dose for a cultivars.

However, adding 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) and 2 mg l^{-1} GA_3 (Gmitter et al. 1990) didn't give good results. This proves that there is not an ideal GA_3 dose for species and varieties and the results may vary according to the species, varieties and embryo development stages.

There is linear relationship between embryo development stage and germination since germination rates from embryos taken 120 DAP at all culture medium was found higher than other. As shown in the results, Carimi et al. (1998) and Viloria et al. (2005) obtained the best germination ratio in citrus embryos from the embryos taken 120 DAP and stated that genetic structure of embryo and embryo development stage affect germination. On the other hand, there are some results showing that good embryo germinations were obtained at 50 DAP (Chen and Wang 1986; Wang et al. 1999), 80 DAP (Tan et al. 2007), 100 DAP (Wang et al. 1981; Deng et al. 1996; Tusa et al. 1996), 105 DAP (Scarano et al. 2005), and 118 DAP (Chagas et al. 2005) after pollination. These differences may be due to the growing location and cultivars used.

The embryo development stage and the including of GA_3 to the culture medium did not affect the trifoliate rate. It seen that trifoliate rates increased with progressive of embryo development stage on culture medium supplemented with 1 mg l^{-1} GA_3.

Table 1. The germination rates of embryos Common mandarin x Carrizo citrange obtained from various embryo development stage and different GA_3 doses (%).

Embryo stage	GA_3 doses			
	Control	**0.5 mg l^{-1}**	**1 mg l^{-1}**	**Means of embryo stage**
80 days	0.00 ± 0.00	0.00 ± 0.00	35.00 ± 0.15	11.67 ± 0.06 **c***
100 days	65.00 ± 0.13	45.00 ± 0.12	85.00 ± 0.08	65.00 ± 0.07 **b**
120 days	80.00 ± 0.11	95.00 ± 0.05	100.00 ± 0.00	91.67 ± 0.04 **a**
Means of GA_3 doses	48,33 ± 0.08 **B**	46,67 ± 0.08 **B**	73.33 ± 0.07 **A**	

* Mean separation within columns and main effects by Duncan's multiple range test, $p \leq 0.05$. Capital letters indicate doses and small letters indicate embryo taking time.

Table 2. Trifoliate rates of Common mandarin x Carrizo citrange seedling (%).

Trifoliate seedling rates (%)								
Control			**0.5 mg l^{-1} GA_3**			**1 mg l^{-1} GA_3**		
80 days	**100 days**	**120 days**	**80 days**	**100 days**	**120 days**	**80 days**	**100 days**	**120 days**
0	20.00	30.77	0	33.33	23.53	20.00	21.43	33.33

The results show that it was necessary to supplement GA_3 in the culture medium at citrus according to the species and varieties for embryo development and the appropriate GA_3 dose may vary according to the species and varieties. However, as obtained germination and trifoliate rates is higher on 120 days prove that the optimal time for embryo rescue at citrus is 120 days after pollination.

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