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Uniform and virus-free citrus rootstocks production via nucellus culture

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Key words: citrus, nucellus culture, uniform rootstocks, virus-free.

Abstract: Prevalence of various virus and virus-like diseases is among the main reasons for the decrease in quality and quantity of citrus crops. These diseases are mainly spread through the propagation method in citrus which is budding. Using nucellus culture of bitter orange and Mexican lime seeds, uniform and virus-free rootstocks could be produced so that the diseases prevalence could be prevented. In order to generate adventitious shoots from nucellus culture in each of the two rootstocks, direct organogenesis method is used. In all conducted experiments, Murashige and Skoog (MS) medium were used. Two plant growth regulators of benzyl adenine (BA) in 0, 1, 1.5 and 2 mg l^{-1} concentration and gibberellins (GA) in 0, 1 and 2 mg l^{-1} concentration were used in the medium and the main effects of each plant growth regulator were studied separately and their interaction on shoot generation were also surveyed. Considering the retrieved data, it was determined that the interaction of BA and GA have a higher impact on shooting, comparing to the cases where each of the regulators is used alone. In Mexican lime rootstock, the best culture medium for generating shoots from nucellus culture is the culture medium containing 2 mg l^{-1} BA and 2 mg l^{-1} GA and in bitter orange rootstock, the highest shooting rate was attributed to the culture medium containing 2 mg l^{-1} GA and 1 mg l^{-1} BA. For the Mexican lime and bitter orange shoots rooting, indole butyric acid (IBA) was used. The concentrations of this plant growth regulator used in Mexican lime were 0, 0.5, 1 and 1.5 mg l^{-1} and for bitter orange were 0, 1 and 1.5 mg l^{-1}. The highest rooting rate for Mexican lime was in culture medium containing 0.5 mg l^{-1} IBA and for bitter orange, it was the culture medium containing 1 mg l^{-1} IBA. The obtained plantlets were gradually adapted with the external environment.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Citrus is among the most important fruit tree groups in tropical and subtropical regions in the world (Jajoo, 2010) and have a significant role in human diet as fruits which contain a high amount of vitamin C and other nutrients such as potassium. Similar to other fruit trees, citrus is commercially propagated through grafting a scion on a proper stock and a grafted tree is generated which retains the traits of stock and scion (Spreen, 2009). Citrus propagation through this method is among the most important limiting factors in generating them, since it leads to the

spread of various viral and virus-like diseases through generation of infected seedling (Rangan *et al.,* 1968). Choosing stock and scion suitable for each environment and then ensuring plants free of any disease play a great role in optimal generation of this crop (Shahsavar, 2005). Hence, it is required to generate healthy and virus-free stock and scion so that a healthy plant combination is generated. Generating healthy and virus-free scion is possible through shoot-tip-grafting (STG) (Murashige *et al.,* 1972; Shahsavar and Khosh-Khui, 1994; Shahsavar, 2005). However, virus-free rootstock production is not problematic since rootstocks are produced by seeds and seeds are not virus diseases vectors, even if the mother plant is infected (Altaf *et al.,* 2001; Singh *et al.,* 2006). Another issue which is important in rootstocks is their uniformity. Propagation from seeds could not lead to production of uniform rootstocks. Although some of the rootstocks are produced by the nucellar embryos which are uniform and similar to the mother plant, there are plants produced by natural embryos which are not completely similar to the mother plant and this could lead to the lack of uniformity among the produced rootstocks. Hence, in rootstock production, methods should ensure virus-free rootstocks, and uniformity, as well. The best method in reaching this objective is through planting nucellar embryos. Fortunately there are nucellar embryogenesis and polyembryonic in most citrus species which produce true to type plants (Wutscher, 1979). In 1958, Rangan carried out the initiation of nucellar embryos in one of citrus genotypes for the first time and micro propagation in these genotypes provided the possibility for production of uniform plant populations (Rangan Swamy, 1958). Nucellus culture is generally considered as an effective method for producing virus-free citrus and virus is not spread through nucellus cultured. Also, the produced plants through nucellus culture and somatic embryogenesis have the potentials to produce plants with the traits of the mother plant (Singh *et al.,* 2006). In 2001, Altaf reported that using nucellus tissue explants, they were able to produce virus-free plants showing minimum differences with the mother tissue. In the conducted research, the presence of cytokinin BA was necessary for shoot regeneration. However, the optimum concentration of BA is depending on explant genotype and other conditions. Rooting of the generated shoots have been reported differently considering used genotype, culture medium and IBA concentration (Chaturvedi and Mitra, 1974; Barlass and Skene, 1982; Duran-Vila *et al.*, 1989; Jakson and Looney, 1999).

The main objective of this research is to study Mexican lime and bitter orange micropropagation using generation of adventitious shoots through nucellus culture of these seeds and reaching a uniform and virus-free rootstock and also determining the effect of plant growth regulators in stimulation and growth of shoots and rooting of these shoots.

2. Materials and Methods

In this research two important citrus rootstocks including bitter orange (*Citrus aurantium* L.) and Mexican lime [*Citrus aurantifolia* (Christm.) Swingle] were used. Immature fruits (100-120 days after pollination) of these species were transferred from Darab Research Station to the laboratory of Horticultural Sciences Department at Shiraz University.

Initially, the fruits were washed with water and a dish washing detergent (Rika®) and rinsed. Subsequently, they were put in 40% Clorox® solution (regular commercial bleach which contains 5-6% sodium hypochlorite) for 15 minutes so that their surface was disinfected. The seeds of each fruit were separated and after washing the seed gel, they were transferred under laminar air flow cabinet. The seeds were left in Clorox® solution for 10 minutes and rinsed with sterilized distilled water so that their surfaces were disinfected. Subsequently, both seed shells were separated before planting. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with various plant growth regulators concentrations were used in this research. In order to study the impact of BA and GA and their interaction on shoot generation from nucellus tissue of Mexican lime and bitter orange, BA with concentrations of 0, 1, 1.5 and 2 mg l^{-1} and GA with concentrations of 0, 1 and 2 mg l^{-1} were used. Therefore, there were 4 treatments and 8 replications per treatment and one explant per replication, totally 32 tubes per treatment. Sucrose and agar concentration used in all culture media were 30 and 8 g l^{-1}, respectively. The pH of the culture medium was set to be 5.75±0.05 after putting in autoclave and disinfected for 15 minutes at 120°C and pressure of 1.5 kg cm^{-2}.

Culturing explants

After separating the area of the seeds which contained zygotic embryo, remaining tissue of the seed

which contained the nucellar embryo was cultured horizontally in 150×250 ml test tubes which had 20 ml of MS basal culture medium. One explant was sown per tube and exposed to 16-h daily of 2000-2500 lux illumination of fluorescent light at 27±1°C for 4 weeks.

Rooting of the generated adventitious shoots

For rooting of the generated adventitious shoots, MS basal culture medium along with IBA was used (0, 0.5, 1, 1.5 mg l^{-1} and 0, 1 and 1.5 mg l^{-1} of IBA for Mexican lime and bitter orange, respectively, in ten populations). The cultured shoots were kept in rooting culture medium for 20 days using day length of 16 h at 27±1°C.

Adaptation of rooted plantlets

For adaptation, the rooted plantlets were transferred to pots containing a mixture of 50% soil and 50% sand and plastic bags were put over them and kept at 25±3°C and at light intensity of 2,000 lux. The pots were initially irrigated with one eigth of the MS salts concentration for 10 days and they were irrigated by tap water subsequently and they gradually adapted with the external environment by forming holes in the plastic bags.

Experiment design and data analysis

At the end of regenerating shoots experiment, the number of shoots, the length of the shoots and the number of leaves were recorded for each explant. At the end of rooting experiment, the numbers of roots, the length of roots, roots fresh and dry weight were recorded. Experiments were conducted in factorial arrangement in a completely randomized design with several replications. The number of replications and the number of explants in each replication are presented at the bottom of each table. Data were analyzed by SPSS software and the means comparison was conducted by Duncan's multiple range test at probability level of 5%.

3. Results

Generating adventitious shoot from nucellus culture of Mexican lime

Explants of nucellar tissue began inflating after being placed on the culture medium in places of cuts and gradually adventitious seedlings and then shoots were emerged in the places of cuts. In this research, the highest number of adventitious shoots (Fig. 1) were retrieved in 2 mg l^{-1} BA and 2 mg l^{-1} GA with the value of 3.33 (Table 1) and there was no significant difference found on the generated adventitious shoots between the media which contained only BA or GA in various concentrations of these growth regulators. Considering the derived results, media containing both growth regulators of BA and GA had more impact on shoot regeneration, comparing to the media containing one of the growth regulators only (Table 1).

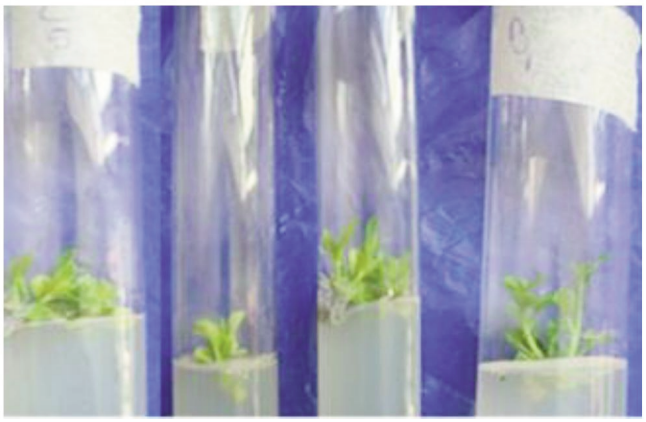

Fig. 1 - Proliferation of shoots of Mexican lime nucellus culture.

The average shoot length in various treatments was different (Table 1). The highest shoot length was derived in treatment of 2 mg l^{-1} of BA and 2 mg l^{-1} of GA with 6.33 cm which had significant difference with the control (0) treatment (Table 1).

The highest rate of leaf generation was 9 leaves in 2 mg l^{-1} of BA and 2 mg l^{-1} of GA treatment, which has a significant difference with the control treatment (Table 1).

Table 1 - The impact of BA and GA growth regulators and their interaction on the number of shoots, length of shoots and number of leaves derived from Mexican lime nucellus culture

Treatment	Number of shoots	Length of shoots	Number of leaves
GA0 mg l^{-1} +BA0 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 0 mg l^{-1}+BA1 mg l^{-1}	2.28 ab	4.4 abc	8.0 ab
GA 0 mg l^{-1} +BA1.5 mg l^{-1}	1.8 abc	3.20 bcd	7.0 ab
GA 0 mg l^{-1} +BA2 mg l^{-1}	1.5 abc	1.75 dec	4.5 bc
GA1 mg l^{-1} +BA0 mg l^{-1}	2.75 ab	5.125 ab	8.2 ab
GA 1 mg l^{-1} +BA1 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 1 mg l^{-1} +BA1.5 mg l^{-1}	2.0 ab	4.167 abcd	5.7 abc
GA 1 mg l^{-1} +BA2 mg l^{-1}	0.0 c	0.0e	0.0 d
GA2 mg l^{-1}+BA0 mg l^{-1}	1.0 bc	1.5 de	2.0 bc
GA 2 mg l^{-1} +BA1 mg l^{-1}	2.33 ab	3.167 bcd	5.1 abc
GA 2 mg l^{-1}+BA1.5 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 2 mg l^{-1}+BA2 mg l^{-1}	3.33 a	6.33 a	9.0 a

GA= Gibberellic acid;
BA= Benzyl adenine;
The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

Rooting of shoots derived from nucellus culture of Mexican lime

Table 2 presents the rooting of separated Mexican lime shoots in culture medium with all concentrations of IBA. The highest amount of roots was 4.3 which was achieved in 0.5 mg l^{-1} of IBA treatment, showing significant difference with the average number of roots in 1 and 1.5 mg l^{-1} and the control treatment. There was no significant difference found between the average root length in concentrations of 0.5, 1 and 1.5 mg l^{-1} and the average length of the control treatment. The highest fresh weight was 0.052 g in concentration of 0.5 mg l^{-1} of IBA that had a significant difference with the control and other treatments. The highest root dry weight was 0.011 g in concentration 0.5 mg l^{-1} of IBA that had no significant difference with the control and other treatments (Fig. 2).

Table 2 - The impact of IBA growth regulator on rooting of shoots derived from Mexican lime nucellus culture

Indole Butyric acid (IBA) mg l^{-1}	Root dry weight (g)	Root fresh weight (g)	Root length (cm)	Number of roots
0	0.0072 a	0.024 b	5.01 a	1.66 b
0.5	0.011 a	0.052 a	4.9 a	4.3 a
1	0.0094 a	0.024 b	6.62 a	2.0 b
1.5	0.007 a	0.018 b	4.93 a	2.28 b

The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

Fig. 2 - Rooting of the *Mexican lime* adventitious shoots.

Generating shoot from nucellus culture of bitter orange

The highest value of adventitious shoot regeneration was in the culture medium containing 1 mg l^{-1} BA and 2 mg l^{-1} GA which had a significant difference with the rest of treatments, including control treatment (Table 3). In this study, no shoot was generated in the culture medium which contained GA only. Also, in culture media which contained BA only, the shoot was generated in 1 mg l^{-1} growth regulator. Considering the retrieved results, culture media containing both growth regulators of BA and GA impact the shoot generation better in bitter orange, similar to Mexican lime (Table 3).

The highest generated shoot length average was in 1 mg l^{-1} of BA and 2 mg l^{-1} of GA treatment which had a significant different with the control treatment (Table 3).

The highest leaf generation was 5 leaves which was related to the 2 mg l^{-1} of BA and 1 mg l^{-1} of GA treatment that had a significant different with the control treatment (Table 3).

Rooting of shoots derived from nucellus culture of bitter orange

Table 4 presents the rooting of separated bitter orange shoots in culture medium with concentrations of 0, 0.5, 1 and 1.5 mg l^{-1} of IBA. The highest number of roots were 3 in 1 mg l^{-1} of IBA treatment, such result did not have any significant difference

Table 3 - The impact of BA and GA growth regulators and their interaction on the number of shoots, length of shoots and number of leaves derived from bitter orange nucellus culture

Treatment	Number of shoots	Length of shoots	Number of leaves
GA0 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 0 mg l^{-1} + BA1 mg l^{-1}	1.33 b	2.6 ab	4 a
GA 0 mg l^{-1} + BA1.5 mg l^{-1}	0 c	0 c	0 c
GA 0 mg l^{-1} + BA2 mg l^{-1}	0 c	0 c	0 c
GA1 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 1 mg l^{-1} + BA1 mg l^{-1}	0 c	0 c	0 c
GA 1 mg l^{-1} + BA1.5 mg l^{-1}	1 b	2 b	2 b
GA 1 mg l^{-1} + BA2 mg l^{-1}	1 b	4 a	5 a
GA2 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 2 mg l^{-1} + BA1 mg l^{-1}	2.25 a	4 a	4 a
GA 2 mg l^{-1} + BA1.5 mg l^{-1}	1.5 b	3.5 a	4.5 a
GA 2 mg l^{-1} + BA2 mg l^{-1}	0 c	0 c	0 c

GA= Gibberellic acid;
BA= Benzyl adenine;
The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

with the average number of roots observed in 1.5 mg l^{-1} treatments, while it had a significant difference with the control. The root length average in each treatment was 10.30 and 8.51 cm respectively for concentrations of 1 and 1.5 mg l^{-1} which showed a significant difference with the control treatment. The highest fresh weight of 0.05 g and the highest dry weight of 0.012 g were observed using concentration of 1 mg l^{-1} of IBA, showing a significant difference with the control treatment.

Table 4 - The impact of IBA growth regulator on rooting of shoots derived from bitter orange nucellus culture

Indole butyric acid (IBA) mg l^{-1}	Root dry weight	Roots fresh weight	Root length	Number of roots
0	0.0 b	0.0 b	0.0 b	0.0 b
1	0.012 a	0.05 a	10.30 a	3.0 a
1.5	0.011 a	0.039 a	8.51 a	2.16 a

The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

4. Discussion and Conclusions

Generating shoot from nucellus culture of Mexican lime and bitter orange

Some seedlings of some citrus cultivars were generated *in vitro* by culturing nucellus explants (Rangan *et al.,* 1968*,* 1969). However, this method has not been successful in all cases (Button and Kochba, 1977). The current study showed that nucellus in Mexican lime and bitter orange species is not capable of generating shoot without plant growth regulators, and application of plant growth regulators could increase their potentials for generating shoot. Usman *et al.* (2005) reported that the number of shoots induced in each explant depends on the citrus type. For instance, in Kinnow mandarin, there is more shoots generated comparing to orange during direct organogenesis. In this experiment, it was indicated that Mexican lime nucellus explants have higher potentials for generating shoot, comparing to bitter orange. Considering the role of plant growth regulators for generating shoot from nucellus, two growth regulators of BA and GA were used in this research. Results have suggested that BA is responsible for generating shoot from nucellus in both species, since in medium without the regulators, there was lower number of shoots in Mexican lime, and there was no shoot in bitter orange. External application of BA is necessary in culture medium (Raj-Bhansal and Arya, 1978). For direct organogenesis, Rattanpal *et al.* (2011) put epicotyl and hypocotyl explants of *Citrus jambhiri* Lush. in culture medium containing BA and this hormone led to generation of shoot in these explants. GA leads to increase the length of the generated shoots (Rattanpal *et al.,* 2011). In studying GA impact, without the presence of BA in the culture medium in this research, it could be claimed that this hormone has been effective on shoot length in both species and the results from this research are in accordance with the results from Saini *et al*. (2010). They reported that adding GA to the culture medium containing BA improves the number of elongated shoots (Saini *et al.,* 2010). Results from this research suggested that using a combination of various levels of BA and GA has been more effective in both species, comparing to the application one of these hormone only. For instance, the highest number of shoots, shoot length and number of leaves in Mexican lime was related to the treatment with 2 mgl^{-1} of BA and 2 mg l^{-1} of GA and in bitter orange, the highest number of shoots and shoot length was related to the use of 2 mg l^{-1} of GA and 1 mg l^{-1} of BA. There are various reports which show that the application of these two plant growth regulators have been effective on shoot generation, such as that of Gill and Gosal (2002) who generated a high rate of shoots in *C. depressa* by applying 1 mg l^{-1} of BA and 2 mg l^{-1} of GA. Also, the highest percentage of shoot generation in explants of *Poncirus trifoliate* was derived when 2 mg l^{-1} of GA and BA were used in culture medium (Usman *et al.,* 2005; Tzatzani *et al.,* 2009).

Rooting of shoots drived from nucellus culture of bitter orange and Mexican lime

The highest rate of rooting for bitter orange was obtained in the medium containing 1 mg l^{-1} of IBA. In Mexican lime the highest rate of rooting was obtained in the medium containing 0.5 mg l^{-1} of IBA. By increasing the concentration of this hormone, there was a decrease in the number of generated roots. Sandra and Morehart in 1998 (in orange) and Singh *et al.* in 2006 (in Mexican lime and tangerine) reported that by the increase in IBA concentration, the rooting rate decreases which are in accordance with the results from this experiment. IBA has been highly successful in rooting in many citrus species, including *Citrus aurantifolia* (Raj-Bhansal and Arya,

1978). In addition to the above mentioned, the results about the impact of IBA on rooting of nucellus shoots in Mexican lime and bitter orange were in accordance with the findings of Khalekuzzaman *et al*. (2008) on *Adhatoda vasica*, Wilson *et al.* (2010) on *Indoneesiella ecohides* and Purkayastha *et al.* (2008) on *Andrographis paniculata*. A great number of lateral roots were generated in Mexican lime in the concentration of 0.5 mg l^{-1} of IBA, while there was no lateral root observed in other treatments.

Transfer and adaptation

The plantlets adapted to the sterilized mixture of equal volume ratio of perlite and vermiculite in 2 months. Since the humidity is high in culture tubes, the humidity for the plantlets is provided by plastic bag during adaptation period and over time the humidity is decreased by forming holes in the plastic bags. All adapted plants were transferred to the greenhouse with 100% success and stayed alive. These results were in accordance with the findings from Dojam *et al*. (2001) in orange in and Jajo (2010) in *Citrus limonia* Osbeck.

Interaction of BA and GA has a higher impact on shooting in Mexican lime and bitter orange rootstocks. In Mexican lime the best culture medium for generating shoots containing 2 mg l^{-1} BA and 2 mg l^{-1} GA and in bitter orange rootstock, the highest shooting rate was attributed to the culture medium containing 2 mg l^{-1} GA and 1 mg l^{-1} BA. The highest rooting rate for Mexican lime was in culture medium containing 0.5 mg l^{-1} IBA and for bitter orange; it was the culture medium containing 1 mg l^{-1} IBA.

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