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Propagation of *Citrus reticulata via in vitro* seed germination and shoot cuttings

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Abstract

Seeds of *Citrus reticulata* were germinated efficiently when they were sown directly after their extraction from fruits harvested in January, and incubated at constant temperature (25 °C). Seed drying decreased both the percentage of seed germination and the number of seedling per seed. Germination of seeds was better on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm^{-3} benzylaminopurine (BAP) than in a soil. Shoot cuttings obtained from germinated seeds were subcultured on B5 medium supplemented with 1 mg dm^{-3} BAP, 0.5 mg dm^{-3} kinetin (KIN) and 0.5 mg dm^{-3} naphthalene acetic acid (NAA), where shoots grew and multiplied. They were rooted on half strength MS medium supplemented with 0.25 mg dm^{-3} BAP, 0.5 mg dm^{-3} NAA and 1 mg dm^{-3} isobutyric acid (IBA). Rooting under light was better than under dark. Seedlings and shoot cuttings with roots were transferred successfully to the soil after three weeks of acclimatization.

Additional key words: mandarin, phytohormones, seed germination, tissue culture.

Introduction

Seed germination is the common procedure for large scale propagation of citrus. It decreased with increase the time between seed extraction and seed sowing (Saipari *et al.* 1998), due to loss of seed water content. Desiccation sensitivity of citrus seeds has been reported by several investigators as an important factor causing poor storability (Fischer *et al.* 1988, Huang and Xiong 1992). Previous studies on germination of citrus seeds indicate that osmotic stress causes decline of seed water content, results in inhibition of seed germination and seedling growth (Zekri 1993). Sankhla and Sankhla (1968) demonstrated that inhibition of seed germination can be counteracted by cytokinins at low concentration. In addition, micropropagation enables production of uniform plantlets in short time through the year, especially useful when seed propagation and conventional vegetative propagation are slow (Onay 2000, Vijaya Chitra and Padmaja 2002)

The present study was undertaken to examine the effect of seed drying and fruit ripening of mandarin on seed germination. Improvement of seed germination of air-dry seed by synthetic medium supplemented with BAP was tested. In addition, micropropagation via shoot cutting of germinated seedlings was investigated.

Materials and methods

Seed extraction: Seeds of *Citrus reticulata* Blanco were extracted from freshly harvested fruits (Bany Hilal, Sohag, Egypt). Seeds were air dried under room condition (20 °C day and 7 °C night) for 0, 1, 2 and 3 weeks. The loss in fresh mass of 25 seeds was determined.

Seeds were disinfected in 5 % commercial bleach solution for 5 min followed by 5 min treatment in 75 % (v/v) ethanol. After three successive rinses in sterile distilled water for 5 min each, the seeds were placed on Murashige and Skoog (1962; MS) agar medium

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Abbreviations: BAP - benzylaminopurine; IBA - isobutyric acid; KIN - kinetin; MS - Murashige and Skoog; NAA - naphthalene acetic acid.
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supplemented with 0.5 mg dm^{-3} benzylaminopurine (BAP) and 3 % sucrose for seed germination. Also, some of these seeds were sown in plastic pots at a depth of 3 cm keeping 5 cm distance between the seeds in sand-clay soil. After three weeks, the percentage of seed germination was determined under the influence of seed drying and temperature treatments (see Tables 1, 2, 3).

Effect of fruit juice on seed germination: Two separate experiments were fulfilled: *1* - Surface disinfected fruits (in 95 % ethanol for 30 s, then flamed for 30 s) incubated in tissue culture room in sterilized backer. *2* - Lopes of sterilized fruit were separated, divided into two halves to bare the seeds on juice vesicles of parenchymatous cells and placed on agar plates of MS medium containing 0.5 mg dm^{-3} BAP under tissue culture room condition. After three weeks, the seeds were investigated to detect seed germination.

Effect of fruit ripening on seed germination: Three different levels of fruit ripening were tested. Green (harvested in November), light-orange (harvested in January) and dark-orange (harvested at the end of March) fruits were used. The seeds were extracted and cultured on MS medium supplemented with 0.5 mg dm^{-3} BAP or sown in soil at constant temperature of 25 °C. The number of seedling per seed was counted and the percentage of seed germination was determined after three weeks incubation.

Shoot culture establishment: Shoot cuttings (about 1.5 cm length) of germinated seeds were subcultured on B5 medium (Gamborg *et al.* 1968) supplemented with 1 mg dm^{-3} BAP, 0.5 mg dm^{-3} KIN and 0.5 mg dm^{-3} NAA or 0.5 mg dm^{-3} BAP alone and incubated in tissue culture room under continuous irradiation (100 μmol m^{-2} s^{-1}) or in dark. The length and the fresh mass of shoots were determined after 3 weeks of incubation. Shoots showing increase in shoot length were cut into segments, each 1.5 - 2.0 cm with one internode or shoot tip. Then they were subcultured on the same type of medium for further growth.

Seedling and plantlet acclimatization for transfer to soil: Shoot cuttings about 1.5 - 2.0 cm length were cultured on half strength MS medium supplemented with 0.25 mg dm^{-3} BAP, 0.5 mg dm^{-3} NAA and 1 mg dm^{-3} IBA and incubated in dark or light (100 μmol m^{-2} s^{-1}). Seedlings or plantlets with extensive root system were washed under running water and potted in a mixture of soil:peat:sand 1:1:1. Two-stage-acclimatization followed under tissue culture room condition: *1*) the pots were covered by plastic bags (25 × 40 cm) for 2 weeks; and *2*) two pores (each about 1 cm radius) were made. The pore size was increased (50 %) each day.

Unless otherwise stated, the following conditions were applied: The agar plates or the plastic pots were maintained in a tissue culture room under white florescent tubes providing an irradiance of 100 μmol m^{-2} s^{-1} during 16-h photoperiod, at a temperature of 25 ± 2 °C and without humidity control. Medium pH was adjusted to 5.8 before adding agar and autoclaving (20 min at 121 °C).

Statistics: Three Petri dishes were used per treatment, and in each dish 10 seeds or explants were cultured. Each experiment was repeated three times. All data were subjected to analysis of variance using statistical package. The significant difference of the average was tested using least significance differences (LSD) at $P < 0.01$ and 0.05.

Results and discussion

The effect of seed drying on seed mass, seed germination and number of seedlings per seed: Mature *C. reticulata* seeds contain relatively high water content (51 %) which declines sharply after their extraction. The seeds lost 32 % of their fresh mass after one week and about 47 % after 2 weeks. The percentage of seed

Table 1. Percentage of germination of seeds subjected to air-drying (0, 1, 2 and 3 weeks at 20 °C day and 7 °C night) and placed on MS basal medium supplemented with 0.5 mg dm^{-3} BAP or on MS medium without BAP. Each value represents the mean ± SD of three experiments; ** - means highly significantly different in relation to control.

Time of drying	Light		Dark	
	+ BAP	– BAP	+ BAP	– BAP
0 (control)	89.0 ± 2.6	87.0 ± 1.0	89.7 ± 2.6	87.0 ± 4.6
1 week	48.7 ± 3.2**	48.0 ± 5.6**	55.0 ± 11.1**	48.3 ± 4.2**
2 weeks	15.3 ± 3.2**	15.0 ± 2.0**	16.0 ± 2.0**	12.0 ± 2.0**
3 weeks	12.0 ± 4.0**	12.0 ± 4.6**	14.0 ± 2.7**	10.0 ± 4.4**
$LSD_{0.05}$	7.6	6.1	9.4	8.9
$LSD_{0.01}$	11.4	9.2	14.3	13.5

germination (Table 1) decreased with increase of water loss due to air drying of seeds during their storage under room condition. This decrease in seed germination was detected on MS medium with or without BAP as well as under dark or light. It was reported also for citrus seeds subjected to osmotic stress (Zekri 1993). BAP did not affect on seed germination under light but improved seed germination under dark. In this work, since it is difficult to prevent ABA accumulation under low water content of seeds (Bengtson *et al.* 1977), it is possible to compensate the loss of *in vivo* cytokinin concentration by applying BAP treatments. This work is in agreement with previous attempts to overcome the seed germination suppression resulting from desiccation using phytohormones (*e.g.* Aharoni *et al.* 1977, Radi *et al.* 1989).

Table 2. Number of seedlings per seed after seed air-drying (0, 1, 2 and 3 weeks) and cultivation on MS basal medium supplemented with 0.5 mg dm^{-3} BAP (agar plates) or sowing in soil at 25 °C. Means ± SD of three experiments; ** - means highly significantly different in relation to control.

Time of drying	Agar plates	Soil
0 (control)	4.0 ± 0.0	3.7 ± 0.6
1 week	2.0 ± 0.8**	1.7 ± 0.6**
2 weeks	1.0 ± 0.0**	0.0
3 weeks	1.0 ± 0.0**	0.0
$LSD_{0.05}$	1.0	0.8
$LSD_{0.01}$	1.5	1.3

Table 3. Percentage of germination of seeds cultured on MS basal medium supplemented with 0.5 mg dm^{-3} BAP (agar plates) or sown in soil and subjected to different temperature treatments. Means ± SD of three experiments; ** - means highly significantly different in relation to control.

Temperature	Agar plates	Soil
20 °C day + 7 °C night	0.0	0.0
20 °C	65.3 ± 9.1**	58.7 ± 5.7**
25 °C	88.0 ± 3.0**	71.0 ± 3.3**
35 °C	81.7 ± 6.1**	63.3 ± 7.0**
$LSD_{0.05}$	9.5	8.1
$LSD_{0.01}$	14.4	16.8

Seed drying affect not only the percentage of seed germination but also the number of seedlings per seed (Table 2). There is no generalization for assumption that desiccation is an obligatory prerequisite for reprogramming of seed development from maturation to germination (Ackerson 1984). Difficulties in water absorption as a result of excessive desiccation and disruption of membrane (Powell and Matthews 1980), could be the reason for the poor germination of dry mandarin seeds. MS medium improved seed germination and increased the number of plantlets per seed, where seeds subjected to two or three weeks of drying in room condition did not germinate in soil (contains full field capacity) but they germinated on MS medium.

Effect of temperature and seed moisture on germination: Seeds cultured on agar plates of MS medium supplemented with 0.5 mg dm^{-3} BAP or sown in soil and subjected to room temperature (7 °C night and 20 °C day) did not show any germination (Table 3). On the other side, at constant temperature 20 °C 65 % of seeds germinated. This fact explains why the seeds do not germinate in soil under the fluctuation of temperature of winter season. On the other side, temperature fluctuations did not produce significant effect on seed germination on Mexican cacti compared to the results obtained at constant temperature (Rojas-Arechiga *et al.* 1998).

Seed germination was affected strongly by seed moisture content. However, no correlation was found between seed moisture content and germination of *Citrus ladanifer* (Perez-Garcia 1997).

Effect of fruit juice on seed germination: Subjection the intact fruits to suitable condition for seed germination did not promote germination of seed still inside the fruit body or in the presence of fruit juice vesicles of parenchymatous cells. It may be due to the presence of high concentration of inhibitory substances in fruit juice of mandarin, which was also detected in tomatoes (Pandey and Sinha 1993). Juice vesicles of parenchymatous cells which did not show any response on MS supplemented with 0.5 mg dm^{-3} BAP, was induced to divide and differentiate as tracheary elements on MS medium supplemented with kinetin or abscisic acid (Khan 1999).

Effect of fruit age on seed germination: Some seeds isolated from green fruits (harvested at the end of November) may have immature embryos (Table 4). Therefore, they were not fit enough to germinate especially in soil (11 %) but they were partially germinated on MS medium (37 %). Such seed required an after ripening period which may be fulfilled successfully when the seeds were germinated under the ideal condition obtained on the synthetic medium. The mass of mandarin seeds was higher when they were extracted from mature fruits harvested in January (0.134 g $seed^{-1}$) than those harvested in November (0.119 g $seed^{-1}$). Our study was in accordance with Khalil (1999) who reported that the high mass of seeds resulted in high rates of seedling emergence, high number of emerged seedlings per seed and more vigorous seedlings. The germination of citrus seeds may be under the control of seed germination inhibitors such as ABA. Increase of ABA concentration during seed development may be due to the synthesis of ABA in embryos or translocation of

Table 4. Number of seedlings and percentage of germination of seeds extracted from fruits at different repining stages and cultured on MS basal medium supplemented with 0.5 mg dm^{-3} BAP (agar plates) or sown in soil at 25 °C. Each value represents the mean ± SD of three experiments. * means significantly and ** means highly significantly different in relation to control.

Fruit harvest	Agar plates germination [%]	number of seedlings [$seed^{-1}$]	Soil germination [%]	number of seedlings [$seed^{-1}$]
Green fruits (control)	36.7 ± 7.2	1.3 ± 0.6	11.7 ± 1.1	2.7 ± 1.2
Yellow orange	88.3 ± 4.1**	3.7 ± 0.6*	72.3 ±10.6**	3.0 ± 1.0
Dark orange	65.0 ± 8.0*	1.7 ± 0.6	56.0 ± 0.6**	1.3 ± 0.6*
$LSD_{0.05}$	18.5	1.5	9.2	1.0
$LSD_{0.01}$	30.6	2.4	15.8	1.7

Table 5. Length and fresh mass of seedling or excised shoots cultured as influenced by medium composition. MS medium was supplemented with 0.5 mg dm^{-3} BAP or 1 mg dm^{-3} BAP in combination with 0.5 mg dm^{-3} KIN and 0.5 mg dm^{-3} NAA. Means ± SD of three experiments; * - means significantly and ** - means highly significantly different in relation to control.

Treatments	Seedling shoot length [cm]	fresh mass [g]	Excised shoot length [cm]	fresh mass [g]
Light + BAP (Control)	3.1 ± 0.3	1.2 ± 0.0	3.3 ± 0.2	1.4 ± 1.0
Dark + BAP	4.3 ± 0.3*	2.0 ± 0.1**	5.1 ± 0.1**	2.4 ± 0.3**
Light + BAP, KIN and NAA	5.2 ± 0.3**	2.5 ± 0.1**	5.3 ± 0.1**	2.5 ± 0.2**
Dark + BAP, KIN and NAA	5.6 ± 0.2**	2.5 ± 0.1**	5.6 ± 0.1**	2.6 ± 0.3**
$LSD_{0.05}$	0.21	0.24	0.13	0.36
$LSD_{0.01}$	0.35	0.40	0.22	0.59

ABA or its precursors from leaves to the developing seeds (Pandey and Sinha 1993). Therefore, the reduction in seed germination of mandarin fruits harvested in March may be due to the presence of seeds in fruit high concentration of seed germination inhibitors for long time.

Shoot culture establishment: The concentration of growth regulators (1 mg dm^{-3} BAP, 0.5 mg dm^{-3} KIN and 0.5 mg dm^{-3} NAA) was chosen from previous studies by various authors (Huang and Xiong 1992, Omura and Hidaka 1992, Singh *et al.* 1994). This combination in B5 medium resulted in maximum shoot growth and shoot proliferation of citrus species. In three weeks, shoots reached about 5 cm length (three times more than in the beginning). Each shoot could be cut into three shoot segments and subcultured on new medium. The chosen combination was better than 0.5 mg dm^{-3} BAP which was used during seed germination experiments (Table 5). Rooting of 85 % of shoots was achieved on half strength MS medium supplemented with 0.25 mg dm^{-3} BAP, 0.5 mg dm^{-3} NAA and 1 mg dm^{-3} IBA in 12 d in tissue culture. At the same conditions, darkness reduced vigorously the percentage of rooting (32 %) and delay the appearance of roots for 5 d, where they appeared in 17 d. After 21 d the plantlets were transferred to soil.

Transfer of the plantlets to soil: Plantlets or *in vitro* grown seedlings were transferred from the Petri dishes into pots. It is well known that plants suffer from a high rate of water loss immediately after transplanting due to the deficiency of epicuticular wax (Sutter and Langhans 1979), the high size of intercellular spaces (Brainerd *et al.* 1981), the slowness of stomatal response to water stress and the poor connection between the adventitious roots and the vascular system of stem. Therefore, acclimatization of plantlets or the seedlings for three weeks under plastic bags was essential prerequisite for successful transfer from Petri dishes to pots. During this time, the plantlets or seedlings undergo morphological and physiological adaptations enabling them to develop sufficient water control (Brainerd and Fuchigami 1981). Direct transfer of the seedlings and plantlets from tissue culture condition to the soil without acclimatization of seedlings resulted in great reduction of transfer efficiency. Acclimatization of plantlets for three weeks resulted in maximum transfer (92 %).

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