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Overcoming Phenolic Accumulation of Date Palm *In vitro* Culture Using α-Tochopherol and Cold Pre-Treatment

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Abstract: Shoot tips were excised from offshoots of superior male date palm and *in vitro* cultured on Muarshige and Skoog (MS) medium. Different antioxidant substances (vitamin E, ascorbic and citric acids) with different concentrations were tested on shoot tips browning, swelling and callus induction. In addition, cold pretreatment at 5°C for three or five days with or without soaking in antioxidants were investigated. Results showed that, soaking of shoot tips in vitamin E before *in vitro* culture enhanced swelling and reduced browning. Moreover, keeping the explants for 3 days at 5°C decreased browning percentage and improved swelling records. Vitamin E as well as cold pretreatments enhanced callus induction significantly.

Key words: Antioxidant · Browning · Callus induction · Cold temperature · Date palm · Swelling · Vitamin E

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) an economically important tree is a monocotyledonous, widely cultivated in arid regions of Middle East and North Africa [1]. It occupies special significance for its distinguished economic, nutritional, esthetic, historic and social values [2]. The multiplication of the species is carried out by using offshoots. Although this method of propagation is vegetative, it limits the expansion of the existing palm groves, especially in North Africa. In addition, it cannot solve two important problems of date palm expansion; the lack of high quality material for date palm and the accumulation of biotic diseases [3] especially Bayoud (lethal disease) to date palm trees [4]. Besides, sexual propagation through seeds has many limitations like seed dormancy, low rate of germination and progeny variation [5]. Recently, *in vitro* culture technique had been employed in this field extensively in order to solve problems related to wide genetic variation by sexual propagation, low number of offshoots (20-30 offshoot/ tree) of elite date palm cultivars, providing a rapid system for production of large number of genetically uniform [6], production of disease-free plants and germplasm conservation [7]. The most commonly used technology approach is somatic embryogenesis, which presents a great potential for the rapid propagation and genetic resource preservation of date palm species. However, several problems still need to be solved and are currently under study [8]; high content of phenolic compounds and its accumulation in culture media which affects vitality of explants and survival percentage and after a while, explants color exchanges into brown and subsequent death of the cultured explants appears. Several attempts had been done in order to reduce *in vitro* explant browning; Baiea [9] found that storing explants of date palm in refrigerator (at 5°C) for 10 days before culturing succeeded in reducing necrosis and browning and increasing development. Bharat *et al.* [10] stated that, 12 hours cold treatments of explants at 4°C prior to sterilization of explants, were found to be the best methods to control browning and therefore to increase the survival rate of cultured explants of the Yali pear. Other methods were followed to overcome *in vitro* explant browning; addition of absorbing agents such as activated charcoal [11] and polyvinylpyrrolidone (PVP) [12], or antioxidant agents such as ascorbic and citric acid [13, 14] have been reported to be effective in preventing *in vitro* explants browning. Ascorbic acid is a naturally occurring organic compound with antioxidant properties. It dissolves well in water to give mildly acidic solutions. Ascorbic acid is one form of vitamin C [15]. In addition, Citric acid is a weak organic acid. It is considered as an excellent cleaning and chelating agent. Meanwhile,

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Tocopherols (or TCP) are a class of chemical compounds of which many have vitamin E activity. It is a series of organic compounds consisting of various methylated phenols. Alpha to delta Tocopherols and Tocotrienols, all of these various derivatives with vitamin activity may correctly be referred to as "vitamin E". Alpha-Tocopherol is the form of vitamin E. Tocopherols and Tocotrienols are fat-soluble antioxidants (http://en.wikipedia.org/wiki/). Anthony *et al*. [16] reported that, Tocopherols are known to show high antioxidant activities and its mechanism based on findings that one mole of Tocopherol can trap more than two mole of radicals and can trap alkyl radicals as well as peroxy radicals. To minimize browning, Murashige [17] has suggested the pre-soaking of explants in ascorbic and citric acid solution and adding them to the culture medium for decreasing the oxidation of the phenolic compounds. Zaid [18] soaked date palm explants in antioxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid) prior to surface sterilization treatment. In addition, he rinsed disinfested date palm explants with sterilized distilled water supplemented with the following antioxidants and adsorbents in g/l: caffeine: 2; sodium dichyldithiocarbonate: 1; and polyvinylpyrolidone (PVP): 1. Addition of combination of adsorbents including citrate adenine, glutamine and PVP retarded browning in date palm explants.

This study aims to investigate the effect of pretreatment of low temperature and antioxidant substances on reducing *in vitro* browning and their effect on the development of date palm explants.

MATERIALS AND METHODS

The present study was conducted in Biotechnology fruit Lab. of Pomology Department, National Research Centre, Dokki, Egypt during the period from 2011 to 2012.

Preparation of Explants: Offshoots of a superior male date palm (*Phoenix dactylifera* L.) were prepared by removing the old leaves and other outer parts until the appearance of white soft leaves surrounding the shoot tip. After that, they were subjected to running tap water for two hours then divided into two groups: first one was soaked in different antioxidant substances (First experiment), second group subjected to cold pretreatment (Second experiment). Under aseptic conditions, all explants in both experiments were surface sterilized using ethyl alcohol (70%) for 1-2 minutes then rinsed once with sterile distilled water and transferred to 50% Clorox (2-5% sodium hypochlorite) and two drops of tween 20 per 100 ml solution for 20 minutes. All traces of the used disinfected were removed by rinsing three times in sterilized distilled water. Shoot tips with 6-8 leaf primordial were excised as explants. The prepared explants were sectioned longitudinally into several pieces and cultured on the Murashige and Skoog basal medium supplemented with 10.0 mg/l 2, 4 D, 3.0 mg/l 2iP, 100 mg/l glutamine, 5 mg/l thiamine HCl, 1 mg/l biotine, 30 g/l sucrose, 2.0 g/l activated charcoal [19] and solidified with Difco agar at 6.0 g/l the pH of the medium was adjusted to 5.7 and autoclaved at 121°C and 15 Ib/in^2 for 20 minutes. After culturing the explants, the jars were directly plugged with polypropylene closure caps. All the cultures incubated in the growth room under controlled conditions, where the temperature was maintained at 27±2°C and the cultures were kept under dark conditions.

Table 1: Antioxidant concentrations

Antioxidant	Concentration
Control	Distilled water only
Ascorbic +citric acid	15 %+10%, respectively
Vitamin E 1	20%
Vitamin E 2	40%
Vitamin E 3	80%

First Experiment

Effect of Antioxidant Treatments: Vitamin E (α-Tochopherol) was employed at different concentrations (Table 1) as antioxidant in comparison to mixture of (150 mg/l ascorbic acid+100 mg/l citric acid). Whereas, date palm explants were soaked in these antioxidants and kept for 24 hours at the refrigerator at 5°C before culturing.

Second Experiment

Effect of Cold Temperature Pre-Treatment: In this experiment, explants were divided into two groups first group soaked in mixture (150 mg/l ascorbic acid and 100 mg/l citric acid), second group soaked in distilled water and both of them were incubated in refrigerator at 5°C for different periods (3 or 5 days).

Total Phenols Accumulation: After 6 weeks of growth, the explants were removed and the total amounts of phenols in the culture media (for excreted phenols from explants to medium) were analyzed according to Folin-Ciocalteu method [20] by using gallic acid as the standard and the results were given as gallic acid equivalents [21].

Statistical Analysis: Data for browning and swelling were collected after six weeks, while data for callus induction were collected after three subcultures with six weeks intervals. Scores were given for browning, swelling and callus induction as follows: Negative results = 1, below average = 2; average = 3, above average = 4 and excellent = 5 according to Pottino [22]. Treatments were arranged in complete randomized design, each treatment was replicated three times, each replicate involved 5 Jars and each contained three explants. Means were compared according to the method described by Snedecor and Cochran [23].

RESULTS

Effect of Antioxidant Treatments Date Palm Explant: Data presented in Table 2 indicated that vitamin E treatments statistically reduced both browning percentage and browning degree as compared with the other treatments. Vitamin E 2 and vitamin E 3 treatments gave the lowest browning percentage as well as browning degree. Concerning total phenols, it is clear that treating explants with various antioxidants reduced total phenols with a significant difference with the control. Moreover, vitamin E treatments showed less phenols accumulation compared with the mixture of ascorbic and citric treatment however, these results were insignificant. Table 3 and Fig. 1 (a, b, c, d and e) show that vitamin E treatments encouraged a significant increase in swelling percentage as well as swelling degree. Vitamin E 1 treatment gave the highest percentage of swelling. Meanwhile, vitamin E 3 treatment gave the highest degree of swelling followed by vitamin E 2 treatment. On the other hand, both the mixture of ascorbic acid + citric acid and control treatments produced the lowest significant effect on swelling parameter under study in comparison with the other treatments. Concerning callogenesis, vitamin E treatments encouraged a significant increase in callus induction whereas; vitamin E 2 treatment gave the highest callus induction followed by vitamin E 3 treatment while the control did not show any response for callus.

Effect of Cold Temperature Pre-Treatment on Date Palm Explant: Table 4 show the effect of cold temperature as pre-treatment on browning of date palm explants. It is noticed that, incubating date palm explants for 3 days in refrigerator at 5°C before *in vitro* culturing reduced browning percentage of the explants as well as browning degree in relation to the other period and the control.

Table 2: Effect of type and concentration of antioxidant on the browning percentage, browning degree and total phenols of date palm explants

Parameter Antioxidant treatments	Browning (%)	Browning degree	Total phenols
Control	53.33	4.40 a	38.04 a
Ascorbic +citric acid	33.33	3.20 b	22.47 b
Vitamin E.1	40.00	2.6 c	13.51 b
Vitamin E.2	20.00	1.27 d	20.11 b
Vitamin E.3	26.67	1.40 d	16.71 b

Table 3: Effect of type and concentration of antioxidant on swelling percentage, swelling degree and callus induction of date palm explants

Parameter Antioxidant treatments	Swelling (%)	Swelling degree	Callus degree
Control	20.00	3.20 d	0.0 e
Ascorbic +citric acid	20.00	3.27 d	0.33 d
Vitamin E.1	100.00	3.67 c	1.07 c
Vitamin E.2	60.00	4.33 b	2.67 a
Vitamin E.3	60.00	5.00 a	1.67 b

Means followed by the same letter with each column are not significantly different from each other at 1% level

Table 4: Effect of cold temperature as pre-treatment on browning percentage, browning degree and total phenols of date palm explants

Parameter Antioxidant treatments	Browning (%)	Browning degree	Total phenols
Control	53.33	4.40 a	38.04 a
3 day – antioxidant	20.00	1.27 c	12.83 b
3 days + antioxidant	46.67	3.00 b	12.16 b
5 days – antioxidant	53.33	3.20 b	17.23 ab
5 days + antioxidant	60.00	4.27 a	37.58 a

Means followed by the same letter with each column are not significantly different from each other at 1% level

Table 5: Effect of cold temperature pre-treatment on swilling percentage swelling degree and callus induction of date palm explant

Parameter Antioxidant treatments	Swelling (%)	Swelling degree	Callus degree
Control	20.00	3.20 c	0.0 d
3 day – antioxidant	100.00	4.53 a	1.67 a
3 days + antioxidant	46.67	3.00 c	1.07 b
5 days – antioxidant	33.33	4.07b	0.5 c
5 days + antioxidant	26.67	3.00c	0.5 c

Means followed by the same letter with each column are not significantly different from each other at 1% level

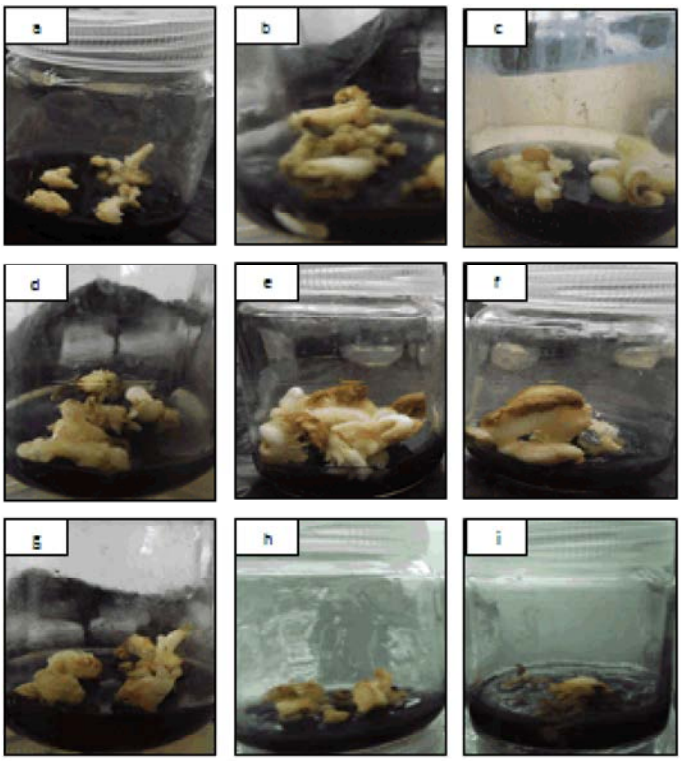

Fig. 1: Show the effect of different antioxidants substances and storage in low temperatures treatments on swelling as the following:
a) Control, b) Mixture of 150 mg ascorbic acid +100 mg/L citric acid, c) Vitamin E.1, d) Vitamin E.2, e) Vitamin E.3, f) Storage for 3days at 5°C, g) storage for 3day at 5°C + antioxidant, h) Storage for 5days at 5°C and i) storage for 5days at 5°C + antioxidant

On the other hand, keeping the explants for 3 days at 5°C + antioxidant treatment as well as 5 days in refrigerator at 5°C with or without antioxidant failed to induce any significant differences when browning was considered. Concerning total phenols, data show that, keeping the explants for 3 days in refrigerator at 5°C with or without antioxidant showed the lowest total phenols compared with the control. Table 5 and Fig. 1 (f, g, h and i) show the effect of cold temperature pre-treatment on swelling. It is noticed that incubating date palm explants for 3 days at 5°C before *in vitro* culturing increased swelling (swelling percentage and swelling degree) in relation to the other periods and the control. In addition, swelling production was not statistically affected by keeping the explants in cold treatment for 3 days + antioxidant or 5 days + antioxidant in comparison with the control under study. Similar results were obtained in callus induction whereas; 3 days of cold treatment gave the highest degree of callus.

DISCUSSION

Based on the aforementioned results, data show that pre-treatment with vitamin E achieved the best swelling and the lowest browning results. This was due to the effect of vitamin E as an antioxidant in reducing oxidation of phenolic compounds as compared with other

pre-treatment and the control. Gupta and Datta [24] stated that exogenous application of antioxidants such as glutathione and α-tocopherol and ascorbate inhibited somatic embryogenesis but stimulated shoot organogenesis. Ascorbic and citric mixture gave lower results compared with V.E treatments. This result is in harmony with the findings of Taha [25] who stated that treated explants with 150 mg/l citric acid and 100 mg/l ascorbic acid prior to surface sterilization treatment, did not gave satisfied results for reducing browning of mango explants. Total phenols analysis shows that there is a significant difference between the control and the other antioxidants treatments. The control treatment showed the highest total phenols and it is found to be correlated to browning appeared with the control. Two new phenolic compounds were isolated from date palm (*Phoenix dactylifera* L.) callus and have been identified to be polar hydroxycinnamic acid derivatives (DHC3 and DHC4). Their accumulation is being in relation with tissue browning that occurs during *in vitro* culture [26]. Wounding explants and *in vitro* technique are considered a stress that leads to release phenols from explants to the medium. The involvement of the phenolic metabolism in resistance of plant to different stress (abiotic or biotic) was in general characterized by an increase in total phenolics content caused by an accumulation of compounds present before the stress and/or the appearance of compounds which had not previously been detected [27]. In addition, vitamin E treatments encouraged a significant increase in swelling and callus induction. This result was in harmony with the findings of Reustle and Ingeborg [28], who stated that the application of 0.5 % PVP reduced browning of the culture media to a low level. In addition, it remarkably improved the formation of microcalli from grapevine protoplasts.

Considering cold pretreatment, results show that incubating date palm explants for 3 days at 5°C without antioxidant before *in vitro* culturing decreased browning (browning percentage and degree) to the lowest level. In addition, it increased swelling production (swelling percentage and swelling degree) as well as callus induction (callus degree) in relation to the other periods and the control. Herath *et al*. [29] indicated that effect of cold pre-treatment of panicles with reduced temperatures (5°C, 7°C, 8°C and 10°C) and exposure for different time durations (at weekly intervals up to 4 weeks) on anther culture of Japonica and Indica rice varieties and their F1 hybrids for high frequency callus induction and plant regeneration was investigated. It was found that cold treatments generally increased callus induction of tested varieties. In addition, cold pretreatment for 3-7 days at 4°C was found to have a positive influence on the embryo induction frequency of rice genotypes anther culture [30]. Tang *et al.* [31] stated that brown callus derived from anther limited the application of anther culture in balsam pear (*Momordica charantia* L.). After pre-treatment at 4°C for 1 day, callus induction rate was the highest and browning rate was the lowest. When anthers pretreated for more than 3 days, it was hardly induced callus. These findings was similar to the current investigation in which incubating date palm explants for 5 days at 5°C gave low response to callus induction. Reducing browning and total phenols accumulation could be due to the effect of cold pre-treatment on phenolic oxidation enzymes such as polyphenol oxidase and peroxidase in which their activity was inhibited with cold temperature. Ohlsson [32] stated that, one of the most commonly used approaches to control enzymatic activity in fresh–cut products is the use of low temperature, which reduced enzymatic browning.

CONCLUSION

It could be concluded that pre-treatment with V.E at 40% is recommended to achieve the lowest browning and the highest callus induction results in date palm cultures. In our knowledge, it is the first time to use vitamin E or Tochopherol as an antioxidant in date palm *in vitro* cultures. In addition, cold pre-treatment for three days at 5°C is recommended when the using of antioxidant is not preferred.

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