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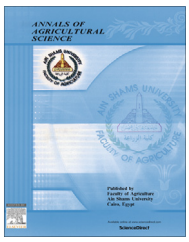

Some biochemical changes and activities of antioxidant enzymes in developing date palm somatic and zygotic embryos *in vitro*

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Abstract Some biochemical changes and activities of antioxidant enzymes in different developmental stages of date palm somatic and zygotic embryos were studied *in vitro*. The levels of endogenous ascorbic acid (AA), dehydroascorbic acid (DHA) and some biochemical components (phenols, flavonoid, free amino acids, proteins, and malondialdehyde (MDA)) and the activities of antioxidant enzymes peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in the different developing stages of somatic and zygotic embryos of date palm cv. Sewi were determined. Zygotic embryo and also embryogenic callus contained high concentrations of AA (9.48 and 9.13 mg 100 g^{-1} f.wt., respectively). While the mature somatic embryos contain the lowest concentration of AA (4.09 mg 100 g^{-1} f.wt.), Zygotic embryo contains the highest significant levels of phenols, flavonoid, free amino acids, proteins and MDA. However, the lowest concentrations of phenols and flavonoids were recorded at the embryogenic callus. The POD activity was the highest level at embryogenic callus and then decreased gradually during the subsequent developmental stages, while, the activities of PPO and PAL were the highest levels at zygotic embryos.

Introduction

Date palm is subject to an intensive exploitation in Mediterranean Africa, the Middle East, West Asia and the United States. In exporting countries as in self-consumer ones, it is advantageous to quickly replace the aged or diseased trees. This replacement should be done with off-shoots offering identical characteristics of fruit quality, flavor and productively with the selection cultivars (Booij et al., 1993). It is well known that date palm is propagated sexually through seeds and vegetatively by off-shoots (Alkhateeb, 2006). *In vitro* plant regeneration of date palm occurs through organogenesis and somatic embryogenesis.

Somatic embryos are used as a model system in embryological studies. However, the greatest importance of somatic embryos is its practical application in large scale vegetative propagation. In some cases, somatic embryogenesis is favored

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over other methods of vegetative propagation by using bioreactors (Von Arnold et al., 2002).

Rapid cell proliferation and active aerobic metabolism, which occur mainly in the presence of auxin and cytokinin, are often associated with the production of reactive oxygen species. Reactive oxygen species (ROS) which include compounds such as superoxide, peroxide, singlet oxygen, and the hydroxyl radical, are an inevitable by-product of aerobic metabolism, being produced during the electron transfer reactions that take place in the mitochondria, chloroplasts, and peroxisomes. ROS are toxic molecules; unless their concentration is regulated, they can cause protein, membrane, and DNA damage and ultimately cell death (Mittler, 2002).

The regulation of the cellular redox state is a crucial point for plant development and responsiveness to environmental stimuli. Several redox metabolites are involved in maintaining the ideal redox balance in plant tissues, among which ascorbate (ASC) plays a pivotal role by acting as a redox buffer as well as a sensor of metabolic changes involving redox reactions (Foyer and Noctor, 2003).

Pullman et al. (2009) estimated the contents of endogenous ascorbic acid (AA), dehydroascorbic acid (DHA), glutathione (GSH) and glutathione disulfide (GSSG) in developmentally staged zygotic embryos and female gametophytes over the sequence of loblolly pine (*Pinus taeda* L.) seed development. Their data refer that, AA acid and DHA peaked in mid-development and then decreased in the embryo. Both glutathione and glutathione disulfide in both embryo and female gametophyte increased until mid-development and then decreased.

It was established that AA is a co-factor of several enzymes involved in the synthesis of ethylene, gibberellins (Prescott and John, 1996) and possibly ABA (Arrigoni and De Tullio, 2000). It was found that ascorbic acid acts within the meristems, where it is required as a factor necessary for the cell cycle progression during cell division (De Tullio et al., 1999). Ascorbate affects also the elongation of plant cells. Kerk and Feldman (1995) found that ascorbate is able to stimulate quiescent cells to divide by stimulate auxin transported to the root meristem.

Taqi et al. (2011) suggest that endogenous ascorbic acid (AA) has been involved in the promotion of plant growth and development. AA plays an important role in resistance to oxidative stresses such as heavy metal, saline and ultraviolet. Rapidly increasing evidence indicates that AA is centrally involved in several physiological processes.

Plants have several L-AA biosynthetic pathways including routes *via* L-galactose and L-glucose (Wolucka and Van Montagu, 2003), but the contribution of each one varies between different species, organs and developmental stages (Cruz-Rus et al., 2011).

When the endogenous AA level is experimentally lowered by lycorine, an inhibitor of the AA *de novo* biosynthesis, both of cell division (Liso et al., 1984) and cell elongation (Córdoba-Pedregosa et al., 1996) are inhibited. These inhibitory effects of lycorine are ascribed to suppression of the biosynthesis of AA by blocking conversion of the precursor L-galactonic acid-γ-lactone to ascorbate and in lycorine treated tissues, addition of galactone–lactose induces ascorbic acid biosynthesis comparable to controls (De Gara et al., 1994).

De Pinto and De Gara (2004) studied the AA level, redox state and its related enzymes, as well as peroxidase (POD) isoenzymes, which were analyzed in the apoplastic and symplastic compartments of different segments of pea (*Pisum sativum* L.) shoots which were indicative of different differentiation levels. Their obtained data showed opposite gradients in the AA system and PODs during cell differentiation.

Studies of lipid peroxidation in plant tissue cultures demonstrate that aldehydes are produced during culture initiation and throughout routine sub-culturing. They also accumulate in cultures which have lost their totipotency, compared to those who have maintained their regeneration potential (Adams et al., 1999). Aldehydic products, formed by *in vitro* cultures may be deleterious as they have the potential to cross-link with key macromolecules which are essential to the maintenance of growth and development. The cytotoxic effects of the aldehydes were, however, partially reversible and on transfer to aldehyde-free media, some of the treated callus cells were able to recover their proliferative and morphogenetic capabilities (Deighton et al., 1997). Adams et al. (1999) demonstrated that Malondialdehyde (MDA) is produced *de novo* by plant cells and that their exogenous addition can inhibit *in vitro* growth and development.

Improved understanding of ascorbate roles in plants will lead to the possibility of enhancing the growth of tissues *in vitro* by increasing ascorbate concentration that will exogenously be supplied to the culture media. It could be that the present report is the first one estimate the endogenous levels of AA, DHA, AA/DHA ratio and total forms of ascorbic acid (TFAA) in date palm somatic embryos grown *in vitro*.

This study aimed to underlie the biochemical and metabolic status during somatic embryogenesis process in date palm which could be important for the development and optimization of strategies for large scale propagation and germplasm conservation.

Material and methods

Plant material and tissue culture protocol

The present study was performed throughout the period from 2013 to 2015. Usual protocol for date palm (*Phoenix dactylifera* L.) somatic embryogenesis was made to obtain the different developmental stages of somatic embryos to determine the chemical analyses in this study. Starting with choosing the healthy off-shoots of the selected cultivar (Sewi) grown at Giza governorate, then, many layers of tough, fibrous leaf bases, must first be removed with machete or hack-saw until the apical region (entire meristem and its three or four leaf primordial) has been exposed. After this, surface sterilization was made under aseptic conditions by immersion for the explants in 0.01% mercuric chloride ($HgCl_2$) for 1 h and thoroughly washed with sterilized distillated water for 3 times. Then, the explants were cut longitudinally into four equal segments and were cultured on MS (Murashige and Skoog, 1962) inorganic salts supplemented with 170 mg l^{-1} $KH_2PO_4 \cdot 2H_2O$ + 100 mg l^{-1} myo-inositol + 0.4 mg l^{-1} thiamine hydrochloride + 200 mg l^{-1} glutamine + 30 g l^{-1} sucrose + 10 mg l^{-1} 2,4-D + 3 mg l^{-1} 2iP + 6 g l^{-1} agar + 1.5 g l^{-1} activated charcoal. After this, media were dispensed into culture jars (200 ml) at the 40 ml/jar and the jars were capped with polypropylene closures and autoclaved at 121 °C 1.5 kg/cm^2 at 20 min. Then, the cultures were incubated in a temperature-controlled room at 27 °C + 2, under total darkness for 4–8 months and the sub-cultures were made