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Full Length Research Paper

The effect of natural antioxidant(s) on date palm (*Phoenix dactylifera* L.) *in vitro*

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Date palm (Phoenix *dactylifera L.*) is one of the most valuable economic resources in the Middle East and North Africa that grow on monocotyledonous trees. To increase crop yield of palm trees, *in vitro* micro-propagation has become an attractive alternative for large-scale production of date palm. A problem that frequently damages tissues in the early micro-propagation is the brown color that advances in the callus culture due to the creation of quinones. Quinones seize plant cellular developments which lead to cellular decay. This study advocates the use of antioxidant factors found in spinach, kale and strawberries within various concentrations (50, 150 and 300 mg/L) with respect to the medium culture, in an attempt to reduce the level of total phenol and browning which occurs, and also to improve growth and development in different *in vitro* stages of date palm (*P. dactylifera L.*). The results indicate that better growth value of callus was achieved using 150 mg/L of kale concentration; allowing the total phenol level to be reduced to 0.9237 mg/g D.W, presenting a significant growth value in comparison to the other treatments in the embryonic callus stage. In the date palm's somatic embryogenesis stage, the results show that the use of 50 mg/L of spinach, 50 mg/L of kale, 150 mg/L of strawberries, achieved a high number of somatic embryos and the total phenol level was reduced to 0.6167 mg/g D.W. Results from date palm shoot proliferation shows that high numbers of shoot (16.3) was achieved using 50 to 300 mg/L of kale; however, total phenol level was reduced to 0.04567 at 150 mg/L of spinach concentration. The fluctuation of reducing total phenol level in date palm was recorded when the explants were grown on medium supplemented with 50 mg/L of kale concentration.

Key words: Date palm, tissue culture, natural antioxidants, browning, quionones.

INTRODUCTION

Date palm (*Phoenix dactylifera L.*) is an important crop that grows in the arid regions of North Africa, Middle East and the Arabian Peninsula. Date palm is a chief nutriment for the locals and plays substantial roles in the industrial, economic and environmental aspects of these areas. Furthermore, date palms have the ability to be manipulated in numerous ways making them a crop that adds ease to its production (Al-Khateeb, 2008).

The ecological aspects of date palm tree is that it has the ability to be grown ideally where permanent water

table is present within the soil surface; minimally 8 to 9 acre feet of irrigation water per year is required for fine palm production. The mean temperature between the flowering and the ripening period should be above 2°C rising to 26°C for at least a month, to ensure proper fruit ripening. It takes about six months for the dates to ripe. There are certain regulators that withhold irrigation during fall and winter. There must be no rain during flowering time; extreme winter temperatures are harmful to this crop. Its cultivation process may be propagated through seeds or off shots. It takes about eight years in Egypt for an offshoot to yield economically (Chao and Krueger, 2007).

Through recent breakthroughs in tissue culturing methods, culture propagation is ideal for the expansion of date production. Date palm *in vitro* plant regeneration occurs through organogenesis and somatic embryogenesis relying on the genotype and hormonal manipulations. The utmost method for date palm regeneration used by various cultivators is somatic embryogenesis from shoot tip derived callus; since it has feasibility in micropropagation scale-up for commercial requirements. Date palm somatic embryogenesis involves series of consecutive stages beginning with callus induction, embryogenic callus multiplication, somatic embryo formation, shoot formation and finally rooting (Al-Khateeb, 2008).

There is a major problem that encounters tissue culturing techniques, which is the “browning color” that advances in the callus. This phenomenon results from physiological changes in the cultured tissues that lead to gradual browning and death of the tissues eventually. The brown color that advances in callus cultures of various plant cultures is due to the creation of quinines, which prevent plant cellular growth. The increase of quinones to a certain level harms *in vitro* growth especially in explants from woody plants (Mustafa et al., 2013).

Phenol oxidation frequently damages tissues in the early stage of micropropagation and advances in browning of explants which slows down growth or leads to death of the explants. Oxidative browning can sometimes be evaded by washing, soaking or stirring the explants in antioxidant solutions as a pretreatment before moving them on the media.

To be precise, adopting certain measures with the culturing of plant parts during winter and spring seasons, incubation of tissues in the dark especially in the first three months and adding charcoal to the medium can reduce this phenomenon (Mustafa et al., 2013; Panaia et al., 2000; Wu and du Toit, 2004).

The proposition of the use of caffeine, activated charcoal and polyvinylpyrrolidone (PVP) in the culture media of Eucalyptus was tested not to assist in avoiding exudation of phenolic compounds (Gill and Gill, 1994). Similarly, the proposition of adding PVP and ammonium citrate aids in reducing the explants of date palm shoots from browning (Mustafa et al., 2013).

This study investigate the alternative ways to avoid the browning phenomenon drawback in date palm (*P. dactylifera L.*) explants during tissue culturing procedures, through the use of natural antioxidants (kale, spinach and strawberries).

MATERIALS AND METHODS

The work was carried out in The Central Laboratory for Research and Development of Date Palm, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt, throughout the year of 2010.

Callus formation

The embryogenic callus produced and established from the shoot tip were transferred and cultured on Murashige and Skoog (1962) basal media supplemented with 200 mg of glutamine, 2 mg/L of glycine, 30 g/L of sucrose, 6 g/L of agar, and 0.1 mg/L of NAA. The formed callus was sub-cultured to fresh media with three different concentrations that is, 50, 150, and 300 mg/L from each extraction juice of spinach, kale and strawberry. For the control, 2 g/L PVP were added to the media instead of antioxidant source. All culture jars were maintained in complete darkness at 27 ± 2°C for four weeks.

The data was excerpted as an average per explant according to Pottino (1981) for growth value of the culture visual calculations; while total phenols were calculated with the use of the spectrophotometer.

Embryoids initiation

The investigation of effect of different concentration of spinach, kale and strawberry extraction juice on callus differentiation and initiation of embryoids was carried out through white friable embryonic nodular callus (0.1 g in weight and 1 to 2 mm in diameter) which were obtained from embryonic callus formation medium. They were transferred and cultured on embryoids differentiation medium which consisted of MS basal media supplemented with 200 mg of glutamine, 2 mg/L of glycine, 30 g/L of sucrose, 6 g/L of agar, 0.1 mg/L of NAA, 0.5 mg/L of ABA with three different concentrations that is, 50, 150, and 300 mg/L from each extraction juice of spinach, kale and strawberry. PVP was used as the control of antioxidant source by adding 2 g/L to the medium culture. All culture jars were kept in the complete darkness at 27 ±2°C for four weeks. After four weeks, data was collected through the counted number of embryos; while total phenols were calculated with the use of the spectrophotometer.

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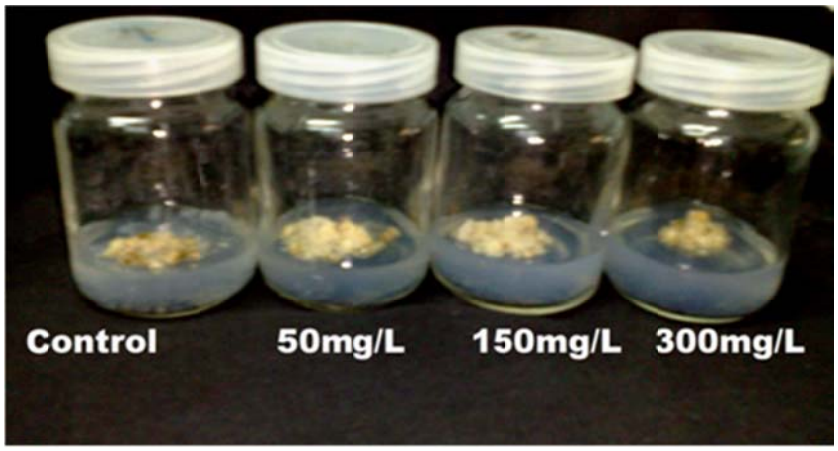

Plate 1. Date palm *c.v. Malakaby* embryogenic callus treated with different concentrations of Spanish juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

Shoot proliferation

The investigation of the effect of different concentration of spinach, kale and strawberry extraction juice on shoot proliferation was conducted by developing two to three shoots in a small cluster. They were developed from germinated mature embryos of *Malakaby*, then cultured on MS basal nutrient media, supplemented with 200 mg/L of glutamine, 2 mg/L of glycine, 30 g/L of sucrose, 6 g/L of agar, 0.05 mg/L of BA, 0.1 mg/L of NAA and three different concentration from each of spinach, kale and strawberry extraction juice (that is, 50, 150 and 300 mg/L), and 2 g/L PVP were added. Small jars (150 ml) were used to dispense the nutrient medium of each treatment and 2 g/L of PVP was used as the control of antioxidant source. All culture jars were kept in the complete darkness at 27± 2°C for four weeks. After four weeks of culturing, data was collected through the counted number of shoots; while total phenols were calculated with the use of the spectrophotometer

Total soluble phenols determination

Phenols were determined by the colorimetric method described by Snell and Snell (1953). A procedure of folin (A.O.A.C, 1980) was adapted for determining the total soluble phenols in the ethanolic extract from 0.05 g of dry materials. Folin-Denis reagent was prepared by transferring 100 g of sodium tungstate to 25 g of sodium molybdate and 700 ml water was added to a 1500 ml flask. The mixture was also supplied with 50 ml of 85% phosphoric acid and 100 ml of concentrated hydrochloric acid attached to reflux condenser, then boiled gently for 10 h, after which 150 g of lithium sulphate, 50 ml water, and a few drops of liquid bromine were added. To remove the bromine excess, the mixture was boiled without attaching the condenser, cooled and diluted to one liter. The total soluble phenols were calculated as mg pyrogallol/g of dry weight.

Statistical analysis

The experiments were carried out using completely randomized blocks design and three replicates. The results were analyzed using variance analysis, while the means were compared using L.S.D. at 5% level. The entire data were subjected to variance analysis with completely randomized blocks according to Snedecor and Cochran (1980).

RESULTS

This study advocates the use of antioxidant factors to reduce the level of total phenol and browning which occurs, and to improve growth and development in different *in vitro* stages of date palm (*P. dactylifera L.*). The effects of the different antioxidants on the growth values and total phenols levels were evaluated. The following data exposes the results obtained.

The effect of the antioxidants with various concentrations on the embryonic callus of date palm

The effect of three different concentrations (50, 150, or 300 mg/L) of spinach, strawberry and kale juice as antioxidants on embryogenic callus browning are presented in Plates 1 to 3 respectively. Generally, by adding the antioxidants, browning were reduced compared to the control. The best result was shown with the use of 150 mg/L of the strawberry extraction and 50 mg/L from spinach and kale extractions as antioxidants and callus appeared healthier with less browning. For callus formation, using the 300 mg/L from the antioxidants affected negatively the callus formation. However, the medium supplemented with 50 mg/L of both strawberry and spinach extraction juices gave almost 20% growth value of the embryogenic callus compared to the control (Figure 1). In the case of using kale extraction juice, 150 mg increased the growth value

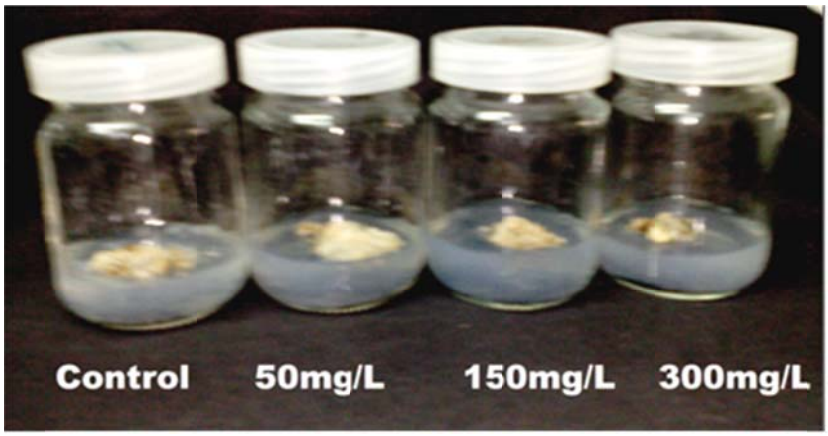

Plate 2. Date palm *c.v. Malakaby* embryogenic callus treated with different concentrations of Strawberry juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

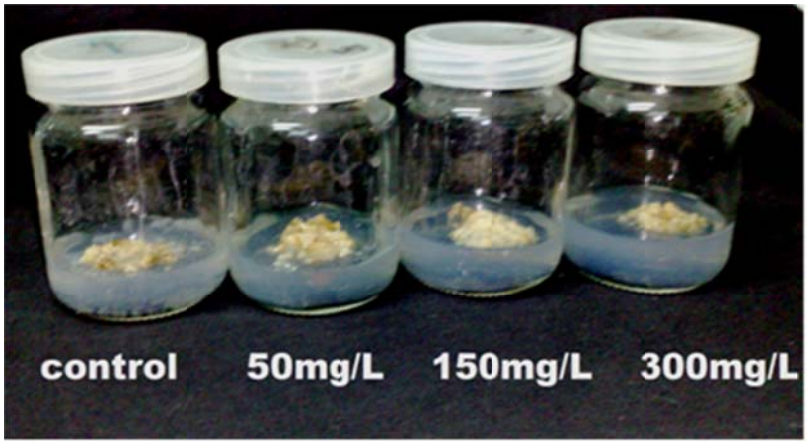

Plate 3. Date palm *c.v. Malakaby* embryogenic callus treated with different concentrations of Kale juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

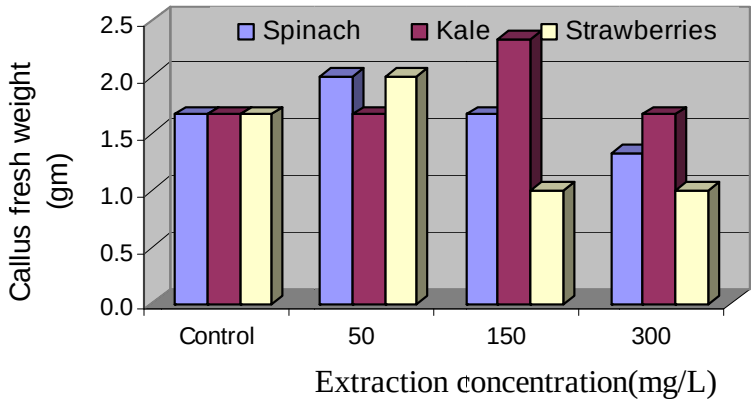

Figure 1. Effect of different concentrations of spinach, kale and strawberry juice as antioxidants on the growth value of embryogenic callus of date palm *c.v. Malakaby.*

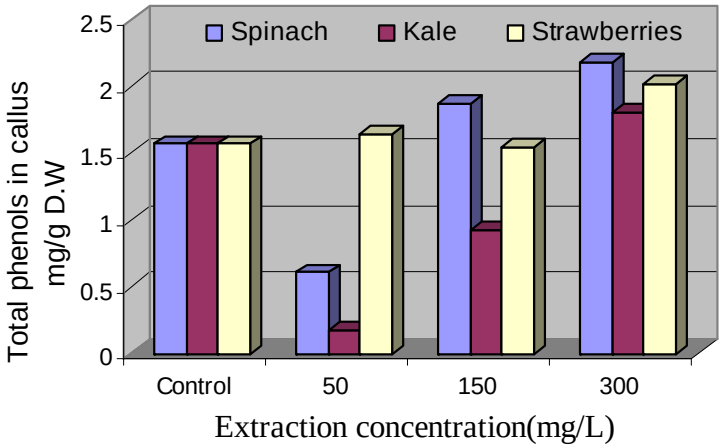

Figure 2. Effect of different concentrations of spinach, kale and strawberry juice as antioxidants on the total phenols of embryogenic callus of date palm *c.v. Malakaby.*

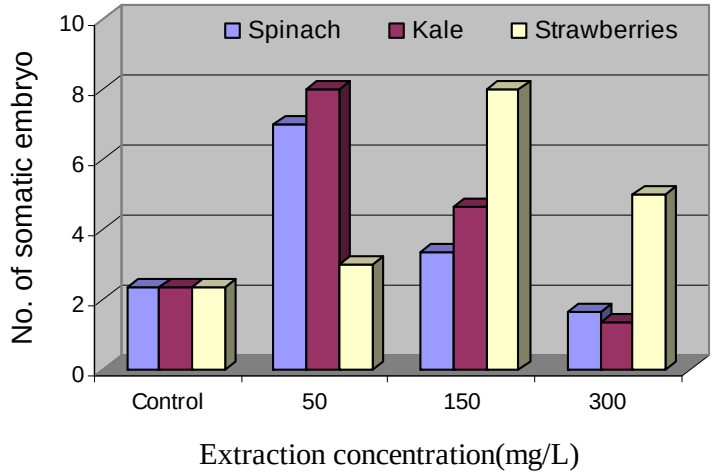

Figure 3. Effect of different concentrations of spinach, kale and strawberry juice as antioxidants on number of embryo of somatic embryo of date palms *c.v. Malakaby*.

by almost 35% (Figure 1). Concerning the total phenol levels, it was clear that treating the embryonic callus cultures with kale at the concentration of 50 and 150 mg/L gave the value of 0.17 mg/g D.W. and 0.92 mg/g D.W, which reduced the total phenol by 79.1 and 41.2% respectively, and had the preeminent results amongst other antioxidants (Figure 2). Overall, for these results, the better type of antioxidant for callus formation was kale at the concentration of 150 mg/L (Figures 1 and 2).

The effect of the antioxidants with various concentrations on the somatic embryos of date palm

In the date palm's somatic embryogenesis stage, the results show that the use of 50 mg/L of spinach and 150 mg/L of strawberries elevated the number of somatic embryos to almost 4 times the control. Meanwhile, using 50 mg/L of kale extraction achieved almost 3 times the number of somatic embryos for date palm with the control (Figure 3). The effect of the three antioxidants on total phenol level is presented in Figure 4. Two of the extraction juices that is strawberries and spinach at the lowest concentration (50 mg/L) gave the significant reduction on the total phenol level to the extent of 60 and 46% with the value of 0.6167 and 0.862 mg/g D.W respectively compared to the control. These percentages become lesser when the concentration increased to 150 and 300 mg/L. For the kale extraction juice the level of total phenol was decreased to the lowest value (0.549

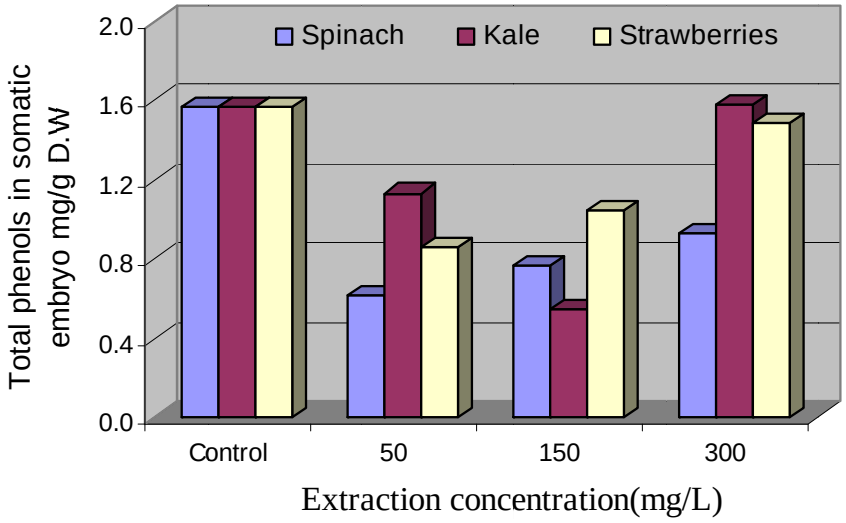

Figure 4. Effect of different concentrations of spinach, kale and strawberry juice as antioxidants on total phenols of somatic embryo of date palm *c.v. Malakaby.*

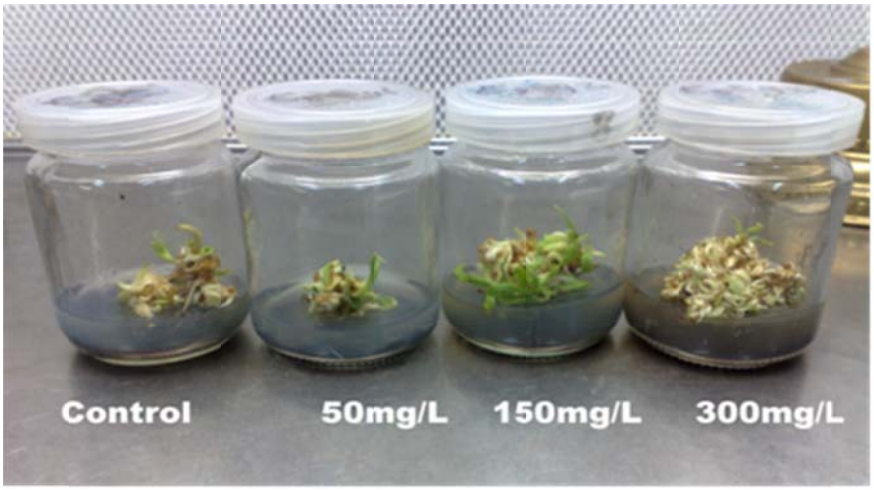

Plate 4. Date palm *c.v. Malakaby* embryogenic somatic embryo treated with different concentrations of strawberry juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

mg/g D.W) over all the nine treatments when concentration of 150 mg/L was added. This significant reduction was calculated as 66% from the total phenol in the control. The level of browning are illustrated in Plates 4 to 6 which reflect the results. When calculating the mean per type of antioxidant, strawberries presented the finest results; while calculating the mean per concentration, 50 mg/L gave the best results. These results suggest that for somatic embryos initiation, the better antioxidant was Kale extraction juice at the concentration of 50 or 100 mg/L (Figures 3 and 4).

The effect of the antioxidants with various concentrations on the shoot proliferation of date palm

Results from date palm shoot proliferation shows increase value with all treatments for the three antioxidants than control (Figure 5). Among the three antioxidants, high numbers of shoot (16 and16.3) were achieved using 50 and 300 mg/L of kale extraction juices followed by the three concentrations of strawberry juice (Figure 5 and Plates 7 and 8), in addition, the spinach

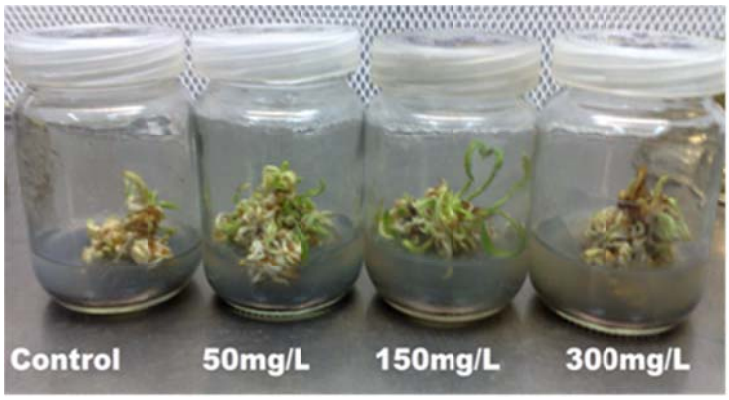

Plate 5. Date palm *c.v. Malakaby* embryogenic somatic embryo treated with different concentrations of kale juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP

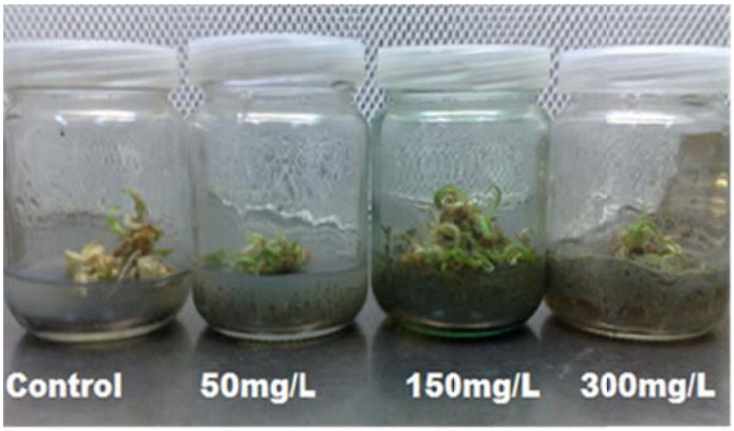

Plate 6. Date palm *c.v. Malakaby* embryogenic somatic embryo treated with different concentrations of spinach juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP

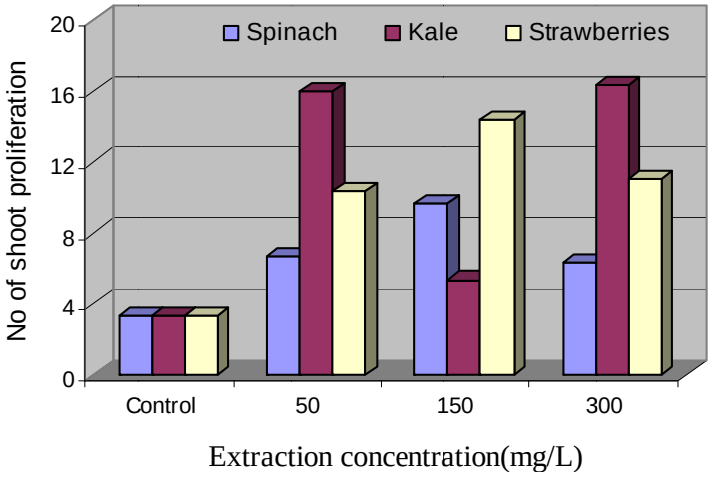

Figure 5. Effect of different concentrations of spinach, kale and strawberry juice as antioxidants on number of shoots of shoot proliferation of date palm *c.v. Malakaby*.

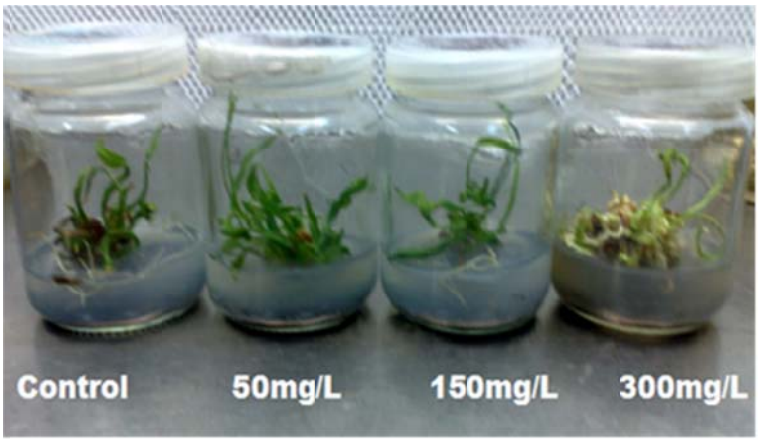

Plate 7. Date palm *c.v. Malakaby* embryogenic shoot proliferation after treated with different concentrations of kale juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

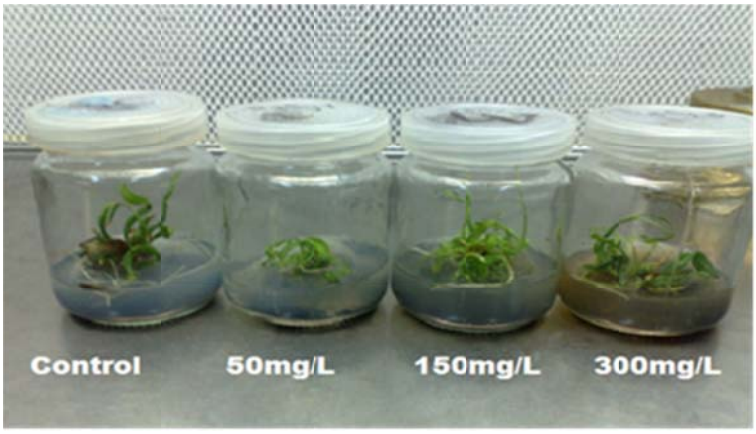

Plate 8. Date palm *c.v. Malakaby* embryogenic shoot proliferation after treated with different concentrations of strawberry juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

antioxidant showed the lowest effect amongst them (Figure 5 and Plate 9). Total phenol level in shoots was highly significantly reduced (95%) to the value of 0.04567 mg/g D.W at 150 mg/L of spinach concentration (Figure 6). Moreover, a significant reduction in total phenol by almost 75% was calculated with two treatments from strawberry antioxidant (150 and 300 mg/L) (Figure 6), meanwhile, no more than 24% of reduction of total phenol level in date palm was recorded when the shoots were grown on the medium containing any of the kale different concentrations (Figure 6). When calculating the mean per type of antioxidant, kale conveyed preeminent results with the shoot proliferation, while combining that with results of total phenol level, it was suggested that treating the shoot proliferation cultures with strawberry at the concentration of 150 mg/L might give the best result (Figures 5 and 6).

DISCUSSION

Commercial cultivation and expansion of date palm (*P. dactylifera L.)* had unsatisfactory upshots due to varieties of undesired contamination, the incidence of contamination in the culture could sometime lead to important losses. An obstacle caused by phenol oxidation during the callus phase, and the callus was subjected to browning.

In this study, callus initiation, somatic embryoids and shoot proliferation have been subjected to three different concentrations of various antioxidants, which consist of spinach, kale and strawberries. The growing or dipping of the explants in the antioxidant concentrations has proved to be efficient with almost negligible or no browning noted. The results were parallel with those of Abdel Sattar's (1999), which describes the effect of reducing

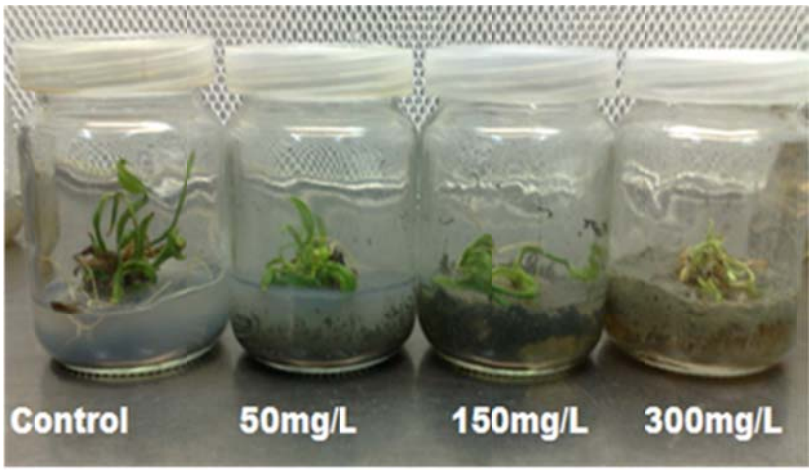

Plate 9. Date palm *c.v. Malakaby* embryogenic shoot proliferation after treated with different concentrations of spinach juice (50, 150 and 300 mg/L) as antioxidants. The control was treated with PVP.

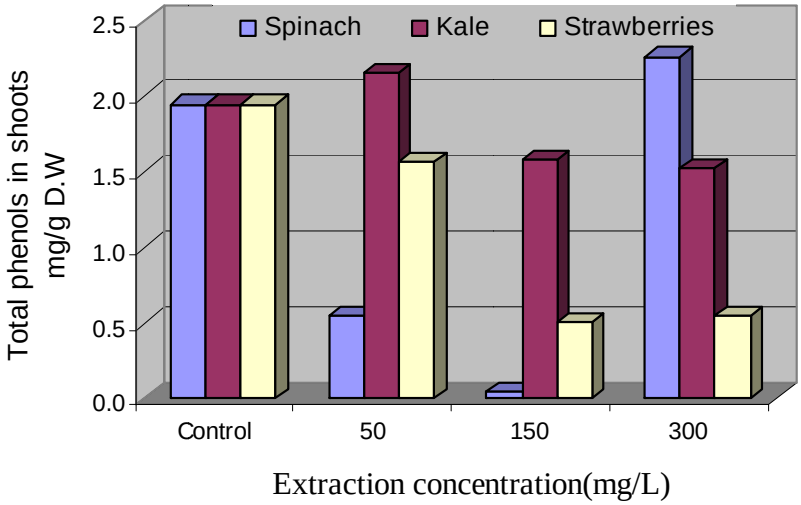

Figure 6. The effect of different concentrations of antioxidants on the total phenols of the shoot proliferation: the figure represents Spinach, kale and strawberry juice as antioxidants on total phenols of shoot proliferation of date palm *c.v. Malakaby*.

the browning on the callus induction rate.

The effect of kale as an antioxidant on the treatment was to reduce the phenolic compounds as compared with the control, which used PVP as the factor of reducing the browning color in the cultures.

It has been tested in various studies conducted in immature date embryo culture that the liquid endosperm of *Cocos nucifera* (coconut milk) showed stimulation in cell division in other cultured tissues and is used as an antioxidant to overcome contamination problems, in comparison to applying the tested antioxidants (kale, spinach and strawberry) (Duhamet and Gautheret, 1950; Morel, 1950; Nickell, 1950; Duhamet, 1951; Henderson et al., 1952; De Ropp et al., 1952; Archibald, 1954; Wiggans, 1954). Also, other complex plant juices and liquid endosperms have been shown to possess stimulatory properties more or less similar to those of coconut milk. These include liquid endosperm from immature corn (Netien et al., 1951), tomato juice (Nitsch, 1951; Straus and La Rue, 1954), immature fruits and seeds (Steward and Caplin, 1952; Steward and Shantz, 1959), orange juice, malt extract, yeast extract, casein hydrolysate, leaf

extracts, sap from a number of plants and tumor extracts (Butenko, 1968) in comparison to date palm. Similarly, Straus (1960) has shown that tomato juice, yeast extract or casein hydrolysate function by supplying a form of organic nitrogen (a mixture of amino acids) while malt extract provide an auxin, kinetin, minositol, urea and arginine to *in vitro* cultured explants (Steinhart et al., 1961). The mentioned experiments serve as a support to the hypothesis that natural antioxidants have positive preeminent impact on the date palm culture and rate of callus induction.

Conclusion

It could be concluded that treatments with antioxidants from natural sources did have favorable results in reducing the total phenol levels, which plays a role in the browning that occurs in cultures. In addition, those observed at high concentrations of antioxidants had no positive effects in reducing the phenol levels. It is recommended to use concentrations between 50 to 150 mg/L of the stated antioxidants for utmost results. Furthermore, it was also revealed that for the date palm cultures kept in the dark, the explants' tissues started numbering and swelling for callus formations at a higher rate than the ones kept in the light.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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