Original Article

The Antioxidant Enzymatic Activity of Date Palm Seedlings Under Abiotic Drought Stress

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ABSTRACT

Objective: the present research was focused to evaluate the date palm resistance to osmotic stress exerted by three concentrations of poly ethylene glycol 6000 in three months seedlings as reflected by the change in phenolic and flavonoid content and the activity of catalase, peroxidase and polyphenol oxidase. **Materials and Methods:** The phenolic and flavonoid content and the target enzyme's activity was estimated at four time points; 0,1,3,7 days. **Results and Discussion:** The phenolic and flavonoid content as well as the activity of catalase, peroxidase and polyphenol oxidase were increased by increasing the poly ethylene glycol and the treatment period. **Conclusion:** Phenolic and flavonoids are non-enzymatic antioxidants with high scavenging potential to the free radical produced from the oxidative stress resulted from the drought tolerance in date palm seedlings. In addition, the increase in the enzymatic activity of catalase, peroxidase and polyphenol oxidase under drought stress is very important to get rid of the free radicals and the reactive oxygen species.

Key words: Date palm, Phenol, Flavonoid, Catalase, Peroxidase, Polyphenol oxidase.

INTRODUCTION

Phoenix dactylifera L. (date palm) belongs to the genus *Phoenix* of (family *Arecaceae*). It has high economic value worldwide especially in the Middle East Countries and North Africa. Its place of origin is probably around Iraq.[1]

Date palm tree is drought tolerant, so it can tolerate the substantial water deficit in the soil or in the atmosphere, which is the constraint to crop productivity and yield stability worldwide.[2] Drought affects a variety of vital physiological and biochemical processes in plants that cause a reduced growth and consequently a reduced final crop yield.[3,4,5]

Al-Khayri and A-Bahrany.[6] stated that water availability is one of the principal limitations of crop production, particularly in the arid and semiarid regions where date palm is predominantly grown.

Due to its huge size, most physiological studies on date palm trees are achieved on seedling which originated from seed or tissue culture.[3,4,7,8] Al-Ka'aby and Abdul-Qadir.[7] studied the effect of water stress on callus induction from shoot tips of date palm (*Phoenix dactylifera* L.) cv. Bream cultured *in vitro*. Saidi *et al.*[9] stated that brittle leaf disease induces an oxidative stress and decreases the expression of manganese-related genes in date palm (*Phoenix dactylifera* L.).

Al-Senaidy and Ismael[10] purified and characterized peroxidase from date palm leaves and suggested that it could be a promising tool for applications in different analytical

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determinations as well as for treatment of industrial effluents at low cost.

Date palm callus cultures exhibiting enhanced tolerance to salinity by exposure to NaCl, potassium chloride (KCl) and calcium chloride (CaCl2), and drought stress assessed in response to varying concentrations of poly-ethylene glycol (PEG-8000) were *in vitro* selected.[11]

El Rabey *et al.*[4,5] analyzed the proteome of date palm under drought and salinity stress condition and defined the genes responsible for drought and salinity tolerance. The response of date palm seedling against drought stress exerted by treatment with three levels of poly ethylene glycol at four-time points (0, 1, 3, 7 days) was studied in the light of phenolic and flavonoid content and the enzymatic activity of catalase, peroxidase and polyphenol oxidase.

MATERIALS AND METHODS

Date palm seedlings

Three months seedlings were obtained by germinating date palm seeds (Sagie cultivar) according to the protocol of Sané *et al.*[8] as follows: seeds were sacrificed with concentrated sulfuric acid (96%) for 5 min, washed 5 times with sterile distilled water, followed by sterilization with 1% mercuric chloride for 3 min, washed 5 times with sterile distilled water, and then imbibed for 48 h in distilled water. Seeds were sterilized a second time with calcium hypochlorite (5%) for 4 min and then washed 4 times with sterile distilled water. The sterilized seeds were germinated between wet layers of tissue papers until the radical reached 1 cm and then transferred to pots containing beet moss and irrigated with tap water until the age of 3 months. The three months-old seedlings were subjected to 3 different drought stress conditions using polyethylene glycol 6000 (PEG): 6.9%, 13.95% and 27.5% of PEG. Samples were harvested at 4-time points, zero point at the start of the experiment, 24 h, 3 days and 7 days.

Chemicals

Ammonium molybdate, 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH)were purchased from Fluka (Germany). Hydrogen peroxide, guaiacol and catechol were purchased from Sigma (USA). Other Chemicals were purchased from Riedel-de-Haen (Germany).

Preparation of methanol extract

10 ml of 80% methanol were added to two g of date palm leaf powder, extracted by shaking at 150 rpm and 25ºC for 24 h, and then filtered through Whatmann filter paper no. 1.

Estimation of total flavonoid content

The modified colorimetric method described by Zhishenet and team.[12] was used in estimation of the total flavonoid content. In this method, catechin was used as a standard for estimation of total flavonoid content. 250 ml of the standard solution (methanol extract) was mixed with 1.25 ml distilled water and 75 m of 5%$NaNO_2$ solution. The mixture was combined with 150 ml of 10% $AlCl_3$ solution after standing for 6 min. A 0.5 ml of 1 M NaOH and 275 ml distilled water was added to the mixture after waiting for 5 min. The absorbance was calorimetrically measured at 510 nm. The total flavonoid content was then quantified using the calibration curve obtained by measuring the absorbance of known concentrations of catechin. The obtained values are expressed as mg catechin equivalent (CE)/g tissues.

Determination of total phenolic content

The method of Velioglu *et al.*[13] was used in the estimation of the total phenolic content. In this method, a 50 ml of the methanol extract was mixed with 850 ml of methanol and 100 ml of Folin–Ciocalteu reagent, and then allowed to stand for 5 min at room temperature. After that, a 50 ml of 20%sodium carbonate was added and allowed to react with the mixture for 30 min, and then the absorbance was measured at 750 nm. The total phenolic content could be calculated from the calibration curve obtained by measuring the absorbance of known concentrations of gallic acid. The obtained total phenolic content is expressed as mg gallic acid equivalent (GAE)/ g tissues.

Antioxidants assay

Radical cation decolorization assay

The ABTS (2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) forms a relatively stable free radical, which decolorizes in its non-radical form. The scavenging activity of $ABTS^{\bullet+}$was determined according to the method of Re *et al.*[14] $ABTS^{\bullet+}$ was obtained by the reaction of 7 mM ABTS in H_2O with 2.45 mM potassium persulfate and stored in dark at room temperature for 16 h. Dilution of $ABTS^{\bullet+}$ was achieved to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). After that, 1 ml of $ABTS^{\bullet+}$ solution was added to the crude methanol extract. The absorbance was measured 1 min after mixing. The percentage of radical scavenging was calculated in relation to a blank containing no scavenger. In addition, the

extent of decolorization was calculated as the percentage reduction of absorbance. The scavenging capability of test compounds was calculated using the following equation:

$$\text{ABTS} + \text{scavenging}\ (\%) = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

The results were plotted by stetting the % of scavenging activity against the concentration of the sample. The inhibition concentration (IC_{50}) is the amount of crude methanol extract required for 50% of free radical scavenging activity. In addition, the IC_{50} value was calculated from the curve as the antioxidant concentration required for providing 50% free radical scavenging activity.

DPPH radical scavenging activity

The scavenging activity free radical of the crude methanol extract was determined following the method of Ao *et al*.[15] using 2,2-diphenyl-1-picrylhydrazyl (DPPH). 100 µl of the methanol extracts were added to 900 µl of freshly prepared 0.1 mM DPPH methanol solution. Methanol was used as a control (an equal amount). It was incubated in dark for 30 min at room temperature, and then the absorbance was measured at 517 nm.

The activity of scavenging percentage was calculated as follows:

$$\text{DPPH radical scavenging}\ (\%) = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

The results were plotted by setting the % of scavenging activity against the concentration of the sample. The IC_{50} value was calculated from the curve as the antioxidant concentration required for providing 50% free radical scavenging activity.

Determination of antioxidant enzymes activity

Preparation of the crude extract

One g of milled date palm leaf was homogenized in 20 mM Tris–HCl buffer (pH 7.2) contained 0.1 M NaCl and 2% Triton-x100 using homogenizer. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was stored at −20°C for further analysis.

Estimation of catalase activity

Catalase activity was determined according to the method of Bergmeyer.[16] 2.5 ml of the substrate solution was made up to 25 mM $H_2O_2$with 75 mM sodium phosphate buffer (pH 7.0) and crude extract. The decrease in absorbance at 240 nm and 25°C was recorded for 1 min. A unit of catalase activity was defined as the amount of the enzyme that causes a change of 0.1 in absorbance per min under standard assay conditions.

Estimation of peroxidase activity

The peroxidase activity was estimated according to the method of Miranda *et al*.[17] In 1 ml tube, the least amount of crude extract (100 µl) was mixed with (900 µl) 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer (pH 5.5). The absorbance was measured at 470 nm after 1 min due to guaiacol oxidation. A unit of peroxidase activity is defined as the amount of enzyme which increased the O.D. 1.0 per min under standard assay conditions.

Estimation of polyphenoloxidase activity

The activity of polyphenol oxidase was assayed with catechol as a substrate according to the spectrophotometric procedure of Jiang *et al*.[18] 100 µl of the enzyme solution was rapidly added to 900 µl of 40 mM catechol solution prepared in 0.01 M sodium phosphate buffer (pH 6.8). The increase in absorbance at 400 nm and 25°C was recorded for 3 min. A unit of polyphenol oxidase is defined as the amount of the enzyme that causes a change of 0.1 in absorbance per min.

Statistical analysis

The standard error, the mean value and all curves were calculated and plotted using Origin 8 program.

RESULTS

Table 1 show that the total phenolic (gallic acid equivalent) and flavonoid (catechin equivalent) content were increased by increasing the PEG concentration and the exposure period compared to the control.

Antioxidant activity of GAE of date palm leaf on reduction of DPPH and ABTS radical scavenging.

The antioxidant activity of gallic acid equivalent as revealed by reduction of DPPH and ABTS radical scavenging of drought stressed date palm is shown in Table 2 and Figure 1 and Figure 2, respectively.

The free radical scavenging activity of DPPH and ABTS is reduced by increasing PEG concentration and increasing exposure time as revealed from the mean value of the correlation coefficients (R^2) between phenolic contents and DPPH and ABTS scavenging activity, respectively and the IC_{50}.

The activity of catalase in date palm leaf under drought stress

Table 3 and Figure 3 (A) show the activity of catalase in the leaf of date palm under stress using the three concentration of PEG under study.

Table 1: The total phenolic and flavonoid contents in date palm leaf under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

Days	PEG	Phenolic content mg GAE/ g tissue	Flavonoid content mg CE/ g tissue
control	0	3.4± 0.024	0.99± 0.002
1 day	6.9%	3.57± 0.032	1.19± 0.003
	13.96%	3.86± 0.046	1.28± 0.002
	27.5%	3.94± 0.041	1.36± 0.004
3 days	6.9%	4.01± 0.053	1.48± 0.003
	13.96%	4.05± 0.055	1.52± 0.004
	27.5%	4.33± 0.052	1.56± 0.005
7 days	6.9%	4.56± 0.053	1.59± 0.003
	13.96%	4.74± 0.056	1.7± 0.002
	27.5%	4.89± 0.054	1.74± 0.0021

GAE, gallic acid equivalent, CE, catechin equivalent. Values are presented as means ± SD (n =5), values are presented as means ± SD (n =5).

Table 2: Antioxidant activity of GAE of date palm leaf on reduction of DPPH and ABTS radical scavenging under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

Days		DPPH		ABTS	
	PEG	R2	IC_{50}(µg GAE)	R2	IC_{50}(µg GAE)
control	0	0.982	23.1± 0.32	0.955	8 ± 0.34
1 day	6.9 %	0.996	11.58± 0.18	0.989	7.19 ± 0.30
	13.96 %	0.985	11.4± 0.21	0.993	6.45 ± 0.26
	27.5 %	0.981	11± 0.16	0.997	5.81 ± 0.31
3 days	6.9 %	0.976	9.864± 0.15	0.989	4.78 ± 0.28
	13.96 %	0.9969	9.75± 0.17	0.973	4.25 ± 0.14
	27.5 %	0.947	9.24± 0.18	0.999	3.82 ± 0.10
7 days	6.9 %	0.941	8.468± 0.075	0.990	2.7 ± 0.094
	13.96 %	0.918	7.34± 0.10	0.992	1.72 ± 0.096
	27.5 %	0.9238	7.049± 0.12	0.993	1.4 ± 0.089

IC_{50}: is the inhibition concentration as µg GAE of the test sample that decreases 50% of DPPH and ABTS radicals. Values are presented as means ± SD (n = 5). R^2 are the correlation coefficients between phenolic contents and DPPH and ABTS scavenging activity, respectively.

The activity of peroxidase in date palm leaf under drought stress

Table 4 and Figure 3 (B) show the change in the activity of peroxidase in the leaf of date palm under stress using the three concentration of PEG (6.9%, 13.9% and 27.5%) under study. The activity of peroxidase was increased by increasing the PEG concentration and the treatment period.

The activity of polyphenoloxidase in date palm leaf under drought stress

Table 5 and Figure 3 (C) show the change in the activity of polyphenoloxidase in the leaf of date palm under stress using the three concentration of PEG (6.9%, 13.9% and 27.5%) under study. The activity of polyphenoloxidase was increased by increasing the PEG concentration and the period of treatment.

DISCUSSION

The current study showed that the activity of catalase, peroxidase and polyphenol oxidase in date palm leaves was increased with the increase in PEG concentration due to the increase of osmotic stress, as well as the increase in the exposure period to drought stress. This result is consistent with that of Huseynova[19] and El Rabey *et al.*[5] on wheat and Kar and Mishra.[20,21,22] on rice. This result also agrees with that of Wang [23] who noticed the increase in the antioxidant enzyme activity during germination of alfalfa under salt and drought stresses subjected to drought stress.

The osmoregulation is considered a physiological adaptation under stress condition, which occurs by reducing cellular water potential via accumulation of a variety of organic and inorganic solutes in the cell and increase

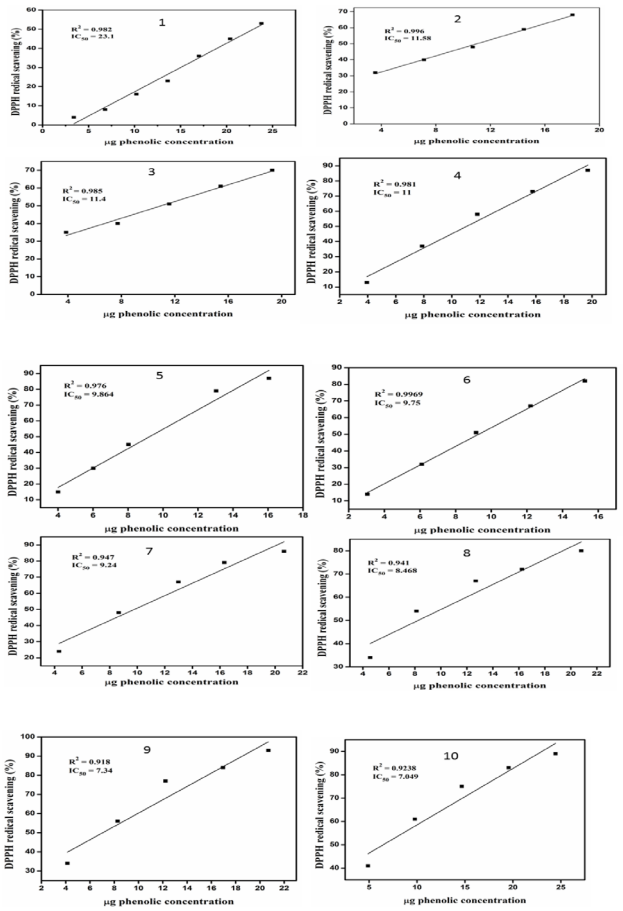

Figure 1: Correlation between concentrations of phenolic compounds of date palm leaf, and their antioxidant activity as determined by DPPH assay.1: control; 2: 1day,6.9% PEG ; 3: 1day,13.9% PEG; 4: 1day,27.5% PEG ; 5: 3days,6.9% PEG; 6: 3days,13.9% PEG; 7: 3days,27.5% PEG; 8: 7days,6.9% PEG; 9: 7days,13.9% PEG; 10: 7days,27.5% PEG.

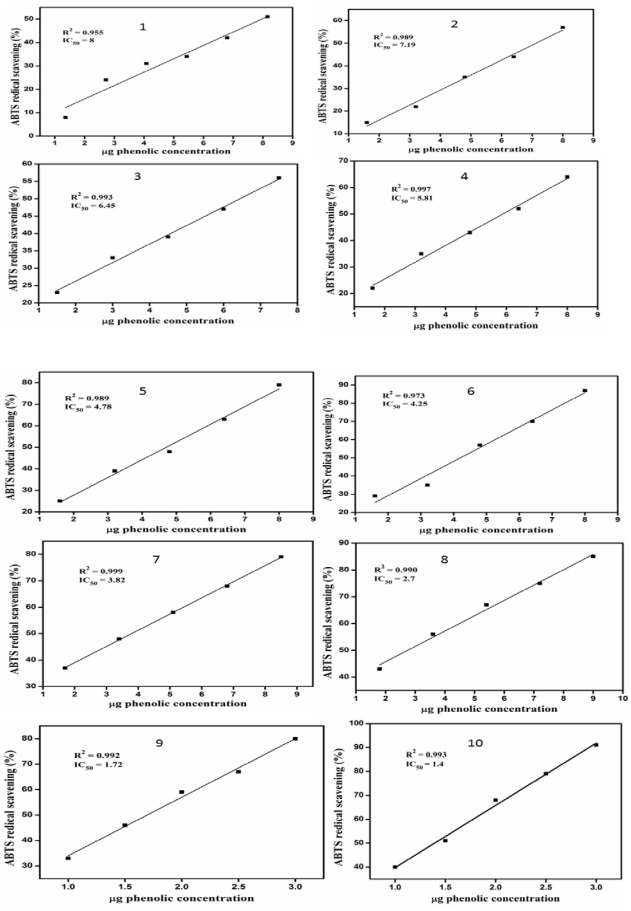

Figure 2: Correlation between concentrations of phenolic compounds of date palm leaf, and their antioxidant activity as determined by ABTS assay.1: control; 2: 1day,6.9% PEG ; 3: 1day,13.9% PEG; 4: 1day,27.5% PEG ; 5: 3days,6.9% PEG; 6: 3days,13.9% PEG; 7: 3days,27.5% PEG; 8: 7days,6.9% PEG; 9: 7days,13.9% PEG; 10: 7days,27.5% PEG.

Table 3: Activity of catalase under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

Catalase	6.9%PEG unit/g/min	13.95% PEG unit/g/min	27.5% PEG unit/g/min
control	54	54	54
1 day	59	62	69
3 days	65	71	77
7 days	78	80	83

Table 4: Activity of peroxidase under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

peroxidase	6.9% PEG unit/g/min	13.95% PEG unit/g/min	27.5% PEG unit/g/min
Control	118	118	118
1 day	119	124	132
3 days	123	137	148
7 days	135	146	159

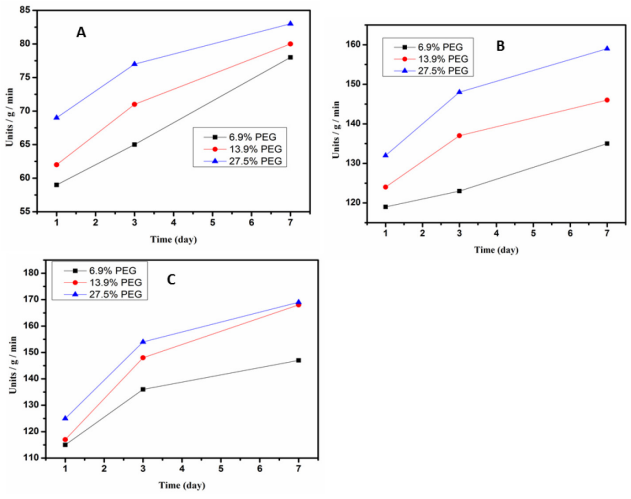

Figure 3: The activity of catalase (A), peroxidase (B) and poly phenol oxidase (C) under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

Table 5: Activity of polyphenoloxidase under drought stress exerted by different concentrations of PEG (6.9%, 13.9% and 27.5%) for 1, 3 and 7 days.

Polyphenoloxidase	6.9% PEG unit/g/min	13.95% unit/g/min	27.5% unit/g/min
Control	106	106	106
1 day	115	117	125
3 days	136	148	154
7 days	147	168	169

the oxidative stress.[2,5] Under drought stress, plants are capable of taking up water from a low water potential medium to sustain normal or near normal physiological processes necessary for growth and development.[2]

The increase in the activity of the target enzymes under drought stress in the current study is in agreement with the theory says that plants produce a variety of antioxidants that counteract the generation of reactive oxygen species (ROS) in response to drought stress.[21,22,23] Phenol and flavonoids of date palm leaves under study were increased as a result of increasing the concentration of PEG and the treatment period. These two compounds represent the non-enzymatic antioxidants scavenging the free radicals resulted from the oxidative stress exerted by PEG treatment.[24] The increase in phenolic and flavonoids content and the enzymatic activity of catalase, peroxidase and polyphenol oxidase together with other responses and adaptations enable plants to sustain growth and development under drought condition.[2,5] In addition, Proteome analysis of date palm under drought stress showed that drought stress elicits the genes responsible for photosynthesis.[3,4] The phenolic and flavonoids content and the enzymatic activity of catalase, peroxidase and polyphenol oxidase were increased by the increase of PEG concentration and the treatment period. Phenolic and flavonoids are non-enzymatic antioxidants with high scavenging potential to the free radical produced by the oxidative stress resulted from the drought tolerance in date palm seedlings. Moreover, the increase in the enzymatic activity of catalase, peroxidase and polyphenol oxidase under drought stress is also important to get rid of the free radicals and the reactive oxygen species resulted from drought stress.[25,26,27]

CONCLUSION

Phenolic and flavonoids are considered to be the non-enzymatic antioxidants with high antioxidant potential and they scavenge the free radical produced during the oxidative stress in the drought-stressed date palm seedlings. Not only the phenolics and flavonoids level but also other antioxidative enzymes like catalase, peroxidase and polyphenol oxidase significant increase which prevent the plant from oxidative damage.

CONFLICT OF INTEREST

No conflict of interest is declared.

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ABBREVIATIONS

DPPH:1,1-Diphenyl- 2-picrylhydrazyl; **ROS:** Reactive oxygen species; **PEG:** polyethylene glycol 6000; **GAE**: Gallic acid equivalent, **CE:** Catechin equivalent; **ABTS:** Ammonium molybdate, 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid).

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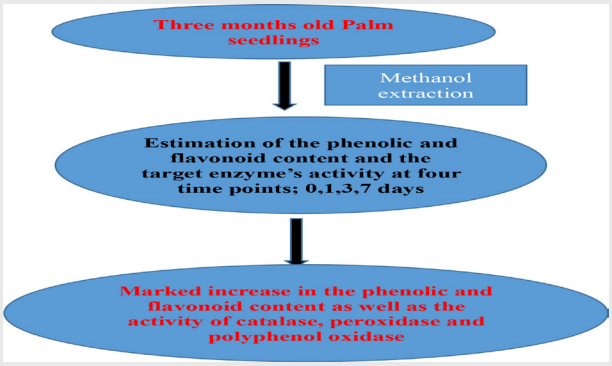
PICTORIAL ABSTRACT



About Authors

Dr. Uzma Faridi: Assistant Professor in University of Tabuk, Saudi Arabia; I did Ph.D. in Biochemistry in 2012 from Lucknow University and CIMAP. The Research Interests include anticancer and antioxidative phytochemicals or extracts.

SUMMARY

- The present study was design to evaluate the antioxidative activity of date palm seedlings under abiotic drought stress conditions.
- For the antioxidant studies three months seedlings were obtained by germinating date palm seeds and different antioxidant enzymatic activities were carried out in the extract methanol extracts of the leaves. Total phenolics, catalase, peroxidase activity and polyphenoloxidase activity were performed.
- Phenolic and flavonoids, the non-enzymatic anti-oxidants with high scavenging properties resulted from the drought tolerance in date palm seedlings. In addition, the increase in the enzymatic catalase, peroxidase and polyphenol oxidase was present which play crucial role under drought stress.

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