MOLECULAR AND PHYSIOLOGICAL BEHAVIOR VARIATIONS OF ARTEMISIA MONOSPERMA FOR SEASONAL TEMPERATURE DIVERGENCE IN THE MIDDLE REGION, SAUDI ARABIA

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Abstract.

Artemisia monosperma were collected from three independent locations in Riyadh during the periods between June 2009 –Feb. 2010 . The goal of this study is to compare the physiological and biochemical strategies during temperature seasonal variation in Artemisia monosperma that is divergent in photosynthetic pigments and antioxidant system. Photosynthetic pigments, including chl a, b and carotenoid were significantly varied according to temperature divergence in different seasons. Tissue malondialdehyde (MDA) content which used as an oxidative stress indices was greatly enhanced by % in Artemisia monosperma collected during winter (at 9ºC) in comparison with plants collected during summer period(at 38ºC). Differences in accumulation of some antioxidant metabolites and enzymes activities during seasonal variations in temperature were investigated. Total ascorbate was markedly accumulated during winter period (especially at 9 & 15 ºC). Also antioxidant enzyme activities were significantly enhanced in Artemisia monosperma during winter period compared with summer period. the different temperatures gave different results, which were quite significant (POD, $P<0.05$; PPO, $P<0.05$) in showing an inverse trend in relation to the concentration of total phenols. Zymogram analysis showed the induction of two isozymes bands in A. monosperma subjected to high temperature 38 ºC and 47ºC.

Keywords: Antioxidants, *Artemisia monosperma*, Arid region, Molecular biology, oxidative stress

Introduction

Desert plants generally follow two main strategies i.e., they tolerate the stresses through phonologic and physiological adjustments referred to as tolerance or avoidance mechanisms contribute to the ability of a plant to survive stress but it also depends on the frequency and severity of the stress periods. Plants in many habitats have various physiological mechanisms for responding to environmental changes, and the ability to tolerate environmental disturbances often contributes to their success in

communities [1]. In addition to genetic adaptation, the survival of a certain species is often determined by its ability to acclimate to environmental changes [2]. Acclimation is known to be a widespread phenomenon in nature, and long-term responses can be observed in the course of a season.

Increases in temperature raise the rate of many physiological processes such as photosynthesis in plants, to an upper limit. Extreme temperatures can be harmful when beyond the physiological limits of a plant. Decreasing photosynthesis seems to be the major cause of the chill induced reduction in the growth of plant in temperate climates [3] . Several indicators support this assumption: periods of low temperature were accompanied by a lower chlorophyll content [4], an increased pool size of xanthophyll cycle pigments, reduced photosynthetic capacity [4].

Plants in arid regions cope with temperature divergence between the prolonged annual hot and dry period in summer and the cooled winter(Fig.1). Plants evolved different survival mechanism including activation of antioxidant system, up-regulation of early light-induced proteins (ELIPs), and xanthophyll-cycle-dependent heat energy dissipation, among others [5, 6].

Leaf antioxidant systems can prevent or alleviate the damage caused by reactive oxygen species (ROS) under stress conditions, and include enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and metabolites including ascorbate acid (AsA) and glutathione (GSH) [7, 8, 9].

Phenolics are ubiquitous secondary metabolites in plants and include a large group of biologically active components, from simple phenol molecules to polymeric structures with molecular mass above 30 kDa [10]. It has been demonstrated that thermal stress induces the production of phenolic compounds, such as flavonoids and

phenylpropanoids [11, 12, 13].

Phenols are oxidised by peroxidase (POD) and primarily by polyphenol oxidase (PPO), this latter enzyme catalysing the oxidation of the *o*-diphenols to *o*-diquinones, as well as hydroxylation of monophenols [14]. These activities of enzymes increase in response to different types of biotic and abiotic stresses [15, 16]. More specifically, both enzymes have been related to the appearance of physiological injuries caused in plants by thermal stress [18, 19].

Artemisia monosperma is a dominant perennial in many areas of Asian deserts. In Saudi Arabia, it is mainly distributed in the, Northeast of Buraydah and also can be found among the sand dunes in different localities in the middle region. *Artemisia monosperma* is observed to be as one of the first species to dominate disturbed sand dunes in Saudi Arabia.

This study was conducted to compare some eco-physiological aspects *of A. monosperma* between winter and summer-grown in the period between 2009 and 2010 by determining their responses to various temperatures ranging from 9-47°C. Changes in some physiological parameters in response to temperature seasonal variations were examined, including photosynthetic pigments (chlorophyll a, b and carotenoid), some oxidative stress indices and changes in antioxidant defense system in *A. monosperma* collected from middle region in Saudi Arabia.

Materials and Methods

In this Field study research, each experiment was realized during a period of 30 days. During all experiments material sampling of *A. monosperma* was randomly collected in either summer (June-August) or winter (December-February) from three independent locations in Riyadh, Saudi Arabia.

Plant sampling

Fully expanded leaves from the current year growth (several leaves from each of the three plants per location) were collected on sunny mornings (0.900 h) of each sampling date from June to February 2009-2010 and frozen at -80 ºC to be used for the analysis of malondialdehyde (MDA) content, antioxidant enzymes and antioxidant metabolites. Fresh leaves were used for the analysis of chlorophyll content performing triplicate assays for each extraction.

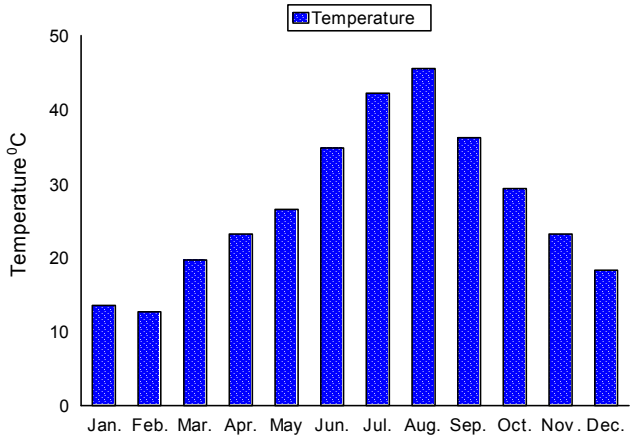

Fig. 1: Mean temperature variation in the middle region of Saudi Arabia (Riyadh) for ten years (1999-2009). Source from the Presidency of meteorology and environment in Saudi Arabia(PME)

Photosynthetic pigments determination

Photosynthetic pigments including chlorophyll a, b and carotenoid were measured in fresh leaf samples according to Lichtenthaler[19].

Extraction and quantification of phenolics

Phenolics of plant material were extracted with methanol. Total phenolic content was assayed quantitatively by A765 with Folin–Ciocalteau reagent [20]. The results obtained were expressed as mg of caffeic acid g^{-1} F.W.

Extraction of antioxidant enzymes

Leaves were extracted 15 min at 4^{o}C in 5 ml of cold extraction buffer (100mM potassium phosphate) pH 7.8, 300 mg polyvinylpyrrolidone, 1% (v/v) Triton X-100 [22]. The extract was centrifuged (30 min 20,000 g, 4^{o}C and equilibrated with 100m M potassium phosphate, pH 7.8. For stabilization of ascorbate peroxidase (APx) the elution buffer contained 5 mM ascorbate. The supernatant was used for the enzyme assays.

Enzymes assay

Superoxide dismutase (SOD) was assayed according to Stewart and Bewely [22]. One unit of SOD activity was the amount of enzyme activity that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme.

Ascorbate peroxidase (APX) activity was assayed according to Asada [23]. The reaction was initiated by the addition of H_2O_2. One unit of APX was the amount of enzyme that oxidized 1μmol of ascorbate minute at room temperature.

Catalase (CAT) activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm [23]. One unit of enzyme activity is equal to 1 μmol of H_2O_2 decomposed per min.

Polyphenol Oxidase (PPO) activity was assayed as described by Nicoli *et al*. [24] with some modifications. The assay mixture consisted of 30 mM caffeic acid in 100 mM buffer (Na_2HPO_4:KH_2PO_4), pH 7.0, through which air was bubbled for 5 min. Catalase (420 units) was added in 0.1 ml H_2O to prevent peroxidation of the substrate. The assay was initiated by the addition of enzyme extract. PPO activity was measured by the change in A_{370} of the assay mixture (30°C) based on the measurement of the disappearance of caffeic acid by enzymatic oxidation.

Guaiacol peroxidase(POD) was estimated according to Adam *et al*., [25], the reaction was started by addition of 300 µl of 13 mM H_2O_2. The increase in absorption was recorded at 470 nm.

Native Page and PPO activity staining

Aliquots (30-50 µg per lane) were loaded on 1.5 mm thick 12.5% non denaturating polyacrylamide gels [26] and were run at 4^{o}C at 20 mA per gel, using a vertical slab gel apparatus (Bio Rad Protein analysis).The gels were stained for PPO activity according to [27]. The identification of PPO isozymes was carried out according to Scandalios *et al*., [28].

Determination of malondialdehyde (MDA)

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation as described by Heath and Packer [29].

Results

Variations in photosynthetic pigments content in *A. monosperma* in response to temperature divergence under field conditions was determined and concerned to chlorophyll a, b and carotenoids.

The photosynthetic performance and pigment contents of *A. monosperma* were markedly changed in response to temperature divergence . (Chl) a and b contents were significantly decreased especially during winter. The decrease in (Chl) a and b were 85% and 74 % in plants collected in winter at 9 ºC compared with plants collected in summer at 47ºC. Also 5-aminolaevulinic acid (precursor of chlorophyll) was decreased in a similar manner, reached to 65 % reduction (Fig.2).

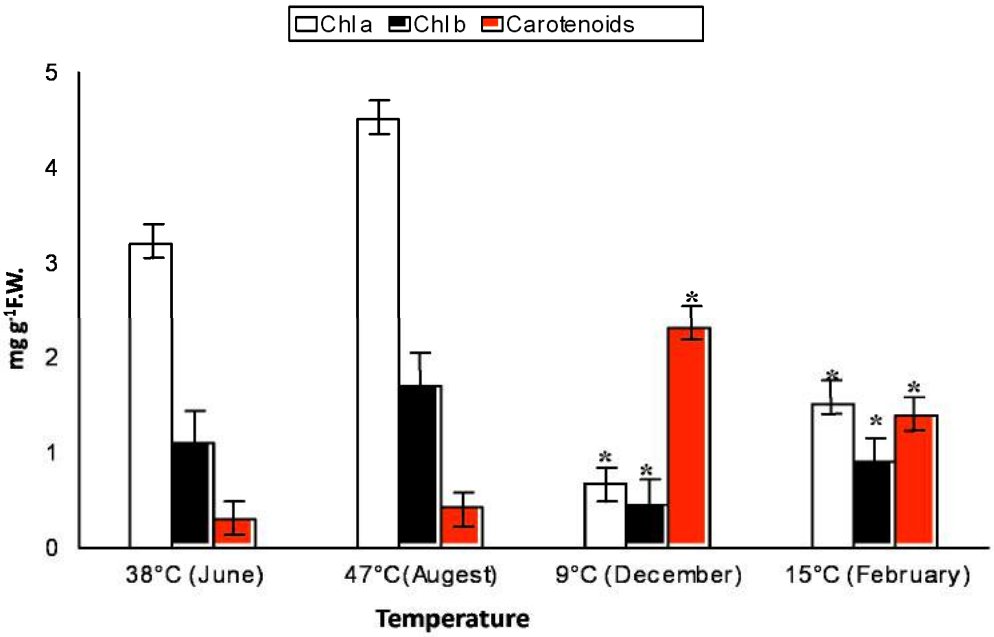

FIGURE2 - Variation in photosynthetic pigments chlorophyll a, b and carotenoids in A. monosperma subjected to temperature divergence in middle region of Saudi Arabia. Data are means ±SE (n=5).

On the other hand, carotenoids increased significantly by 3.1 and 7.7-fold increase in *A. monosperma* plants collected in winter (9ºC) compared with plants collected during summer (at 38ºC and 47ºC) in the experimental period. The chl a/b ratio showed a significant increase during summer period in the experimental periods, reached approximately 2-fold increase in comparison with plants collected in winter (Fig.2).

Based on ANOVA analysis, the levels of chlorophyll a content was insignificantly affected by location (p=0.47), whereas, significantly affected by temperature (ANOVA, p=0.05). The levels of chlorophyll a rose as temperature increased from 9ºC in winter (December) to around 47°C in summer (August) (Fig.2). Similarly, both chl b and carotenoids were affected significantly by temperature rather than plant

location. On the other hand carotenoids showed an opposite pattern to that for chl a and b, where their contents decreased significantly in summer.

Oxidative stress indices such as malondialdehyde (MDA), which used as indicator of membrane stability, was increased significantly during winter season especially at 9ºC and reached to 96% higher than those plants collected during summer at 47ºC (Table 1).

Temperature divergence (ANOVA, p=0.05) was significantly influenced the levels of total phenolic compounds between different seasons. In winter plants contained highly significant amount and reached to 3.66 mg g^{-1} FW at 9 ºC the temperatures near to the extreme conditions. Whereas, the phenolic content of summer collected plants was lowered significantly and reached to 0.84 mg g^{-1} FW at 47ºC (Table 1, Duncan's multiple range test, $p<0.05$). In summer, the levels of accumulated phenolic compounds quantified in plants collected at 47°C was about 4.5-fold lowered than that collected at 9°C (Table 1). This extreme lower temperature induced phenolic compounds accumulation was markedly observed at 9ºC and 15 ºC in winter season. Both reduced and oxidized forms of ascorbate were hardly affected by temperature divergence and increased significantly under low temperature in winter. For instance total ascorbate in *A. monosperma* collected in winter at 9ºC was 1.6-fold more than plants collected in summer at 47ºC. Oxidized ascorbate (DHA) increased significantly in *A. monosperma* plants collected in winter (9°C) and reached to 65% of total ascorbate (DHA+ASA) in comparison with 26% in plants collected in summer (47°C) (Table 1). Concerning DHA/ ASA ratio, plants collected at 47ºC showed a great decrease in this ratio reached to 0.34, whereas, plants subjected to lower temperature in winter was significantly increased in this ratio and reached to 1.8

at 9ºC. Moreover the ratio at 38ºC was 16 % in comparison with *A. monosperma* collected at 38ºC(Table 1).

TABLE 1- changes of 5-amino laevulinic acid, malondialdehyde (MDA), total phenols, total ascorbate and dehydroascorbat/ ascorbate ratio in A. monosperma plants subjected to temperature divergence in the middle region of Saudi Arabia. Data are means ±SE (n=5).

Sampling date	Temp.	**5-ALA mg g^{-1} FW**	MDA nmol mL^{-1} FW	Total Phenols mg caffeic acid g^{-1} FW	Total ascorbate µmol g^{-1} FW	DHA/ASA
15 June	38ºC	136.4±3.38	2.98±0.056	0.79 ± 0.033^{a}	28.7±2.23	0.35
15 Aug	47ºC	168.4±3.48	3.45±0.059	0.84 ± 0.036^{a}	39.5±3.72	0.34
15 Dec	9ºC	59.6±1.87	6.78±0.062	3.66 ± 0.09^{c}	62.7±4.53	1.8
15 Feb	15ºC	79.6±1.87	5.64±0.060	2.76 ± 0.06^{d}	52.6±3.87	2.2

According to an analysis of variance, the influence of temperature divergence on antioxidant enzymes activities was not uniform for the studied enzymes. Over all harvest dates, the activities of SOD, APX and CAT in *A. monosperma* plants collected at9ºC and 15ºC were significantly ($p<0.05$) higher than in plants collected at 38ºC and 47 ºC. Whereas a marked depression for PPO and POD in *A. monosperma* collected at the same temperatures (Table 2). The activity of the antioxidant enzymes, namely SOD, CAT and APX, was unaffected by the plant location, whereas their activities were significantly affected by harvest date temperature (Table 2). The activity of CAT was markedly higher in winter at 9ºC than at 15ºC, regardless the

harvest location. In contrast, the enzymes SOD and APX showed slight differences for the same temperatures from the same locations.

With respect to oxidative enzymes, POD and PPO, the different temperatures gave different results, which were quite significant (POD, $P<0.05$; PPO, $P<0.05$) in showing an inverse trend in relation to the concentration of total phenols. In our experiment, POD and PPO activities at 9ºC were decreased with respect to the highest activities recorded at 38°C, by 66 and 71%, respectively (Table 2).

TABLE 2- Changes in antioxidant enzymes activities; SOD, APX, CAT, PPO and POD of crude extract of *A. monosperma* in response to temperature divergence in the middle region of Saudi Arabia. Data are means ±SE (n=5).

Sampling date	Temp.	U mg protein min $^{-1}$			PPO µmol caffeic acid mg^{-1} protein min^{-1}	POD µmol guaiacol mg^{-1} protein min^{-1}
		SOD	APX	CAT		
15 June	38ºC	14.7±1.12	4.32±0.66	3.27±0.06	38.8±3.86	37.3±2.98
15 Aug	47ºC	16.6±1.22	5.31±0.71	4.87±0.07	28.2±2.19	25.3±2.07
15 Dec	9ºC	22.9±2.08	20.8±2.14	18.74±1.32	11.4±0.83	12.7±0.99
15 Feb	15ºC	19.0±2.03	17.5±1.65	13.58±0.98	22.3±2.30	16.9±1.23

Zymogram analysis indicates the appearance of two new bands in *A. monosperma* subjected to high temperature 38 ºC and 47ºC. The low intensity of the bands detected at 9 ºC and 15ºC denotes the low activity of these isoforms in the crude extract of *A. monosperma* during winter.

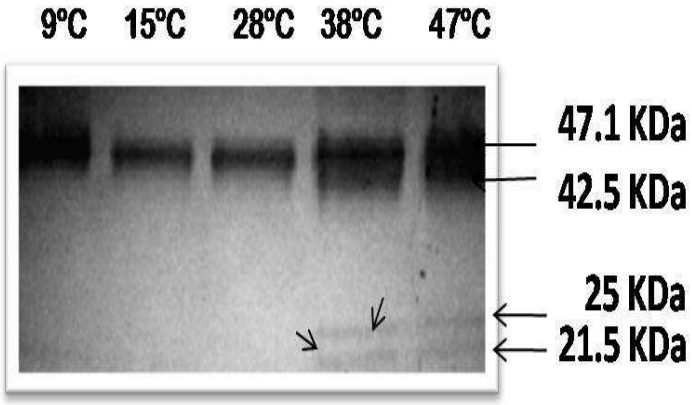

FIGURE 3- Zymogram of PPO activity of crude extract of *A. monosperma* subjected to temperature divergence in the middle region of Saudi Arabia. Two sample applications were performed for each detection. Arrows indicate the detected PPO isoenzymes.

On the other hand the detected *A. monosperma* PPO isoforms in summer plants indicates the higher activity of these isoenzymes in addition to the induction of two new isozymes compared with winter plants(Fig. 3).

Discussion

The amount of chlorophyll a and b is known to parallel photosynthetic activity in; that is, the increase in photosynthetic pigments, especially chlorophyll a, leads to a rise of photosynthetic capacity and the fixed carbon capital available for growth.

In the present study it has been shown that photosynthetic pigments of *A. monosperma* were decreased significantly in response to lowering stress temperature in winter season. Changes in the amount of chlorophyll a rather than chlorophyll b coincided with changes in net photosynthesis in response to lower temperatures. It has been proposed that remperature variations resulted in different oxidative stress responses in *A. monosperma*. Accordingly, we found that chlorophyll a/b ratio and 5-

aminolaveulinc acid (5-ALA) of the studied plant were lower in winter than in the summer in all studied locations(Table 2).

It has been suggested that winter induction of antioxidant enzymes activities may be correlated with the production of reactive oxygen species (ROS), resulting in oxidative stress. Our results are consistent with [15, 30] who explained the occurrence of oxidative stress was parallel with the higher content of some oxidative stress indices such as MDA. Moreover the total ascorbate content was significantly enhanced in *A. monosperma* parallel with the decrease in temperature at 9ºC and 15ºC in winter season. Ascorbate content was directed toward the formation of oxidized form of ascorbate (DHA).

POD and PPO activities were lowest in *A. monosperma* at 9°C and at 15°C in comparison with activities on plants collected at 38 ºC and 47 ºC (Table 2). Moreover, the relationship between POD and PPO activities and soluble phenolics concentration in *A. monosperma* plants appear to indicate that temperature divergence caused oxidative stress, by subjecting the plants to a super-optimal and suboptimal temperatures [31].Some studies have reported that these enzyme activities increase in response to different types of stress, both biotic and abiotic [15, 16]. More specifically, both enzymes have been related to the appearance of physiological injuries caused by thermal stress [17, 32]. The metabolism of phenolic compounds includes the action of oxidative enzymes such as POD and PPO, which catalyze the oxidation of phenols to quinones [14, 33].

In contrast, our results suggested that enzymes that oxidize soluble phenolic compounds, such as POD and PPO, were inhibited in winter, especially at 9ºC. Consequently, soluble phenolic compounds were accumulated perhaps as results of an acclimation mechanism to overcome cold stress. An opposite correlation between

PPO and POD activities and formation of phenolic compounds has been demonstrated in many studies [15, 17].

According to our results we can conclude that, significant induction of PPO activity in plants collected during summer period may be correlated with the appearance of two new bands of PPO isozymes especially at 38 and 47ºC for heat acclimation. In addition to the adaptive responses of *A. monosperma* to temperature divergence attained higher concentrations of phenolic compounds and carotenoids due to their adaptive mechanisms under arid conditions.

Conclusion

We conclude that *A. monosperma* may develop a acclimated mechanism against super-optimal temperature divergence stress caused at 9ºC and 15ºC. This acclimated mechanism appears to consist of the accumulation of total ascorbate, total phenolic compounds as a defense against oxidative stress caused by the stress. Moreover, plant responded to this stress by increment of carotenoids content in addition to enhancement of antioxidant enzymes activities for adapting to this stress.

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