Iranian Medicinal Plants Society

Ethno-Pharmaceutical products

Journal homepage:Http://js.kgut.ac.ir

Ministry of Science Research and Technology
Graduate University of Advanced Technology

Effects of drought on osmotic adjustment, antioxidant enzymes and pigments in wild *Achillea tinctoria* populations

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Article information

Article history:
Received: 8 Apr. 2014
Accepted: 15 Aug. 2014
Available online: 15 Sep. 2014
EPP 2014; 1 (2): 43-54

Keywords:
Anthemis tinctoria
peroxidase
proline
soluble sugars
water stress

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Abstract

Drought stress is one of the most important factors limiting the survival and growth of plants in the different habitats of Iran. Detailed knowledge about the ecophysiological responses of native plants to drought stress could contribute to the success of breeding and re-vegetation programs. Six wild populations of *Anthemis tinctoria*, were assigned to four drought treatments, i.e. well-watered (100% field capacity), mild drought stress (75% field capacity), moderate drought stress (55% field capacity), and severe drought stress (35% field capacity). Relative water content, dry matter content, osmotic solutes (proline and soluble sugars), antioxidant enzymes (peroxidase and polypolyphenol oxidase), total protein content, and pigments content were investigated. Severe drought stress largely increased accumulations of osmotic solutes and peroxidase activity of the most populations, but significantly decreased relative water content, dry matter content, total protein content and polyphenol oxidase activity in the all populations. Drought stress significantly decreased pigments content, but increased the ratio of carotenoids to total chlorophylls in the studied populations. The positive relationships were observed among antioxidant enzymes activities, and between contents of osmotic solutes and antioxidant enzymes activities. These findings suggest that populations are characterized by a significantly different tolerance to drought, when drought stress occurs. Based on these findings it may conclude that the population 27480 is more tolerant to osmotic stress due to specific antioxidative mechanisms, while the population 18041 was the least tolerant to sever drought stress. It seems that the population 27480 has a higher adoption potential to arid and semi-arid conditions which makes it a candidate of choice in breeding programs.

Introduction

Genus *Anthemis* L. includes annual and/or perennial plants. This genus is comprised of over 100 species, found primarily in the Mediterranean region in Asia Minor, in the western part of Central Asia and in Iran. *Anthemis tinctoria* is a relatively drought-tolerant herbaceous perennial plant that is best suited to cottage rather than a formal garden (Halevy., 1999). It has medicinal and cosmetic uses, and extensively grows in drought-prone environments (Bartram., 1995). Due to over collection, essentially in the flowering period, land conversion and also land degradation, the *Anthemis* species are now considered at risk for local extinction, which affect greatly their financial income and subsequently their livelihoods. Many healers has recognized that these species become very scarce recently, hence in order to ensure the sustainable utilization, and to meet the growing demand of these wild species, it has become necessary, to develop rapid methods of their commercial cultivation. Seeds culture is an alternative and easy method of commercial propagation, which is restricted in Iran by water deficiency and water scarcity. More than 82% of Iran's territory is located in arid and semi-arid zones, and faces shortage of water (Amiri & Eslami., 2010). The water constraint constitutes one of the main environmental problems for development and crop productivity of plants. Selection of drought tolerant species and varieties is the best economic approach for exploitation, and rehabilitation of arid and semiarid regions (Shannon., 1985; Alonso et al., 1999; Ghoulam et al., 2001). The effectiveness of such approach depends on availability of genetic variation, and its exploitation by screening and selection of the powerful plants under drought stress (Al-Khatib et al., 1992; Ali et al., 2007).

Osmotic adjustment in terms of accumulating compatible solutes, has been considered as an important physiological adaptation for plants to resist

drought . Osmotic adjustment facilitate extracting water from dry soils and maintaining cell turgor, gas exchange and growth in very dry environments (White et al., 2000; Chaves et al., 2003). Proline and soluble sugars are two kinds of the most important compatible solutes in plants (Chaves et al., 2003; Ben Ahmed et al., 2009; Hessini et al., 2009). Besides their roles in osmotic adjustment, they may protect membranes from damages and stabilize the structures and activities of proteins and enzymes (Iyer & Caplan., 1998; Samuel et al., 2000; Villadsen et al., 2005; Lee et al., 2008; Ben Ahmed et al., 2009; Hessini et al., 2009). Drought stress usually leads to oxidative stress due to stomatal closure (Lei et al., 2006; Ozkur et al., 2009), which causes the over-reduction of photosynthetic electron chain (Bacelar et al., 2007; Ben Ahmed et al., 2009) and high formation of reactive oxygen species (ROS) in chloroplasts and mitochondria (Asada., 1999; Fu & Huang., 2001). ROS could disrupt normal metabolisms of plants through oxidative damages to lipids, proteins, nucleic acids, and photosynthetic pigments and enzymes (Smirnoff., 1993; Fu & Huang., 2001; Ozkur et al., 2009).

In order to overcome oxidative stress, plants have developed enzymatic and non-enzymatic antioxidant defense mechanisms to scavenge ROS (Smirnoff., 1993). Peroxidase (POD) is one of the antioxidant enzymes which scavenge accumulation of hydrogen peroxide (H_2O_2) in tissue (Reddy et al., 2004). Besides, non-enzymatic antioxidative carotenoids (Car) such as B-carotene and xanthophylls can also quench ROS and stabilize photosynthetic complexes (Adams et al., 1999; Bassi & Caffarri., 2000; Munné-Bosch & Peñuelas., 2003).

Plants also contain a number of natural secondary products with antioxidant properties, including different phenol compounds (Rice-Evans et al., 1997). There is no universal pattern for phenolic compound activity in the drought stress of different plants and organs (Bagniewska-Zadworna et al., 2007). Enzymes, such as Peroxidase (POD) and Polyphenol oxidase (PPO), may oxidize phenol compounds and thus take part in the regulation of the phenolic concentration in plants. Recent studies have also indicated that phenol oxidizing enzymes may participate in the response to various abiotic stresses including drought (Sofo et al., 2005; Veljović-Jovanović et al., 2006 and 2008). Investigations of intra-specific variation to drought provide an opportunity to understand species specific adaptations, relative importance and variation of physiological adaptations within species. Therefore, achieving a greater perception of intra-specific variation to drought, is of value both from a scientific perspective i.e. identification of traits associated with greater stress resistance, as well as from an applied perspective i.e. identification of superior planting stock for specific restoration needs and breeding programs. In order to provide more detailed knowledge for the selection of plant populations of *A. tinctoria* and contribute to the success of breeding and re-vegetation programs, we have compared osmotic adjustment, antioxidant enzymes and pigments content of six populations of *A. tinctoria* under experimental drought conditions. The specific objectives of this study were 1) evaluating relatively short-term physiological/biochemical responses to drought stress, and 2) determining whether there is a significant intra-specific variation in drought tolerance among populations of *A. tinctoria*.

Materials and Methods

- **Plant material, growth, stress conditions**

Seeds of six populations of *Anthemis tinctoria* from Iran (27480, 27507, 9787, 18047, 19943 and 18041; Table 1), provided from the Iranian Natural Resources Gene Bank at Research Institute of Forests and Rangelands (RIFR), was evaluated in the present study. Plants were grown in plastic pots (16 cm height and 18 cm diameter) containing peat and sand (5:1) in a greenhouse (temperature: 25 °C ± 2 and relative humidity: 60% ± 5). Prior to drought stress treatments, all plants were well-watered. After 60 days, when the plants well established, drought stress was applied in 35% field capacity (FC), 55% FC, 75% FC and 100% FC (well-watered). During 30 days lasting experiment, the soil water potentials and corresponding soil water contents used in the study were calculated from soil water retention curves. The pots were kept at the designated drought stress levels by weighting. The study was carried out in a greenhouse at the RIFR in Tehran, Iran. During the experiment, the minimum and maximum temperatures inside the greenhouse were 16.2 °C and 33.5 °C, respectively.

- **Relative water content and dry matter content**

To determine relative water content (RWC), 3 leaves from each plant were weighed immediately (FW) after harvesting the plant. Leaves were then placed in distilled water for 4 hr and then turgid weight (TW) was measured. Then the leaves were dried in oven at 80°C for 24 hr to obtain their dry weight (DW). RWC was calculated by the using the equation: RWC = FW-DW/TW-DW ×100 (Weatherley., 1950 and 1951). Dry matter content (DM) was calculated by the using the equation: Dry matter = 100-(FW-DW/FW ×100).

- **Proline and soluble sugars**

Proline was determined by the ninhydrin method (Bates et al., 1973). Soluble sugars were determined by the anthrone method (Yemm & Willis., 1954). Three replicates per species and treatment were

obtained from the youngest fully expanded leaves of different individuals during midday after 30 days of treatments.

- **Antioxidant enzymes assay**

Fresh tissue samples (0.5 g) were ground in a mortar and pestle with 1.2 ml of sodium phosphate buffer (50 mM, pH 7.8) containing 1.33 mM EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged at 13,000g for 20 min at 4ºC. The supernatants were used as the crude enzyme source to assay enzymatic activities. Peroxidase (POD) activity was determined, using guaiacol as substrate Fu and Huang (2001). The reaction mixture consisted of 100 µl of crude enzyme extract, 140 µl of 0.3% (v/v) guaiacol (in 50 mM sodium phosphate buffer, pH 6.4). The increase in absorbance at 470 nm was measured after 60 µl of 0.3% (w/v) H2O2O (in 50 mM sodium phosphate buffer, pH 6.4) were added. Polyphenol oxidase (PPO) activity was determined, using pyrocatechol as substrate Fu and Huang (2001). The reaction mixtures were 250 µl 50 mM sodium phosphate buffer (pH 6.4) containing 10 mM pyrocatechol and 50 µl of crude enzyme. Increase in absorbance was measured at 470 nm (for POD) and 389 nm (for PPO) at 1 min intervals up to 3 min using a UV -Vis spectrophotometer. Enzyme specific activity is defined as units (one POD and PPO activity unit defined as absorbance at 470 and 389 nm, respectively, changes per minute) per gram of fresh weight of leaves. Total content of foliar protein was measured according to Bradford (1976), using bovine serum albumin as a standard. The activity of each enzyme was expressed on protein basis. Three replicates per population and treatment were obtained from the youngest fully expanded leaves of different individuals during midday after 30 days of treatments.

- **Pigments**

Chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophylls (Chla+b), and carotenoids (Car) were determined spectrophotometrically using 80% acetone as a solvent (Lichtenthaler., 1987). Three replicates per species and treatment were obtained from the youngest fully expanded leaves of different individuals during midday after 30 days of treatments.

- **Statistical analysis**

All data were subjected to two-way analysis of variance (ANOVA) to determine differences among populations and treatments for each variable. The significant differences between means were determined using Duncan's test at $P < 0.01$ level. Data were tested for normality by Kolmogorov–Smirnov test. When necessary, data were transformed to meet the assumptions of ANOVA. Linear regression coefficients between contents of osmotic solutes and activities of antioxidant enzymes were calculated. Statistical tests were performed with MiniTab 16 (SPSS., Chicago, USA).

Results

Analysis of variance showed highly significant differences among drought treatments (Table 2). However populations were significant in proline content, antioxidant enzymes, Car and ratio of Car/total chlorophyll. The interaction between population and drought treatment was also significant in proline content and antioxidant enzymes (Table 2).

Table 1.The environmental data of the wild populations of *A. tinctoria*.

Populations	Latitude (N)	Longitude (E)	Elevation from seed level (m)	Annual average precipitation (mm)	Annual average maximum temperature (ºC)	Annual average minimum temperature (ºC)
27480	36˚ 42'	45˚ 17'	1840	352	18	7
27507	36˚ 04'	45˚ 30'	1670	856	16	10
9787	36˚ 00'	45˚ 54'	1600	707	18	7
18047	37˚ 32'	45˚ 05'	1315	256	18	6
19943	38˚ 05'	46˚ 17'	1361	245	19	8
18041	37˚ 32'	45˚ 05'	1315	256	18	6

Table 2.Results from the ANOVA on different studied characteristics.

Source of variation	df	F												
		Proline	Soluble sugars	POD	PPO	Total proteins	RWC	DM	Chl_a	Chl_b	Car	Chl_{a+b}	Car/ Chla+b	Chla/ Chlb
Population (Pop.)	5	2.76*	1.87	5.01**	2.35*	3.44**	2.18	0.92	0.79	1.01	2.65*	1.28	2.34*	0.71
Drought treatment (Treat.)	3	61.70**	10.96**	36.42**	2.39*	100.64**	37.52**	13.37**	42.86**	4.45**	11.26**	17.23**	25.44**	0.58
Pop. × Treat.	15	3.61**	0.42	3.46**	2.37*	3.55**	1.14	1.08	0.73	0.34	0.95	0.40	0.55	0.41
CV		45.99	23.76	35.22	21.92	16.87	8.99	12.81	5.07	24.99	10.84	9.98	15.19	23.39

*, **, significant at 0.05 and 0.01 level, respectively.

- **RWC and dry matter**

Mean comparisons at different stress levels indicated that lowering of water potential causes a decrease in RWC and dry matter, which was higher under well-watered condition (Fig. 1a, b). Populations and interaction of drought stress and population had not significant effect on RWC and dry matter. As can be seen from figures 1a and b, similar variations in RWC and dry matter were obtained in all populations.

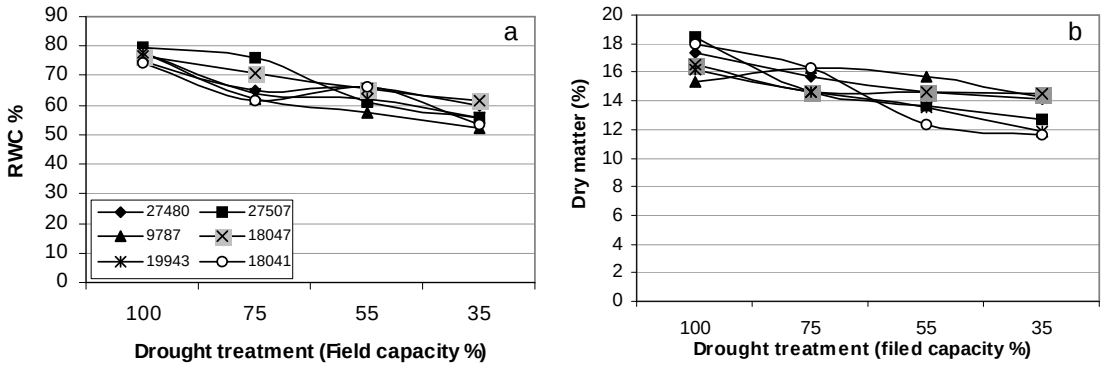

Fig. 1. The percentages of relative water content (RWC) and dry matter of six *A. tinctoria* populations of four drought treatments (n = 3).

- **Proline and soluble sugars**

Drought treatments significantly increased proline content in all six populations under mild and/or moderate stress, increased that in four populations under severe stress, but decreased that in the populations 19943 and 18041 under severe stress (Fig. 2a). The largest increase in proline content was recorded in the population 27480 while increase was lower in the populations 27507, 9787 and 18047. Under sever stress the population 27480 seems to be the most tolerant; and the populations 27507, 9787 and 18047 the moderate tolerant; and populations 19943 and 18041 were the least tolerant. The accumulation of soluble sugars was also affected by drought stress. Among the six populations, the accumulation of soluble sugars exhibited similar responses to drought intensities (Fig. 2b), exhibiting highest values under severe stress.

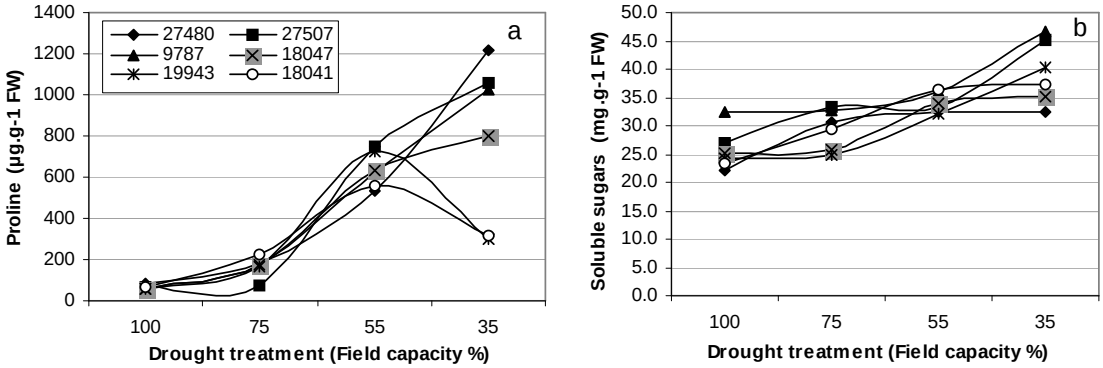

Fig. 2. The contents of proline and soluble sugars of six *A. tinctoria* populations of four drought treatments (n = 3).

- **Antioxidant enzymes**

Drought stress affected antioxidant enzyme activities of all populations with different responses to drought. The activities of POD, PPO, and total protein contents are presented in figures 3a-c. Drought stress significantly increased POD activity in all six populations under mild and/or moderate stress, but decreased that in 18041 as the least tolerant population, under severe stress (Fig. 3a). The largest increase in POD activity was recorded in the population 27480 as most tolerant population. Drought stress increased PPO activity in all six populations except population 9787 under mild and moderate stress, increased that in the population 27480 as the most tolerant population, under severe stress, but decreased that in the five other populations (27507, 9787, 18047, 19943 and 18041 under severe stress (Fig. 3b). Under well-watered condition, the total proteins were significantly higher in all six populations. As drought stress intensified, total protein content increased in all populations. However, drought stress caused less decreases of total protein content in the population 27480 as the most tolerant population, than in the other five populations (Fig. 3c).

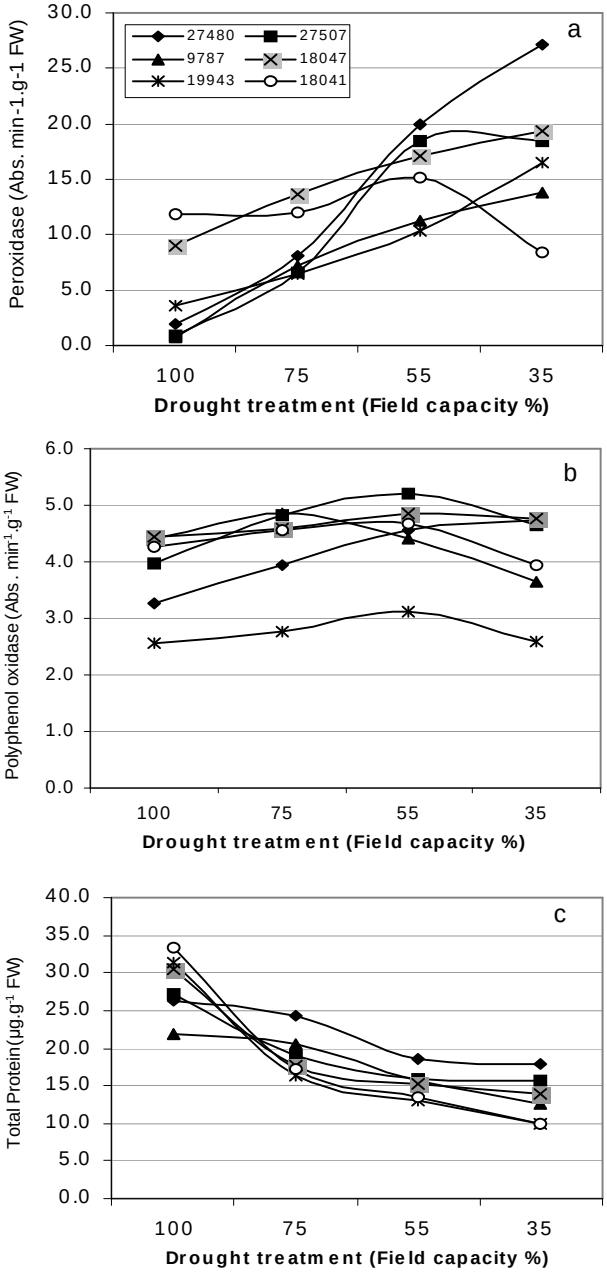

Fig. 3. Activities of POD and PPO, and total protein content of six *A. tinctoria* populations of four drought treatments (n = 3).

- **Photosynthetic pigments**

Pigment contents were affected by drought stress. The Chla, Chlb, and Chla+b contents in all six populations gradually decreased as drought stress intensified. The values of Chla, Chlb, Chla+b exhibited similar responses to drought intensities (Fig. 4a-c), exhibiting highest values under well-watered condition. Among the six populations, the Car exhibited similar responses to drought intensities (Fig. 4d), exhibiting highest values under severe stress. As drought stress intensified, the ratio of Car/Chla+b increased in the populations 27507, 9787, 19943 and 18041, decreased in the populations 27480 and 18047 under moderate stress, which showed an increase under severe drought stress (Fig. 4e).

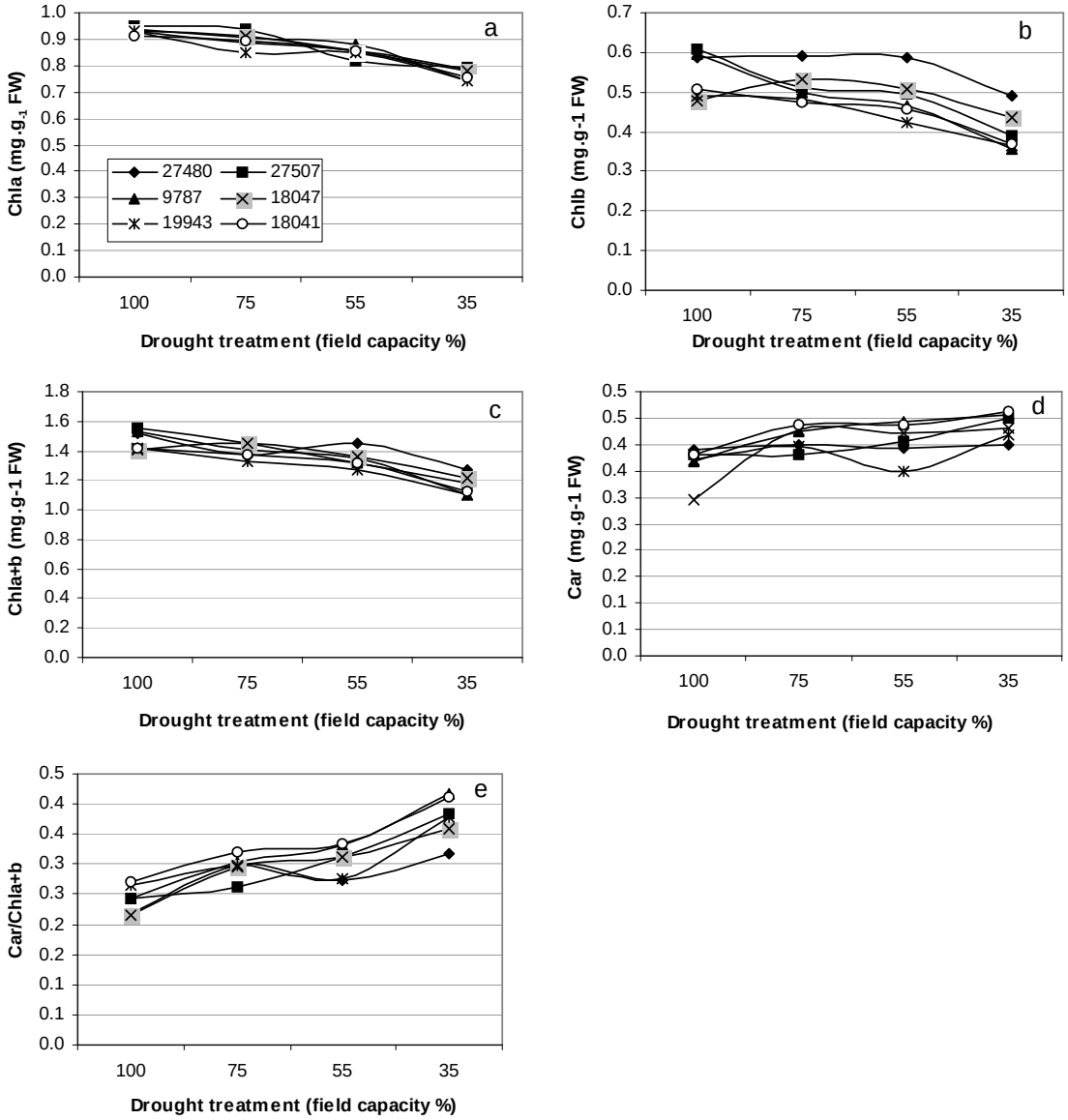

Fig. 4. Chlorophrll a (Chl*a*), Chlorophrll b (Chl*b*), Total chlorophylls (Chl*a*+*b*), Cartenoids (Car) and the ratio of Cartenoids to total chlorophylls (Car/Chl*a*+*b*) of six *A. tinctoria* populations of four drought treatments (n = 3).

- **Relationships between osmotic solutes and antioxidant enzymes**

Proline content was linearly and positively correlated with soluble sugars in 27504, 9787, 18047 and 18041 (Fig. 5a). Proline content was correlated with POD and PPO activity in most populations except 18041 (Fig. 5b and c). Soluble sugars content was positively correlated with POD activity in all six populations except population 18041 (Fig. 5d) and was positively correlated with PPO activity in population 20480, whereas was negatively correlated in population 9787 (Fig. 5e). POD and PPO activities were linearly and positively correlated in 27480, 27907, 18047 and 18041 (Fig. 5f).

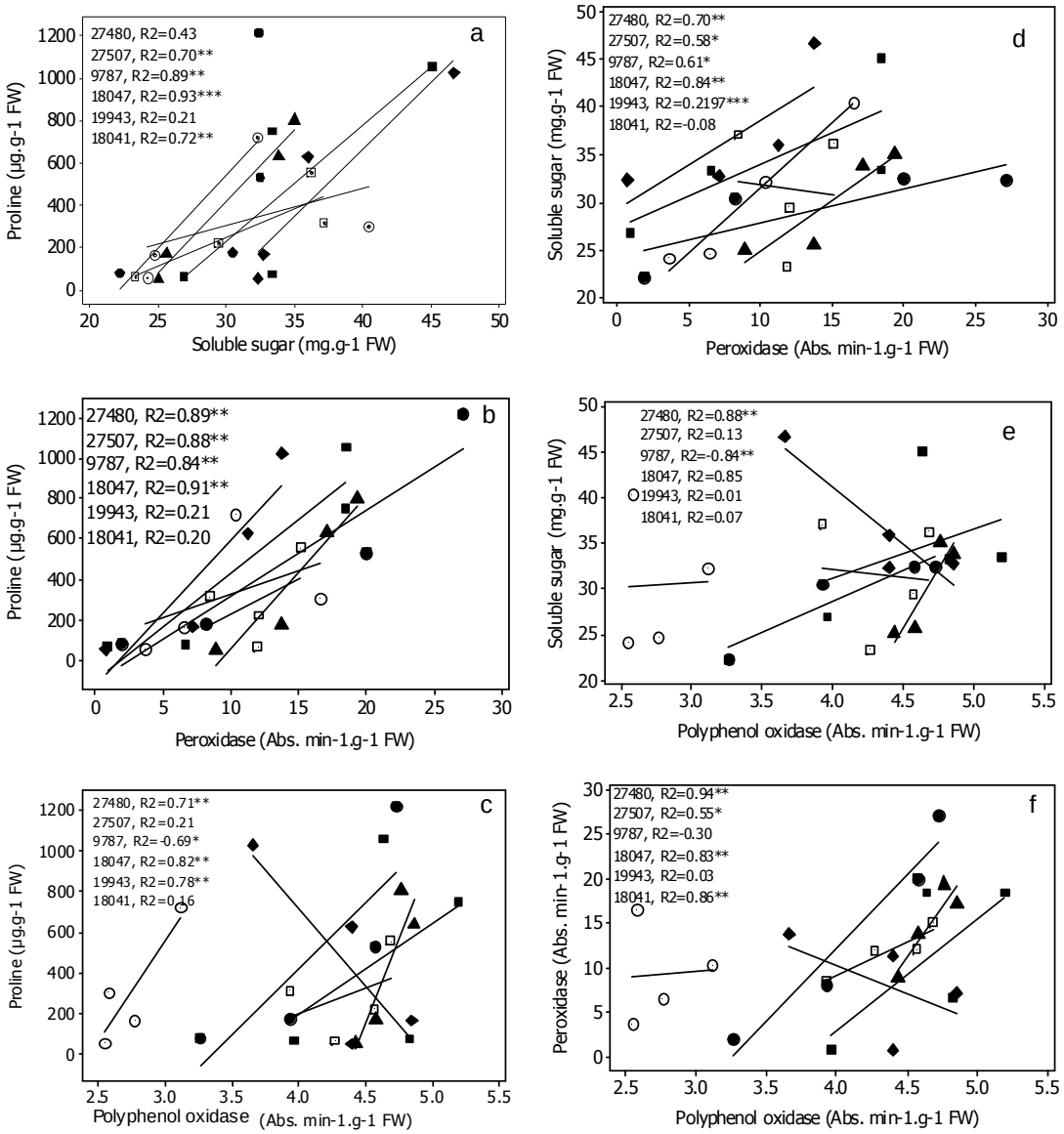

Fig. 5. Correlations between proline content and soluble sugars content (a), POD activity (b), PPO activity (c); between soluble sugars content and POD activity (d), PPO activity (e); between POD activity and PPO activity (f). Population 27480 (•), 27507 (■), 9787 (◆), 18047 (▲), 19943 (○) and 18041 (□). Values are means of three replicates per population and treatment. The solid lines represent the best-fit linear regressions for each species: *P < 0.05; **P < 0.01; ***P < 0.001.

Discussion

The RWC and dry matter were affected by drought stress. The RWC and dry matter in all six populations gradually decreased as drought stress intensified. The values of RWC and dry matter exhibited similar responses to drought intensities, exhibiting highest values under well-watered condition. Experimental data on the other species indicated that drought resistance cultivars or genotypes had higher RWC (Abraham et al., 2004; Shahrokhi et al., 2011) and dry matter (Batlang., 2006; Shahrokhi et al., 2011). The high RWC of resistant genotypes was probably the result of their better ability for water uptake at low soil water potential (Volaire et al., 1998). It is well documented that a critical component of the dehydration tolerance for grasses is cell membrane stability (Crowe et al., 1987; Volaire & Lelievre., 2001).

Under drought conditions, the accumulations of proline and soluble sugars seemed to be associated with drought tolerance in many plant species. The rate of proline accumulation was significantly higher in drought-tolerant cultivars than drought-sensitive cultivars of wheat (Nayyar & Walia., 2003), mulberry (Reddy et al., 2004), and olive (Ben Ahmed et al., 2009). Soluble sugars also contributed to improving drought tolerance of peas (Sánchez et al., 1998), sugar beets (Choluj et al., 2008) and black poplars (Regier et al., 2009). In two mango cultivars, a cultivar, which exhibited more active accumulations of soluble sugars and proline, also revealed higher resistance to drought than the other one (Elsheery & Cao., 2008). In our study, proline content appeared to increase sharply in all six populations under mild and moderate drought stress. However, severe drought stress caused serous metabolic damages and largely decreased proline accumulation in the two populations (19943 and 18041). On the contrary, severe drought stress did not reduce proline content in others and even significantly increased that in population 27480. The responses of soluble sugars to drought intensity showed the similar trends in the six populations. These results suggested that the population 27480 as most tolerante and the populations 27507, 9787 and 18047 as moderate tolerant had higher capacity of osmotic adjustment in terms of accumulating proline, especially under severe drought, which could maintain water absorption under such harsh conditions (White et al., 2000; Chaves et al., 2003).

In the current study, severe drought stress largely decreased the activities of PPO in all six populations except population 27480 as the most tolerant population. On the contrary, in response to severe drought, POD activity did not decrease in the all populations except population 18041 as the least tolerant population even significantly increased, indicating that the scavenging function of antioxidant enzymes was not impaired by severe stress (Fu & Huang., 2001). It has been reported that under drought conditions, the activity of POD increased to a greater extent, resulting in lower levels of lipid peroxidation and electrolyte leakage, in a drought-tolerant clone than in a drought-sensitive one of *Coffea canephora* (Lima et al., 2002). The drought-resistant *Phaseolus acutifolius* also revealed higher activities of POD than the drought-susceptible *P. vulgaris* (Türkan et al., 2005). Khanna-Chopra and Selote (2007) attributed lower membrane injury to the higher activity of POD in a drought-tolerant wheat cultivar than in a drought sensitive cultivar under severe drought stress. High activities of antioxidant enzymes also improved drought tolerance of cultivars of mulberry (Reddy et al., 2004), tea (Upadhyaya et al., 2008) and olive (Ben Ahmed et al., 2009). It seemed to be that higher activity of POD provided higher protection against oxidative stress in the 27480 population under severe drought stress, as judged from higher increases of proline and total protein content in the population 27480.

In current study, total protein content appeared to decrease significantly in all six populations under sever drought stress. Although, the responses of total protein content to drought intensity showed the similar trends in the six populations, the population 27480 had higher amount of total protein content. Contrasting reports regarding the changes in protein contents are available in literature. For example water stress was found to cause a significant reduction in soluble protein content in moth beans (Garg et al., 2001) and *Vigna radiata* L. (Farooq & Bano., 2006). The decrease in total soluble proteins under drought stress in different populations of *A. tinctoria* is consistent with other findings (Bensen et al., 1988; Riccardi et al., 1998; Ti-da et al., 2006).

The observed positive correlations among activities of POD and PPO in the most studied populations (Fig. 5) suggested that these enzymes might be involved in the elimination of the reactive oxygen species (ROS) within the peroxide/phenols/ascorbate system in drought stress (Takahama & Oniki., 1997; Sgherri et al., 2003 and 2004). The intimate relationships between enhanced or constitutive antioxidant enzyme activities in response to drought stress were also observed in many other species (Türkan et al., 2005; Chen & Cao., 2008; Zhu et al., 2009b). The positive relationships between contents of osmotic solutes (proline and soluble sugars) and antioxidant enzyme activities (POD and PPO) were also observed in the current study. It was reported that proline accumulation could activate the antioxidant defense mechanisms (Türkan et al., 2005; Ben Ahmed et al., 2009). Since proline and soluble sugars could stabilize the structures and activities of enzymes (Chaves et al., 2003), the high accumulations of proline and soluble sugars in the population 27480 under severe drought stress may

largely permit their high activities of antioxidant enzymes.

The limitation of water supply induced chlorophyll degradation in present experiment (Fig. 4). Reduction of pigments content, as a result of either slow synthesis or fast breakdown, has been considered as a typical symptom of oxidative stress (Smirnoff, 1993). Authors explained this phenomenon as a photo protection mechanism through reducing light absorbance by decreasing pigments content (Munné-Bosch & Alegre., 2000; Galmés et al., 2007; Elsheery & Cao., 2008). Carotenoids are also responsible for scavenging of singlet oxygen (Knox & Dodge., 1985) and hence their comparative levels in a genotype will determine its relative tolerance. However significant differences in carotenoid contents were not recorded among all six populations. Since Car played an important role in photoprotection (Demmig-Adams & Adams., 1996; Adams et al., 1999; Munné Bosch & Penuelas., 2003), the increased ratio of Car/Chla+b in some populations under drought conditions (Fig. 4) indicated a higher need of photo protection by Car (Baquedano & Castillo., 2006; Elsheery and Cao, 2008). As drought stress intensified, all six populations exhibited lower RWC and dry matter. Severe drought stress largely decreased accumulations of soluble sugars and activity of PPO enzyme in all six populations, but significantly increased proline content and POD activity in the most and moderate tolerant populations. The positive relationships were observed among activities of antioxidant enzymes, and between contents of osmotic solutes and activities of antioxidant enzymes. Drought stress decreased chlorophyll content but increased cartenoids content and the ratio of Car/Chla+b in the studied populations.

Differences under drought stress show that populations are characterized by a significantly different tolerance to drought. Based on these findings it may conclude that the population 27480 is more tolerant to osmotic stress due to specific antioxidative mechanisms, while the population 18041 was the least tolerant to sever drought stress. It seems that the population 27480 has a higher potential to adapt to arid and semi-arid conditions which makes it a candidate of choice in breeding programs.

Acknowledgement

This work was supported by the Research Institute of Forests and Rangelands (RIFR), Iran, Project no. 12-09-09-9001-90001.

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