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Divergent impacts of moderate and severe drought in the antioxidant response of *Calendula officinalis* L. leaves and flowers †

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Abstract: We studied the impacts of moderate and severe drought in different pot marigold (*Calendula officinalis* L.) genotypes, evaluating the antioxidant performance of leaves and flowers concerning the levels of proline and malondialdehyde, the activity of antioxidant enzymes (catalase, peroxidase, and ascorbate peroxidase), as well the impacts in flower production. Overall, we found a high resilience to moderate drought. However, severe drought significantly affected flower production, even though antioxidants were present at high levels. Results indicate significant variation in drought tolerance among pot marigolds, providing an opportunity to identify valuable tolerance traits.

Keywords: abiotic stress; antioxidants; drought; flower tolerance; marigold

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1. Introduction

Drought stress is a major abiotic stress, limiting crop production and yield [1,2]. Drought decreases photosynthesis and chlorophyll synthesis, and alters nutrient metabolism, ion uptake, and translocation, ultimately limiting plant vegetative growth. Drought also has an impact on flower number and size, also affecting the viability and durability of flowers [3]. Abortion of reproductive organs is a major limiting factor for flower production under water stress. Thus, understanding its impacts has become a major aim for the sustainability of the floricultural industry.

Drought triggers the overproduction of reactive oxygen species (ROS), which are toxic and cause damage to proteins, lipids, carbohydrates, and DNA, leading to oxidative stress and, ultimately, plant death [4,5]. To cope with the effects of drought, plants activate an antioxidant system that includes a variety of ROS scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX), and catalase (CAT), as well as non-enzymatic metabolites such as carotenoids, flavonoids, and proline [6]. Malondialdehyde (MDA), produced by membrane lipids in response to ROS is often used as a drought indicator to evaluate the degree of membrane damage and the level of drought tolerance since plants with low amounts of MDA are generally considered to be more tolerant to drought [7]. Nevertheless, the antioxidant response is highly dependent on the level and duration of drought, the species, and even within genotypes of the same species [8–10].

Calendula officinalis L. (pot marigold or calendula), from the plant family Asteraceae, is a widely used plant in ornamental horticulture, as well as in traditional healing treatments due to its wide range of secondary metabolites, flavonoids, and carotenoids contents [11]. It has recently been proposed as an oilseed crop since the oil from its seeds has high amounts of fatty acids [11]. Nevertheless, it is highly affected by drought, although some genotypes show some tolerance to water scarcity [12]. For instance, drought caused a significant decline in the number of leaves and leaf area in *Calendula* cv. Orange King [13]. In measurements performed under field conditions, drought increased the activity of CAT, POX, and APX, as well as the levels of MDA, and leaf and root proline while reducing seed yield in several pot marigold genotypes [12]. Nevertheless, a high variation in responses was found between genotypes [12]. Drought can be a significant constraint in this species, but studies performed under controlled conditions remain scarce. Consequently, this study aims to understand the impacts of moderate and severe drought in four different pot marigold genotypes under a controlled environment. Specifically, we aim to understand the response of the pot marigold antioxidant machinery to drought namely, in the levels of proline and malondialdehyde, and the enzymatic activity of catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) in leaves and flowers. We also aim to detect the impact of drought on the production of flowers.

2. Material and Methods

2.1. Plant experimental design

The antioxidant performance of *Calendula officinalis* was tested in four different genotypes: cv. Indian Prince, Golden Emperor, Orange Prince, and Sun Glow. Seeds were grown in 2 L capacity pots, in a controlled environmental chamber under a long-day photoperiod (16 h light), temperature of 23/19 °C (light/dark period), and relative humidity of 72-76%. Plants were watered every two days using Hoagland nutritive solution. One-month *Calendula* plants were subjected to different water treatments: control, 100% field capacity (FC), moderate drought, 60% FC, and severe drought, 35% FC following [12] during three weeks. Each treatment consisted of 10 biological replications, per cultivar.

2.2. Non-enzymatic activities in leaves and flowers

Proline was determined according to acid-ninhydrin and toluene methods [14]. Absorbance was determined at 520 nm. Results were expressed in micrograms of proline per gram of dry weight. Lipid peroxidation was quantified according to [15] and measured in terms of malondialdehyde content (MDA). The absorbance was measured at 532 nm. Results were expressed as nmol of MDA per gram of dry weight.

2.3. Antioxidative enzyme activities in leaves and flowers

The activity of catalase (CAT) was determined in a 1.5 mL reaction mixture with 50 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2, and the enzyme following [16] and measured at 240 nm. The results were expressed in CAT units mg^{-1} of protein. Peroxidase activity (POX) was determined as described by [17] measuring the absorbance at 430 nm. Results were expressed in units mg^{-1} of protein. Ascorbate peroxidase (APX) was determined according to [18] measured at 290 nm. Results were expressed in units APX mg^{-1} of protein. Data was expressed in dry weight (DW).

2.4. Impacts of drought on the production of flowers

For each genotype and experimental treatment, we quantified the total number of flowers produced. The dry matter content was acquired by drying samples to constant weight using a thermo-ventilated oven at 65°C.

2.5. Statistical analysis

Mean values (± SE) were calculated from the 10 replicates per cultivar, using IBM SPSS v.22. To analyze the effects of salinity we used a multivariate ANOVA or a t-test after checking the homogeneity of variance using Levene's Test for Equality of Variances. Significant differences between means were also followed by Tukey’s test for post-hoc comparisons (at the 5% significance level).

3. Results and Discussion

3.1. Non-enzymatic activities in leaves and flowers

Moderate drought had no significant effects on the levels of proline of the leaves of *Calendula* genotypes ($F_{2,11}$= 0.341, *P*=0.871) while some slight significant increases were already reported in the flowers of Golden Emperor and Sun Glow (respectively, ($F_{2,11}$= 11.872, $P<0.05$ and $F_{2,11}$= 10.201, $P<0.05$; Figure 1A). In contrast, severe drought significantly increased the levels of proline in all genotypes, either considering leaves or flowers (Figure 1A). The accumulation of proline under drought stress has been reported in many plant species [19], including *Calendula* [12]. Proline is mainly synthesized in leaves and transported to other areas to balance osmotic pressure and scavenge ROS, allowing plants to cope with drought [20]. Under stress, proline also maintains cell turgor or osmotic balance, stabilizes membranes, and prevents oxidative burst in plants. Thus, the increase found in this study suggests its role in protecting *Calendula* cultivars from the effects of severe drought.

The levels of MDA showed no significant differences between control conditions and moderate drought when recorded on leaves ($F_{2,11}$= 0.121, $P<0.05$; Figure 1B). However, slight increases were already felt in the flowers of the Golden Emperor and Sun Glow genotypes (Figure 1B). The highest increase in MDA was recorded under severe drought, in all genotypes, especially in the Golden Emperor and Sun Glow genotypes (Figure 1B). ROS induces lipid peroxidation giving rise to MDA, an indicator of membrane damage, especially during stress. Overall, the more the plant is stressed, the higher its MDA content. Thus, this stress marker indicates that flowers of some *Calendula* genotypes were already affected by moderate drought while the severe drought has strong negative effects on lipid peroxidation, on leaves and flowers, of all genotypes. The level of MDA can be used in future studies to evaluate the degree of plasma membrane damage and the ability of *Calendula* plants to tolerate drought stress.

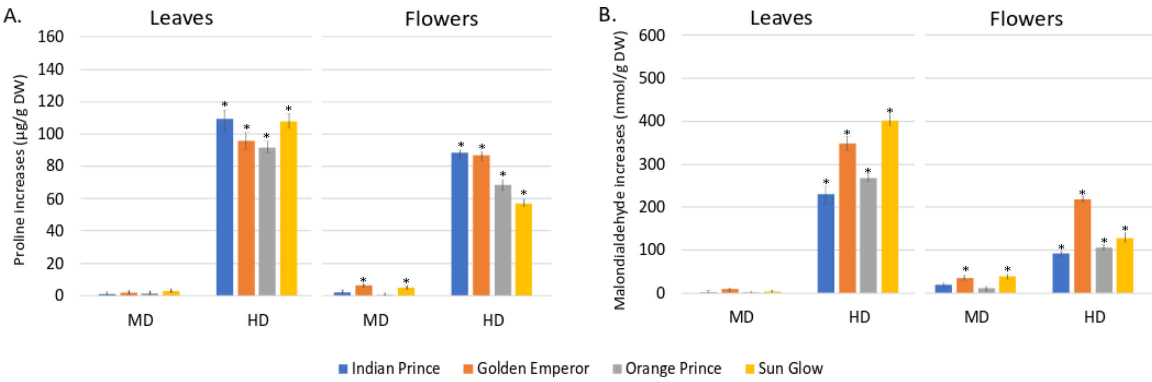

Figure 1. Effects of moderate drought (MD) and severe drought (SD) related to control conditions recorded on the leaves and flowers of four *Calendula officinalis* genotypes. A. Increases in proline content related to control conditions (µg g DW^{-1}). B. Increases in malondialdehyde levels related to control conditions (MDA; nmol g DW^{-1}). Mean values ± SE (n = 10). Asterisks indicate significant differences between MD or SD vs. control conditions, for the same species (t-test at $P<0.001$) considering only leaves or flowers.

3.2. Antioxidative enzyme activities in leaves and flowers

Under moderate drought, the enzymatic activities of catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) measured on leaves showed no significant differences with control conditions, in all genotypes (Table 1). Nevertheless, some genotypes already showed a significant increase in the activity of CAT (Golden Emperor, Orange Prince, and Sun Glow) and POX (Indian Prince) on their flowers, already under moderate conditions (Table 1).

The activities of all enzymes increased significantly under severe drought, in all genotypes and considering their levels in leaves or flowers (Table 1). However, a significant variation in enzyme activities was found between genotypes (always $P>0.05$). The highest enzymatic activities on leaves were reported in the genotype Indian Prince under severe drought, while the highest enzymatic values were recorded on flowers of the genotype Orange Prince, also under severe drought (Table 1). An increase in the activity of antioxidant enzymes was also found in other *Calendula* genotypes submitted to drought, although a high variation was found between genotypes [12]. Together with the increase in the content of proline, the higher antioxidant enzyme activities found under drought suggest a good antioxidant mechanism to cope with drought. Altogether, these enzymatic and non-enzymatic components help to reduce the oxidative stress in *Calendula* triggered by drought, as reported in other plants [18,21]. *Calendula* plants can keep ROS under control by this efficient and versatile scavenging system. This would help to protect cellular structures and functions as well as to maintain water balance and the efficiency of physiological processes.

Table 1. Enzyme activities of catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) expressed as mg of protein/DW under control water conditions, moderate drought (MD) and severe drought (SD) on four *Calendula officinalis* genotypes. Results indicate measurements on leaves/flowers. Results are expressed as means ± SE (n=10). Different superscripts indicate significant differences between water levels for the same species (ANOVA followed by a Tukey test at P<0.001) considering only leaves or flowers.

	Control	**MD**	**SD**
CAT			
Indian Prince	11.23 ± 2.21 ^{a} / 8.14 ± 1.17 ^{a}	11.66 ± 3.44 ^{a} / 8.11 ± 1.19 ^{a}	28.37 ± 4.22 ^{b} / 10.14 ± 1.29 ^{a}
Golden Emperor	14.25 ± 2.74 ^{a} / 7.89 ± 1.15 ^{a}	14.23 ± 3.01 ^{a} / 8.55 ± 2.20 ^{b}	21.99 ± 4.05 ^{b} / 10.31 ± 2.17 ^{c}
Orange Prince	12.07 ± 2.22 ^{a} / 7.99 ± 1.11 ^{a}	12.03 ± 2.66 ^{a} / 9.01 ± 1.25 ^{b}	16.28 ± 3.31 ^{b} / 14.20 ± 1.99 ^{c}
Sun Glow	10.03 ± 2.00 ^{a} / 6.45 ± 1.11 ^{a}	10.20 ± 2.11 ^{a} / 7.25 ± 1.19 ^{b}	17.25 ± 3.07 ^{b} / 9.29 ± 2.33 ^{c}
POX			
Indian Prince	1.24 ± 0.22 ^{a} / 0.33 ± 0.07 ^{a}	1.51 ± 0.44 ^{a} / 1.03 ± 0.98 ^{b}	3.25 ± 0.22 ^{b} / 2.07 ± 0.01 ^{c}
Golden Emperor	0.98 ± 0.21 ^{a} / 0.41 ± 0.11 ^{a}	1.03 ± 0.11 ^{a} / 0.48 ± 0.67 ^{a}	2.71 ± 0.56 ^{b} / 1.06 ± 0.23 ^{b}
Orange Prince	1.08 ± 0.22 ^{a} / 0.37 ± 0.13 ^{a}	1.13 ± 0.66 ^{a} / 0.41 ± 0.21 ^{a}	2.08 ± 0.71 ^{b} / 1.03 ± 0.01 ^{b}
Sun Glow	1.12 ± 0.19 ^{a} / 0.56 ± 0.212 ^{a}	1.18 ± 0.18 ^{a} / 0.23 ± 2.21 ^{a}	2.01 ± 0.98 ^{b} / 1.20 ± 0.21 ^{b}
APX			
Indian Prince	9.44 ± 2.01 ^{a} / 4.55 ± 1.04 ^{a}	9.51 ± 2.03 ^{a} / 4.23 ± 2.01 ^{a}	18.23 ± 3.05 ^{b} / 5.20 ± 2.24 ^{b}
Golden Emperor	10.21 ± 2.33 ^{a} / 3.03 ± 1.89 ^{a}	10.25 ± 2.26 ^{a} / 2.99 ± 2.28 ^{a}	16.39 ± 2.56 ^{b} / 4.56 ± 1.98 ^{b}
Orange Prince	11.23 ± 2.04 ^{a} / 6.21 ± 2.23 ^{a}	10.99 ± 3.01 ^{a} / 6.15 ± 1.99 ^{a}	14.99 ± 2.27 ^{b} / 8.05 ± 2.01 ^{b}
Sun Glow	8.05 ± 2.31 ^{a} / 4.02 ± 1.89 ^{a}	8.12 ± 2.01 ^{a} / 4.11 ± 2.17 ^{a}	11.13 ± 2.27 ^{b} / 6.73 ± 2.35 ^{b}

3.3. Impacts of drought on the production of flowers

Although flowers are crucial for the floriculture industry, studies on understanding the impacts of abiotic stresses, especially concerning *Calendula* species, are remarkably scarce. Flower production decreased by 5.01% in Indian Prince, 4.39% in Golden Emperor, 6.99% in Orange Prince, and 6.81% in Sun Glow, under moderate drought. However, a harsh effect was felt under severe drought decreasing the production of flowers by 23.67% in Indian Prince, 25.66% in Golden Emperor, 37.53% in Orange Prince, and 39.88% in Sun Glow. In general, drought decreases flower production in many species [22] since flower development and related reproductive processes are very sensitive to stress, and also because resources are allocated for plant survival under stress conditions. Therefore, it is not surprising to find that drought has a strong impact also on *Calendula* plants. However, as impacts vary between genotypes, it is crucial to conduct further tests on drought tolerance by screening additional *Calendula* plants. The effects of stress on floral development or abortion would help to develop high-yield cultivars that can cope with environmental changes using traditional and molecular breeding approaches.

3. Conclusions

Calendula plants showed a high resilience to moderate drought, in contrast with severe drought which had a harsh impact on most genotypes. The levels of proline and MDA can be used in future studies as stress markers to understand the impacts of drought in these plants. The antioxidant machinery studied increased under the harsh effect, but did not prevent negative effects on flower production, which was significantly affected by the drought. As drought showed a negative impact on flower production, future studies should focus on understanding the effects on flower development and fertility. Additionally, a high variation was found between pot marigold genotypes suggesting differences in drought tolerance, which can be used to screen useful tolerance traits. Apart from adjustments in the antioxidant system, it should be noted that drought tolerance depends on additional plant features, that should be measured to characterize the severity of drought. Useful traits for future studies should include, for example, net photosynthesis, abundance of osmoprotectants, ABA content and membrane integrity. It would also be useful to identify genes that respond to abiotic stresses in *Calendula* plants.

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