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Comparison of ROS formation and antioxidant enzymes in *Cleome gynandra* (C_4) and *Cleome spinosa* (C_3) under drought stress

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ABSTRACT

Differences between antioxidant responses to drought in C_3 and C_4 plants are rather scanty. Even, we are not aware of any research on comparative ROS formation and antioxidant enzymes in C_3 and C_4 species differing in carboxylation pathway of same genus which would be useful to prevent other differences in plant metabolism. With this aim, relative shoot growth rate, relative water content and osmotic potential, hydrogen peroxide (H_2O_2) content and NADPH oxidase (NOX) activity, antioxidant defence system (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) enzymes and their isoenzymes), CAT1 mRNA level, and lipid peroxidation in seedlings of *Cleome spinosa* (C_3) and *Cleome gynandra* (C_4) species of *Cleome* genus exposed to drought stress for 5 and 10 day (d) were comparatively investigated. Constitutive levels of antioxidant enzymes (except SOD) were consistently higher in *C. spinosa* than in *C. gynandra* under control conditions. CAT1 gene expression in *C. spinosa* was correlated with CAT activity but CAT1 gene expression in C. *gynandra* at 10 d did not show this correlation. Drought stress caused an increase in POX, CAT, APX and GR in both species. However, SOD activity was slightly decreased in *C. gynandra* while it was remained unchanged or increased on 5 and 10 d of stress in *C. spinosa*, respectively. Parallel to results of malon dialdehyde (MDA), H_2O_2 content was also remarkably increased in *C. spinosa* as compared to *C. gynandra* under drought stress. These results suggest that in *C. spinosa*, antioxidant defence system was insufficient to suppress the increasing ROS production under stress condition. On the other hand, in *C. gynandra*, although its induction was lower as compared to *C. spinosa*, antioxidant system was able to cope with ROS formation under drought stress.

1. Introduction

Drought stress caused by soil and atmospheric water deficiency is one of the most significant environmental factors affecting plant growth and productivity worldwide. It disrupts major components of the photosynthesis process. Drought stress decreases intracellular CO_2 concentration due to stomatal closure, and this causes suppression of the carboxylation reaction catalyzed by Rubisco, thus increasing photorespiration. A decrease in the photosynthetic fixation of CO_2 reduces the consumption of ATP and NADPH during the Calvin cycle and this causes over-reduction in the photosynthetic electron transport chain due to insufficient electron acceptor $NADP^+$. When ferredoxin is overreduced during photosynthetic electron transfer, electrons that have a high-energy state are transferred from the PSI to molecular oxygen forming the superoxide anion radical $O_2^{\bullet -}$ via the Mehler reaction [1], which triggers chain reactions that generate more harmful oxygen radicals like $OH^{\bullet}$. As a result of drought stress, these reactive oxygen species (ROS) cause damage to cell structures, proteins, lipids, carbohydrates, and nucleic acids, leading to cell death. However, in spite of their damaging effects, under steady state conditions, ROS play a major physiological role in intracellular signalling and regulation, as secondary messengers [2].

To protect themselves against these toxic ROS, plants have evolved efficient systems which include ROS-scavenging antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), and non-enzymatic antioxidants including ascorbic acid, glutathione, carotenoids, and tocopherols [3]. Among these, SOD decomposes $O_2^{\bullet -}$ to H_2O_2 which is further scavenged by POX in extracellular space and cytosol; and mainly by CAT in peroxisomes. H_2O_2 is also decomposed by APX, one of the Asada-Halliwell enzymes, in different cell compartments. Hence, reacting directly or indirectly with ROS, enzymatic and non-enzymatic antioxidants

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contribute in maintaining the integrity of cell structures and the proper functions of various metabolic pathways [4]. The degree to which the activities of antioxidant enzymes and the amount of antioxidants are elevated under drought stress shows a remarkable variation among plant species [5,6] and even between two cultivars of the same species [7,8]. In recent years, many researchers have established that the induction of the cellular antioxidant machinery is important for tolerance to drought stress [9].

There are significant differences in the tolerance of plants to drought stress depending upon intensity and duration of stress, plant species, and the plant's stage of development. C_4 plants, in contrast to C_3 plants, are reported to be better adapted to water stress [10,11] due to its (i) distinctive leaf anatomy (Kranz anatomy) (ii) having two carboxylation pathways (phosphoenolpyruvate carboxylase (PEPC) and RUBISCO) in mesophyll and bundle sheats that limit photorespiration and increase carboxylation efficiency [12] and (iii) requiring a lower CO_2 saturation point for photosynthesis than C_3 plants [13].

Moreover, in previous studies, differences in salt and drought tolerance between C_3 and C_4 species that belong to different genera were also reported [10,14,15]. For instance, the antioxidant defence mechanisms in the leaves of C_3 (wheat) and C_4 (maize) plants under salt and drought stress have been topics of different researchers [14,15]. However, we are not aware of any research on comparative ROS formation and antioxidant defence systems in the seedlings of C_3 and C_4 species of the same genus; this would be useful to prevent other differences in plant metabolism under stress conditions. This logic led us to consider the feasibility of using the *Cleome* genus, which has been postulated as potential topic of C_4 research [16] since it contains the C_3 (*Cleome spinosa*) and C_4 (*Cleome gynandra*) species and would be useful for comparative studies. The *Cleome* genus is part of the Capparaceae family (which includes the subfamilies Capparoideae and Cleomoideae) and a sister to the Brassicaceae family. Moreover, *Cleome* is a genus closely related to *Arabidopsis* (C_3) and their gene sequences are similar [17]. There are three known C_4 species of *Cleome* [18] and of these *C. gynandra* has been the most studied [19–22]. For these reasons, *C. gynandra* (C_4) and *C. spinosa* (C_3) were selected as research materials in the present study. Under stress and non-stress drought conditions, the following physiological parameters were investigated in the seedlings of *C. spinosa* and *C. gynandra* species of *Cleome* genus: relative shoot growth rate (RGR), relative water content (RWC), and osmotic potential; ROS formation (H_2O_2 content, and NADPH oxidase (NOX) activity); antioxidant defence systems (SOD, CAT, POX, APX and GR enzymes and isoenzymes); CAT mRNA expressions; lipid peroxidations.

2. Materials and methods

2.1. Plant material and drought stress applications

C. spinosa and *C. gynandra* seeds were sterilized in 1% NaOCl (sodium hypochlorite) solution (5 min) and then rinsed five times in distilled sterile water. Seeds were germinated at alternating temperatures 20/30 °C (16/8 h) darkness for 5 days [23]. After germination period, seedlings were transferred into the pots filled with 7:2:1, peat moss soil (Klasmann Potgrond P):vermiculate:perlite mixture. Seedlings were grown for three weeks under controlled conditions (light/dark regime of 16/8 h at 25 °C, relative humidity of 70%, photosynthetic photon flux density of (PAR) 350 $\mu mol\, m^{-2}\, s^{-1}$) and were subirrigated every other day with a half-strength Hoagland's solution [24]. As drought treatment, watering was withheld for 10 days. Seedlings were harvested on the 0, 5 d and 10 d of treatment and then stored at −80 °C until further analysis.

2.2. Growth analysis

5 random plants for each group were used for the growth analyses and were separated to seedlings and root fractions on the 0, 5 d and 10 d of stress treatment. Shoot length, fresh weights (FW) and dry weights (DW) – after the samples were dried at 70 °C for 72 h of seedlings were measured to calculate the relative growth rate (RGR) of seedlings according to Hunt et al. [25].

2.3. Leaf relative water content (RWC)

After harvest on 5 d and 10 d of drought treatment, six leaves were obtained from *Cleome* plants for each species and their FW was determined. The leaves were floated on de-ionised water for 6 h under low irradiance and then the turgid tissue was quickly blotted to remove excess water and their turgid weights (TW) were determined. DW was determined after leaves were dried in the oven. The relative water content (RWC) was calculated by the following formula [26]:

$$\mathrm{RWC}(\%) = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

2.4. Chlorophyll fluorescence

After harvest on 5 d and 10 d of stress treatment, chlorophyll fluorescence parameters (PSII maximum efficiency, Fv/Fm) of leaves of six plants for each group were measured by Plant Efficiency Analyzer of Hansatech, UK. This parameter provided an estimate of the maximum photochemical efficiency of the photosystem II (PSII).

2.5. Leaf osmotic potential ($\Psi\Pi$)

Leaf osmotic potential was measured on the 5 and 10 d of treatment by Vapro Vapor pressure Osmometer 5520. The data were collected from six sample leaves per replicate. These results were converted to MPa according to Santa-Cruz et al. [27] by multiplying coefficient of 2.408×10^{-3}.

2.6. Lipid peroxidation

The level of lipid peroxidation in samples was determined in terms of malondialdehyde (MDA) content according to the method of Madhava Rao and Sresty [28]. Content of MDA, which is an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1}\, cm^{-1}$.

2.7. Enzyme extractions and assays

All assays were performed at 4 °C. For protein and enzyme extractions, 0.5 g of samples were grounded to fine powder by liquid nitrogen and then homogenized in 1.25 ml of 50 mM Tris–HCl, pH 7.8, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% (w/v) Triton-X100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT). For APX activity determination, 5 mM ascorbate was added into homogenization buffer and PVP (2%, w/v) was used instead of DTT. Samples were centrifuged at 14,000 × *g* for 30 min, and supernatants were used for the determination of protein content and enzyme activities. Total soluble protein contents of the enzyme extracts were determined according to Bradford [29] using bovine serum albumin as

a standard. All spectrophotometric analyses were conducted on a Shimadzu (UV 1600) spectrophotometer.

POX (EC1.11.1.7) activity was based on the method described by Herzog and Fahimi [30]. The reaction mixture contained 3,3′-diaminobenzidine-tetra hydrochloride dihydrate solution containing 0.1% (w/v) gelatine and 150 mM Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2. The increase in the absorbance at 465 nm was followed for 3 min. A unit of POX activity was defined as mmol H_2O_2 decomposed ml^{-1} min^{-1}.

CAT (EC 1.11.1.6) activity was estimated according Bergmeyer [31], which measures the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0) with 0.1 mM EDTA and 3% H_2O_2. The decrease in the absorption was followed for 3 min and 1 mmol H_2O_2 ml^{-1} min^{-1} was defined as 1 unit of CAT.

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada [32]. The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA Na_2, 0.1 mM H_2O_2 and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as 1 mmol ml^{-1} ascorbate oxidized min^{-1}.

GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell [33]. The assay medium contained 25 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, and 0.12 mM NADPH·Na_4 and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). One unit of GR was defined as 1 mmol ml^{-1} GSSG reduced min^{-1}.

NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang [34]. The assay medium contained 50 mM Tris–HCl buffer, pH 7.5, 0.5 mM XTT, 100 μM NADPH·Na_4 and 20 μg of protein. After the addition of NADPH, XTT reduction was followed at 470 nm. The corrections of background production were determined in the presence of 50U SOD. Activity was calculated using the extinction coefficient, 2.16×10^4 M^{-1} cm^{-1}. One unit of NOX was defined as 1 nmol ml^{-1} XTT oxidized min^{-1}.

The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein.

2.8. Identification of isoenzymes

Samples containing equal amounts of protein were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli [35] except that sodium dodecyl sulphate was omitted.

For the separation of SOD isoenzymes, samples containing 50 μg protein per well were subjected to non-denaturing polyacrylamide PAGE in 4.5% stacking and 12.5% separating gels under constant current (120 mA) at 4 °C. SOD activity was detected by photochemical staining with riboflavin and NBT as described by Beuchamp and Fridovich [36]. The unit activity of each SOD isoenzyme was calculated by running a SOD standard from bovine liver (Sigma Chemical Co.). The different types of SOD were differentiated by incubating gels in inhibitors of SOD before staining, such as 2 mM KCN to inhibit Cu/Zn-SOD activity and 3 mM H_2O_2 to inhibit Cu/Zn-SOD and Fe-SOD activities as described by Vitória et al. [37] (Mn-SOD activity is resistant to both inhibitor treatments).

POX isoforms were detected according to Seevers et al. [38]. The electrophoretic separation was performed on non-denaturating polyacrylamide mini gels using 10% separating gel under constant current (30 mA). The gels were loaded with 20 μg protein. The gels were incubated for 30 min at 25 °C in 200 mM Na-acetate buffer (pH 5.0) containing 1.3 mM benzidine and 3% hydrogen peroxide. The gels were then stored in 7% acetic acid.

NOX isoenzymes were identified by NBT reduction method as described by Sagi and Fluhr [39]. Non-denaturing PAGE was performed at 4 °C in 7.5% polyacrylamide minigels and 30 μg protein was loaded per lane. Gels were stained in 50 mM Tris–HCl buffer (pH 7.4), 0.2 mM NBT, 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$, in the dark for 20 min. After then 0.2 mM NADPH. Na_4 was added and the appearance of blue formazan bands was observed.

Gels stained for SOD, POX and NOX activities were photographed with Vilber Lourmat gel imaging system and then analyzed with Bio-Profil Bio-1D Windows Application V11.9 software package (Vilber Lourmat, Marne la Vallée, France). In densitometric analyses of POX and NOX gels, activities of control plants was taken as 100% and % of control values for each treatment are shown. The values are average of data from three independent gels ± S.E. Within each isoenzyme, means with the same letter are not significantly different at $p < 0.05$ by Tukey.

2.9. Determination of H_2O_2 content

Determination of H_2O_2 content was performed according to Liu et al. [40]. Samples used in detection of H_2O_2 content were stored in liquid nitrogen immediately after harvesting until analyses. 0.5 g of samples were grounded with liquid nitrogen and were homogenized in 3 ml of cold (−20 °C) acetone and centrifuged at 3000 × *g* at 4 °C for 10 min. 1 ml of the supernatant was mixed with 0.1 ml of titanium reagent (prepared in concentrated hydrochloric acid containing 20% (v/v) titanium tetrachloride) then 0.2 ml of ammonium hydroxide was added to precipitate the titanium-peroxide complex. Reaction mixture was centrifuged at 16,000 × *g* at 4 °C for 10 min, and the pellet was washed with cold acetone (−20 °C). The pellet was dissolved in 2 ml of 1 M H_2SO_4. The absorbance of the solution was measured at 410 nm against water blank. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

2.10. Gene expression analysis

Total RNA from samples were isolated using the RNeasy Mini Kit (Qiagen) from 5 and 10 day old control and stress treated seedlings of *C. gynandra* and *C. spinosa*. Traces of genomic DNA were removed by DNAse treatment (DNase RQ1, Promega). cDNA synthesis was performed by the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Primers for Catalase1 (CAT1) (forward: 5′-TGTGTGGAACAACAACTCCTCCCT-3′; reverse: 5′-AAAGAAACCCTTTGCACTGGCTCC-3′; product 154 bp), *Cleome* elongation factor 1 (forward: 5′-GCTGCTGCAACAAGATGGATGCTA-3′; reverse: 5′-TGGGAACGAACGGGATCTTGTCA-3′; product 130 bp) were used in PCR reaction. CAT1 primers were designed from *C. spinosa* ESTs (accession no.: GR933582) which are homolog of *Arabidopsis* CAT1 gene (accession no.: AT1G20630). The amounts of RNA in each reaction were normalized with *Cleome* elongation factor 1 (*EF1*) gene (accession no.: GR933669) which was homolog of *Arabidopsis* EF1 gene (accession no.: AT5G60390). The conditions for PCR amplification were as follows: 95 °C 5 min, 40 cycles at 94 °C for 25 s, 55 °C for 25 s and 72 °C for 30 s. Maxima™ SYBR Green qPCR Master Mix was used (Fermentas, Germany) to perform the quantitative PCR (qRT-PCR). PCR products of CAT1 and EF1 were run on the gel, purified and sequenced directly. Sequencing was done using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit on a ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The identities of the sequences were verified in BLAST. Three independent experiments were performed for qRT-PCR assays with Bio-Rad iQ5 Real-Time PCR system. qRT-PCR data analyses were performed with Bio-Rad iQ5 software using Pfaffl's model [41]. *C. gynandra* (5 d control plants) was used as a reference point, relative expression

Table 1
Effects of drought stress on relative water content, relative growth rate, osmotic potential and Fv/Fm (maximum quantum yield of PSII) of *C. gynandra* and *C. spinosa*. The different letters are significantly different ($p < 0.05$) values. 5C: 5 d control; 5S: 5 d stress; 10C: 10 d control; 10S: 10 d stress.

	5C	5S	10C	10S
	C. gynandra			
RWC (%)	86.63 ± 1.5a	85.79 ± 1.6a	87.47 ± 1.5a	86.14 ± 1.7a
RGR (mg mg^{-1} d^{-1})	0.1198 ± 0.03a	0.1009 ± 0.02a	0.1112 ± 0.03a	0.092 ± 0.01a
Osmotic potential (Mpa)	−0.732 ± 0.007a	−0.719 ± 0.003a	−0.796 ± 0.005b	−0.794 ± 0.021b
Fv/Fm	0.805 ± 0.005a	0.804 ± 0.001a	0.805 ± 0.009a	0.802 ± 0.003a
	C. spinosa			
RWC (%)	87.1252 ± 1.3a	83.2335 ± 1.6b	85.1965 ± 1.8a	78.8732 ± 1.2c
RGR (mg mg^{-1} d^{-1})	0.1895 ± 0.04a	0.1457 ± 0.05b	0.1841 ± 0.05a	0.1176 ± 0.06c
Osmotic potential (Mpa)	−0.704 ± 0.002a	−0.727 ± 0.034a	−0.797 ± 0.006b	−0.870 ± 0.014c
Fv/Fm	0.859 ± 0.001 a	0.858 ± 0.004a	0.860 ± 0.001a	0.854 ± 0.003b

levels were calculated with respect to this reference value (set to 1) for CAT1 gene in each stress conditions.

2.11. Statistical analysis

The experiments were repeated two times independently, and each data point was the mean of three replicates ($n = 6$). All data obtained were subjected to a one-way analysis of variance (ANOVA), Tukey's post-test was used to compare the groups of the same plants species. Comparisons with $p < 0.05$ were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the means.

3. Results

3.1. Physiological parameters

3.1.1. Relative growth rate (RGR)

RGR of both species were decreased on the 5 and 10 d of drought stress (Table 1). However, the RGR of *C. spinosa* which was decreased by 21% on the 5 d and by 36.2% on the 10 d was more affected than the RGR of *C. gynandra* which was decreased by 15.2% on the 5 d and by 17.3% on the 10 d of stress treatment.

3.1.2. Relative water content (RWC)

There were no substantial differences on RWC on the 5 d and 10 d of drought stress conditions in *C. gynandra* (Table 1). However, significant changes on RWC were determined in *C. spinosa*, in this species RWC showed a decrease by 3.9% (from 87.12% to 83.23%) on the 5 d and by 6.3% (from 85.19% to 78.87%) on the 10 d of stress treatment.

3.1.3. Leaf osmotic potential ($\Psi\Pi$)

$\Psi\Pi$ did not differ between control and drought stress-treated plants either on the 5 d or 10 d stress in *C. gynandra* (Table 1). On the other hand, in *C. spinosa*, while $\Psi\Pi$ was not changed at 5 d, it was decreased at 10 d of stress treatment, as compared to control groups.

3.1.4. Chlorophyll fluorescence

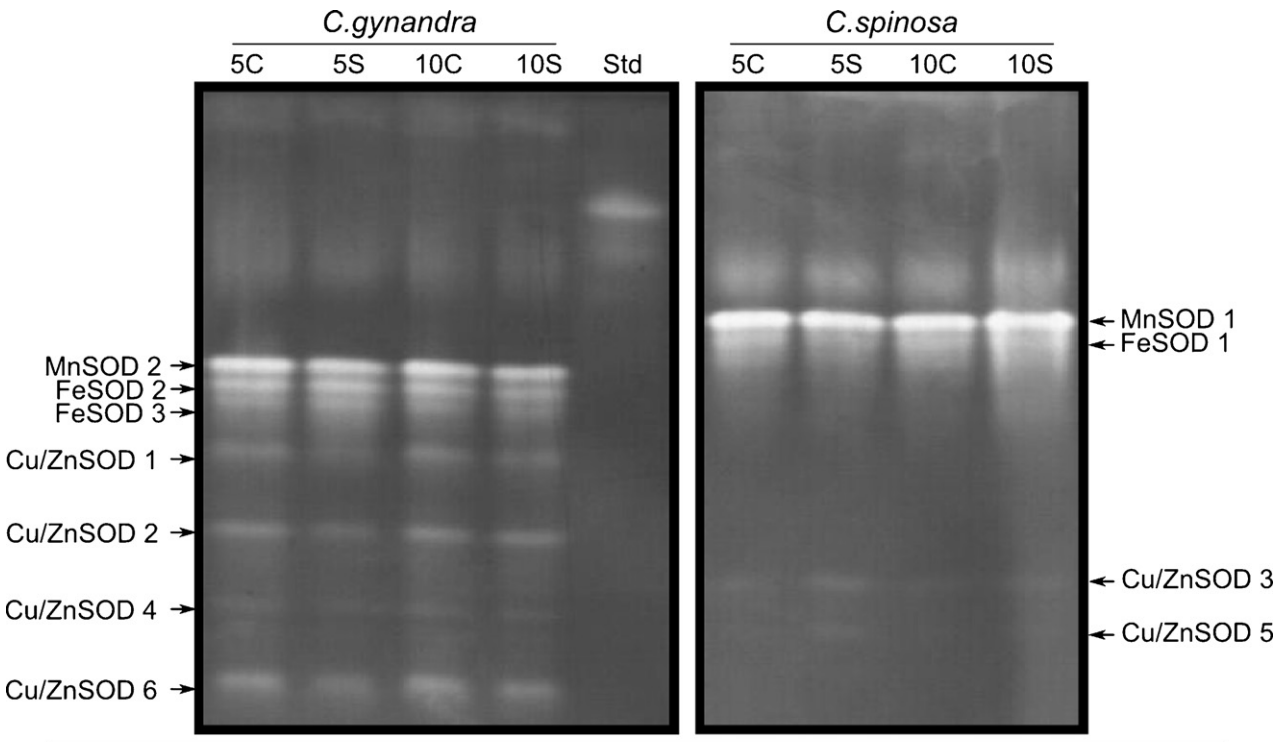
No significant impact of stress treatment on chlorophyll fluorescence of *C. gynandra* was found in present study. Similarly, chlorophyll fluorescence of *C. spinosa* was not changed on 5 d of stress. However, it was decreased from 0.860 (±0.001) to 0.854 (±0.003) on the 10 d of stress treatment (Table 1).

3.2. Enzyme and isoenzyme activities

The activity of SOD was significantly higher in *C. gynandra* than in *C. spinosa* under control conditions (Fig. 1). The activities of SOD were decreased by 12% and 19% on the 5 and 10 d of drought stress in *C. gynandra*, respectively. On the other hand, on the 5 d, drought stress did not create a significant difference in SOD activity in *C. spinosa*. However, it caused an enhancement (22.68%) of SOD activity in *C. spinosa* on the 10 d of stress, as compared to controls. SOD activity was always significantly higher in *C. gynandra* than in *C. spinosa* on the 5 d of drought, but on the 10 d, SOD activities of both species were similar despite of enhancement or decreasing in SOD activities of C_3 and C_4 plants by drought stress.

Drought-induced changes in the isoenzyme pattern of SOD were detected by Native-PAGE electrophoresis. As shown in Fig. 1, SOD isoenzymes were identified as one Mn-SOD, two Fe-SOD and four Cu/Zn-SOD in *C. gynandra* and one Mn-SOD, one Fe-SOD and two Cu/Zn-SOD in *C. spinosa*. Densitometric analyses of activity gels in control seedlings showed the Mn-SOD accounted for 28.75% and 64.13%; Fe-SOD for 31.73% and 30.75% and Cu/Zn-SOD for 39.52% and 5.12% in *C. gynandra* and *C. spinosa*, respectively. Under control conditions, the activities of Cu/Zn-SOD isoenzymes in *C. gynandra* and Mn-SOD isoenzyme in *C. spinosa* were major SOD isoenzymes. During the first 5 d of exposure to drought stress, although total SOD activity in *C. gynandra* was decreased, activity of Fe-SOD2 was increased. At 10 d of stress, the decrease in *C. gynandra* coinceded with the decrease in all SOD isoenzymes (except Cu/Zn-SOD2). The number of isoenzymes in *C. gynandra* did not change in both treatment time. On the other hand, unlike *C. gynandra*, in *C. spinosa*, at 10 d of stress, intensities of Fe-SOD and Mn-SOD isoenzymes showed concomitant increase with total activity in *C. spinosa*, but Cu/Zn-SOD isoenzymes were not determined under same conditions, as compared to control groups. At 5 d of stress, the isoenzymes showing correlated increasing enzyme activity were Cu/Zn-SOD3 and Cu/Zn-SOD5 which were determined only in this treatment group of *C. spinosa*.

POX activity was enhanced by 36.4% on 5 d and 26.9% on 10 d in C. *gynandra*. *C. spinosa* also exhibited a slightly increasing pattern with 17.2% and 13.2% increases on 5 d and 10 d of drought stress, respectively (Fig. 2A). In both species, total of 10 POX activity bands were observed (Fig. 3). There were no identical isoenzymes identified between two species. While POX2, 5, 8, and 10 were determined in *C. gynandra*, POX1, 3, 4, 6, 7, and 9 were identified in *C. spinosa*. Among isoenzymes measured POX2 had no significant difference, but POX5 showed a 21.9% increase on the 5 d of stress. On the 10 d, although POX2 expression was decreased by 13%, no clear POX5 induction was observed under drought stress. However, POX8 and 10 were observed as faint bands in control and also in 5 d of stress plants, but their intensities were increased (73.1% and 90.7%, respectively) by drought stress, on the 10 d. On the other hand, in *C. spinosa*, POX1 was decreased by 10.2% on the 5 d of stress, but it was increased by 5.4% on the 10 d of drought stress. Unlike POX1, POX3 was increased by 11.4% on the 5 d and decreased by 8.3% on the 10 d of stress. Interestingly, POX4 was determined in only control groups. POX6 showed decreasing patterns by 10.6% and 36.5% on the 5 d and



C.gynandra

	5 d		10 d	
	Control	Stress	Control	Stress
MnSOD4	100 (0.413 u ± 0.013a)	99.7 (0.411 u ± 0.015a)	100 (0.394 u ± 0.011a)	75.9 (0.299 u ± 0.017b)
FeSOD2	100 (0.237 u ±0.01a)	120.6 (0.286 u ± 0.009b)	100 (0.0.203 u ± 0.02a)	97.5 (0.198 u ± 0.013a)
FeSOD3	100 (0.218 u ± 0.009a)	96.9 (0.211 u ± 0.015b)	100 (0.134 u ± 0.009a)	78.3 (0.104 u ± 0.01b)
CuZnSOD1	100 (0.128 u ± 0.004a)	47.5 (0.061 u ± 0.003b)	100 (0.116 u ± 0.005a)	76.8 (0.089 u ± 0.008a)
CuZnSOD2	100 (0.129 u ± 0.007a)	56.4 (0.073 u ± 0.008b)	100 (0.115 u ± 0.01a)	108.4 (0.125 u ± 0.009b)
CuZnSOD4	100 (0.053 u ± 0.002a)	82.1 (0.043 u ± 0.003b)	100 (0.055 u ± 0.003a)	56.0 (0.031 u ± 0.007b)
CuZnSOD6	100 (0.256 u ± 0.012a)	68.1 (0.174 u ± 0.010a)	100 (0.245 u ± 0.012a)	69.7 (0.171 u ± 0.009b)
Total MnSOD	0.413	0.412	0.395	0.299
Total FeSOD	0.456	0.498	0.338	0.304
Total Cu/ZnSOD	0.568	0.353	0.533	0.417
Total Unit	1.437	1.263	1.266	1.020

C.spinosa

	5 d		10 d	
	Control	Stress	Control	Stress
MnSOD3	100 (0.64 u ± 0.033a)	102.9 (0.656 u ± 0.022a)	100 (0.510 u ± 0.031a)	118.8 (0.606 u ± 0.027a)
FeSOD1	100 (0.305 u ± 0.017a)	46.3 (0.141 u ± 0.0014b)	100 (0.360 u ± 0.011a)	114.4 (0.412 u ± 0.015b)
CuZnSOD3	100 (0.0509 u ± 0.009a)	181.4 (0.092 u ± 0.006b)	nd	100 (0.049 u ± 0.006a)
CuZnSOD5	nd	100 (0.079 ± 0.005 u)	nd	nd
Total MnSOD	0.638	0.657	0.511	0.607
Total FeSOD	0.306	0.142	0.361	0.412
Total Cu/ZnSOD	0.051	0.172	0.000	0.050
Total Unit	0.995	0.970	0.871	1.069

Fig. 1. Activity staining and % induction of SOD isoenzymes in the crude extract of seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d are shown. Samples applied to the gels contained 50 μg of protein. Lanes: std.: 0.5 unit Bovine Liver SOD; 5C: 5 d control; 5S: 5 d stress; 10C: 10 d control; 10S: 10 d stress. The values in parenthesis show the SOD activity as units mg^{-1} protein. The different letters are significantly different ($p < 0.05$) % inductions within the same species.

10 d of stress, respectively. While POX7 was slightly enhanced (8.2%), there was not any significant change in POX9 on the 5 d of drought stress. On the other hand, activities of POX7 and POX9 were enhanced by 28.4% and 40.7% on the 10 d with increased intensity of stress.

In control plants, the activity of CAT was distinctly higher (2.07 and 2.15 fold on the 5 d and 10 d, respectively) in *C. spinosa* than in *C. gynandra* (Fig. 2B). On the 5 d of drought stress, unlike *C. gynandra*, CAT activity of *C. spinosa* was increased by 45.75% as compared to control groups. On the other hand, on the 10 d, CAT activities of both species were increased significantly under drought stress, but it was more pronounced in *C. spinosa*. However, CAT activity induction was higher in *C. gynandra* than in *C. spinosa* on the 10 d of drought stress.

Expression level of catalase did not change in *C. gynandra* on the 5 d and 10 d of stress when compared to control levels. But there was a significant increase in expression levels between 5 d and 10 d plants both in control and stress groups. On the other hand, *C. spinosa* showed 8.4 fold increase on the 5 d and 4.5 fold increase on 10 d when compared to its controls (Fig. 4).

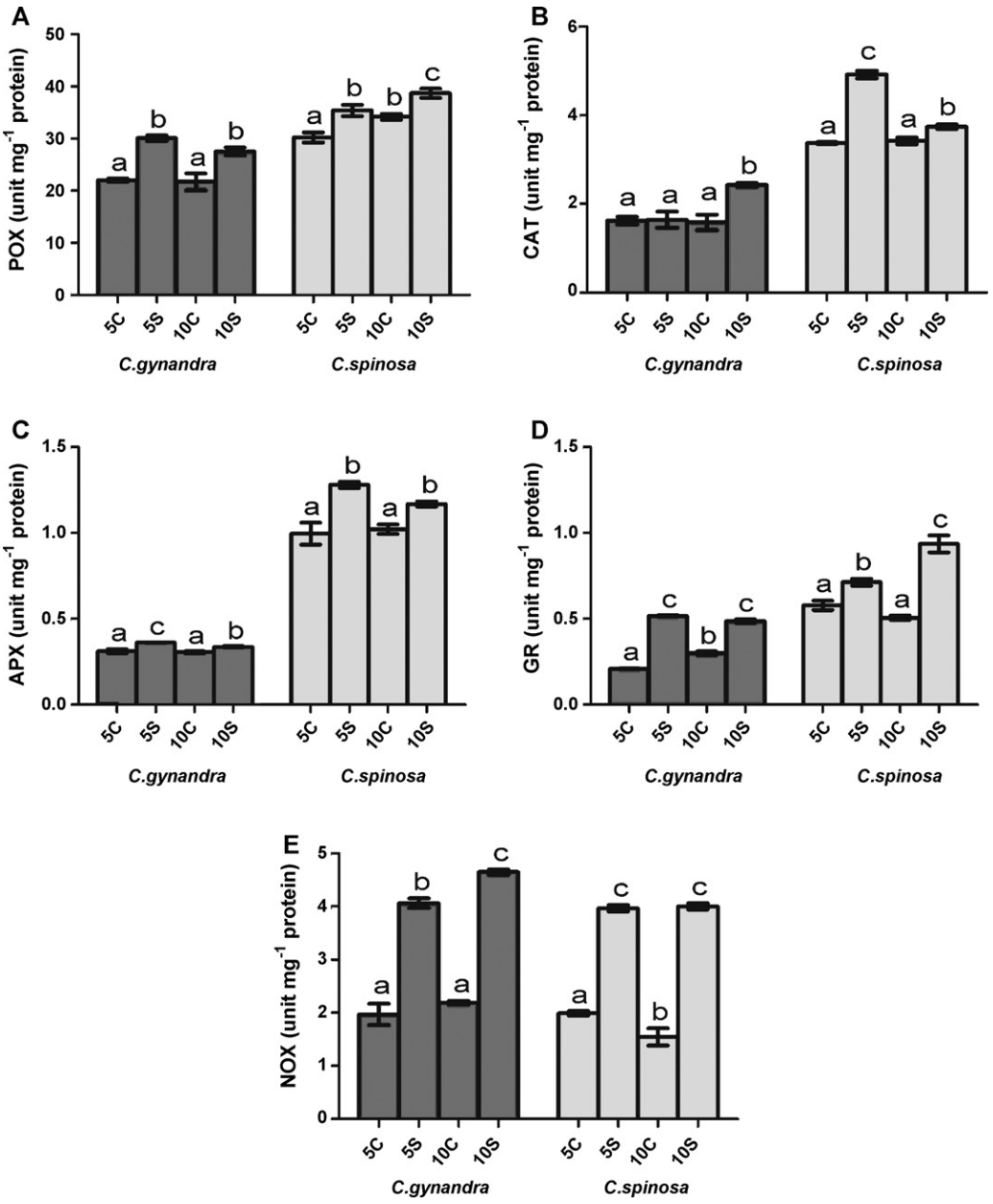


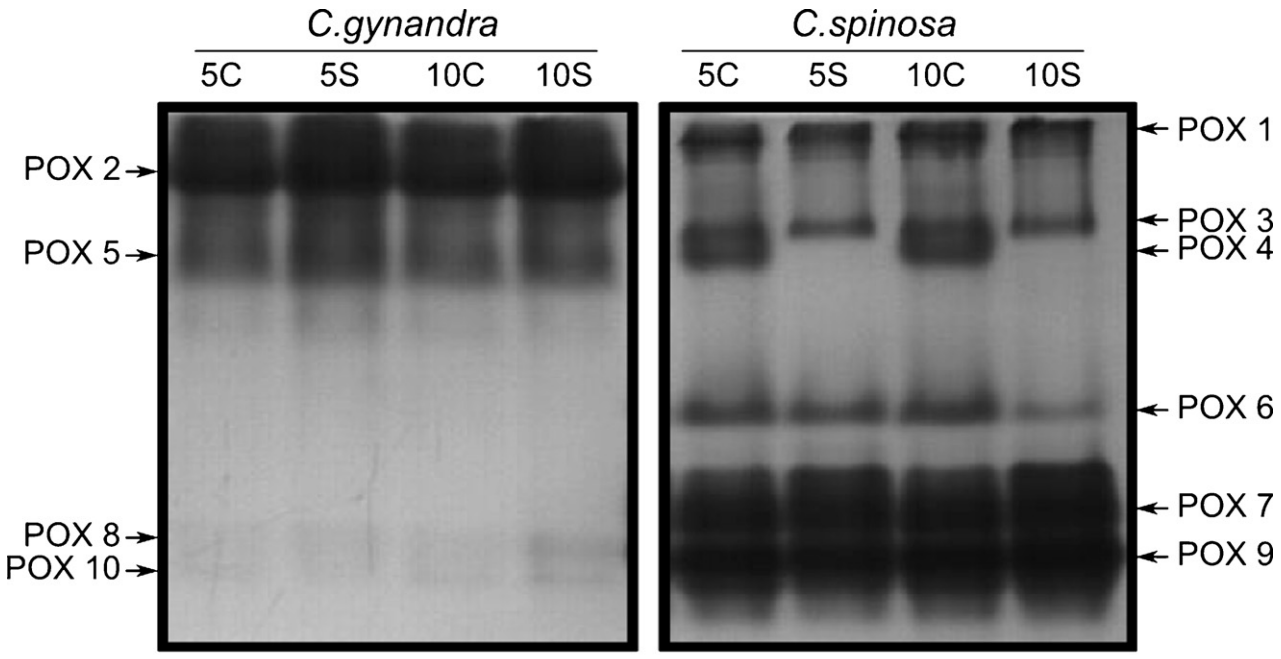
Fig. 2. The effects of drought treatment on POX (A), CAT (B), APX (C), GR (D) and NOX (E) activities in seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d are shown. Data represents the average of two experiments with three replicates. Vertical bars indicate ± SE and values sharing a common letter are not significantly different at $p < 0.05$.

Similar to CAT, the activity of APX in control plants was higher in *C. spinosa* compared to *C. gynandra* (Fig. 2C). APX activity was enhanced in *C. gynandra* by 12.42% and 10.17% on the 5 and 10 d, respectively, whereas in *C. spinosa*, the enhancements were 28.54% and 14.39% respectively, as compared to control groups. Although APX activity increased in *C. gynandra* by 5 d and 10 d of drought stress, it never reached a high level as in *C. spinosa* in the same conditions.

There were remarkable differences between constitutive levels of GR activities in both species (Fig. 2D). The constitutive levels of GR in *C. spinosa* was higher than in *C. gynandra*. GR activity was greatly increased by 147% on 5 d and 62% on the 10 d of stress treatment in *C. gynandra* as compared to controls. *C. spinosa* also exhibited an increasing pattern by 23.3% on the 5 d and 84.5% on the 10 d of drought stress.

To measure the effects of drought stress on ROS production, NOX activity was measured in *C. gynandra* and *C. spinosa* (Fig. 2E). NOX activity was induced on the 5 and 10 d of stress treatments in both species. On the 5 d, NOX activity was induced by 93% and 99% in *C. gynandra* and *C. spinosa*, respectively. However, on the 10 d a stronger induction of NOX was observed in *C. spinosa* by 158% compared to 109% induction in *C. gynandra* compared to their control groups.

For the determination of isoenzyme pattern and regulation of NOX, native activity gels were performed (Fig. 5). 5 NOX isoenzymes were determined in both species. While NOX1 isoenzyme was identified in both species under control and drought conditions, NOX2 and NOX5 in *C. gynandra* and NOX3 and NOX4 in *C. spinosa* were determined. NOX1 activity was increased by 191.8% and 136.6% at 5 d, and 253.8% and 226.3% at 10 d of drought stress in *C. gynandra* and *C. spinosa*, respectively. In *C. gynandra*, NOX2 was observed as a faint band at control groups but their intensities were increased by drought stress on the 5 and 10 d. NOX5 was observed in all treatment groups of *C. gynandra* that showed



C.gynandra				
	5 d		10 d	
	Control	Stress	Control	Stress
POX2	100 a	105.9 ± 3.4 a	100 a	87 ± 4.4 b
POX5	100 a	121.9 ± 5.2 b	100 a	93.4 ± 4.1 a
POX8	nd	nd	100 a	173.1 ± 6.8 b
POX10	nd	nd	100 a	190.7 ± 5.6 b
C.spinosa				
	5 d		10 d	
	5C	5S	10C	10S
POX1	100 a	89.8 ± 2.9 b	100 a	105.4 ± 3.3 a
POX3	100 a	111.4 ± 3.7 b	100 a	81.7 ± 3.2 b
POX4	100	nd	100	nd
POX6	100 a	89.4 ± 3.1 b	100 a	63.5 ± 2.4 b
POX7	100 a	108.2 ± 2.2 b	100 a	128.4 ± 6.1 b
POX9	100 a	104.9 ± 2.4 a	100 a	140.7 ± 7.3 b

Fig. 3. Activity staining and % induction of POX isoenzymes in the crude extract of seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d are shown. Samples applied to the gels contained 20 μg of protein. Lanes; 5C: 5 d control; 5S: 5 d stress; 10C: 10 d control; 10S: 10 d stress. The different letters are significantly different ($p < 0.05$) % inductions within the same species.

a strong induction (191.2% and 137.9%, on the 5 and 10 d, respectively) under drought stress compared to their controls. Similar to these, in *C. spinosa*, NOX3 and NOX4 isoenzymes were also observed in all treatment groups. While, activities of NOX3 and NOX4 were induced by 120.2% and 121.5% on the 5 d and 224.7% and 174.8% on the 10 d of drought stress, respectively.

3.3. Lipid peroxidation

The level of damage related to oxidative stress was determined by monitoring the differences in lipid peroxidation referring malondialdehyde (MDA) formation (Fig. 6A). There were no significant changes in lipid peroxidation levels of *C. gynandra* whereas *C. spinosa* showed a significant increase by 35% and 81% on the 5 d and 10 d of stress treatment, respectively.

3.4. H_2O_2 content

There was no significant change in H_2O_2 content of *C. gynandra* at 5 d or 10 d of the drought stress. On the other hand, H_2O_2 content was increased by 25.9% in *C. spinosa* on 5 d of stress compared to its control group (Fig. 6B).

4. Discussion

Investigating interactions between salt/drought tolerance and antioxidant defence of C_3 and C_4 plants, each belonging to a separate genus set, have been topic for different researches. In these limited number of studies it was found that antioxidants play an important and efficient role, making maize (C_4) more resistant to environmental stresses than wheat (C_3) [14,15] and inherent lev-

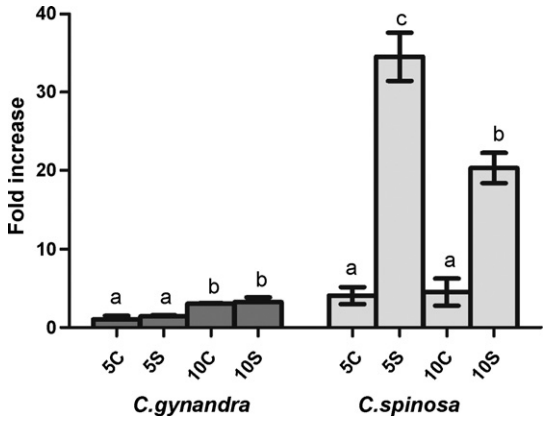


Fig. 4. Expression levels of CAT1 mRNA in seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d of stress. *C. gynandra* (5 d control plants) was used as a calibrator, relative expression levels were calculated with respect to this reference value (set to 1) for CAT1 gene in each stress conditions. Data represents the average three replicates. Vertical bars indicate ± SE and values sharing a common letter are not significantly different at $p < 0.05$.

els of antioxidants in sorghum (C_4) were not consistently higher than in sunflower (C_3) under stress [5]. However, none of these studies were conducted on C_3 and C_4 species of the same genus; which would be useful to prevent involvement of other differences in plant metabolism and anatomy under stress conditions. Taking this into consideration, for the present study, *C. gynandra* (C_4) and *C. spinosa* (C_3), both species from *Cleome*, were selected and the relationships between ROS formation, antioxidant defence and drought tolerance were determined.

Stress conditions have negative effects on RGR. It significantly decreases in C_3 plants and intolerant plants when stress is present [42]. In our experiment, both species showed a decrease in RGR with stress. However, the effect on RGR of *C. spinosa* was approximately 2 fold higher than that of *C. gynandra* for the same drought conditions. Similarly, Nayyar and Gupta [15] also showed the same decreasing growth pattern when drought conditions were applied to maize and wheat.

RWC is generally used to evaluate the level of water balance in plants and is a reliable means by which the state of osmotic stress is measured. Being dependent upon stress duration, C_4 plants are more capable of dealing with water deficiency conditions than C_3 plants [15]. In this study, neither 5 d nor 10 d of drought stress affected the RWC of the *C. gynandra* plants; however, stress reduced the RWC of *C. spinosa* which was more serious after 10 d of drought stress treatment. Ozfidan et al. reported that drought stress decreased osmotic potential in *Arabidopsis thaliana* seedlings depending on the duration of stress [43]. In agreement with this result, the osmotic potential of *C. spinosa* also decreased after stress.

Fv/Fm, one of the chlorophyll fluorescence parameters, is an indicator of the stress damage which is related to the PSII machinery, the subject of photoinhibition by stress. The photosynthetic efficiency of PSII is reduced by stress conditions such as drought and salt exposure [44]. Compared to C_3 plants, C_4 plants more successfully manage to avoid the stress-related inhibition effects of excess photons [15]. In the present study, no significant effect of drought stress in *C. gynandra* was observed while a slight effect was observed in *C. spinosa*. According to Dwyer et al. [45], C_4 plants tend to have lower quantum yields and typically higher light saturation points than C_3 plants, which could make saturation more difficult to achieve. They were unable to achieve saturation with a single flash but were successful with a multiple flash system. The reason why the Fv/Fm of *C. gynandra* was lower than that of *C. spinosa* may depend on the system used to measure chlorophyll fluorescence.

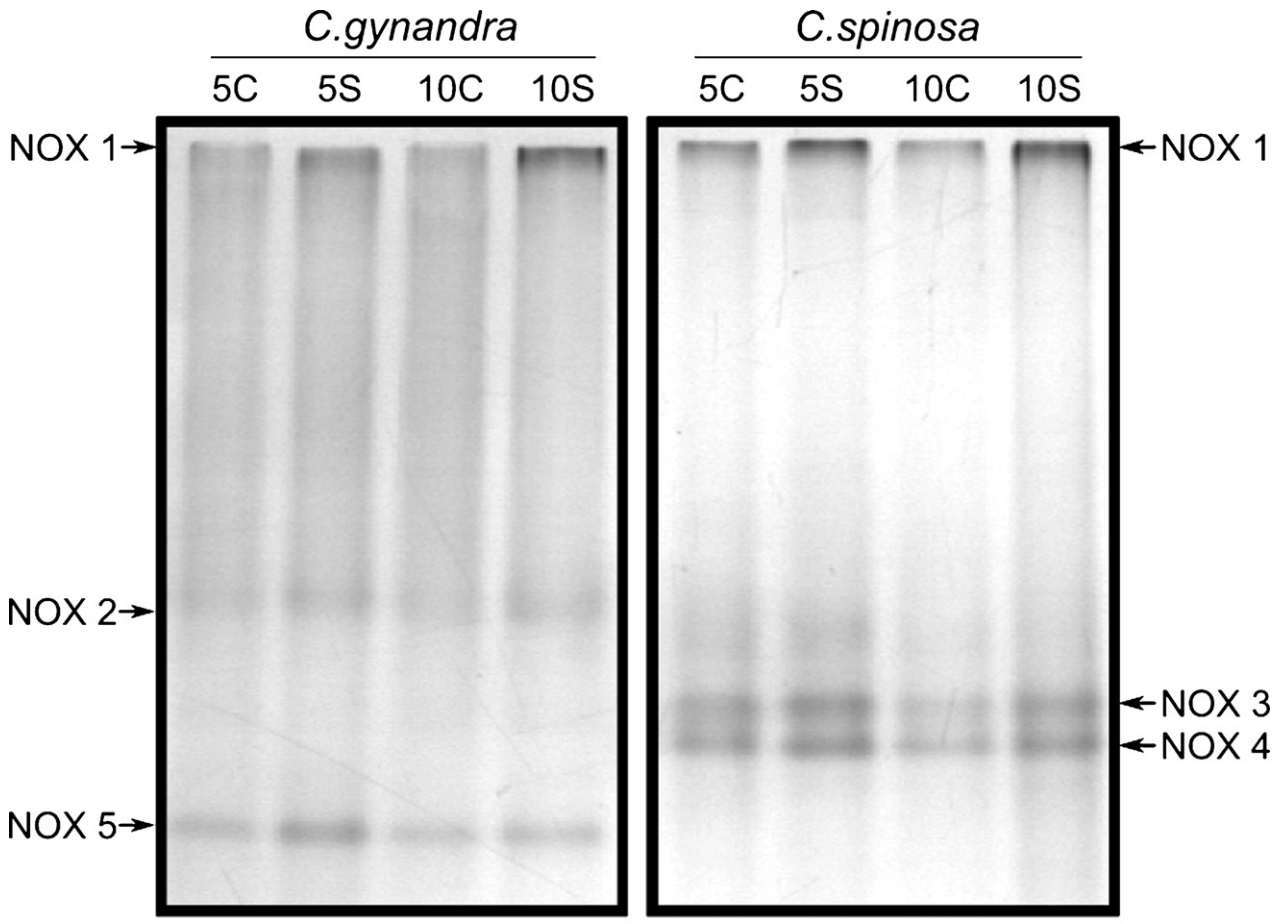
SOD is one of the most important enzymes used against oxidative stress in the plant defence systems [46]. The induction of this enzyme reflects its important role in the defence mechanism of plants [47]. However, Cavalcanti et al. [48] found that SOD activities are not required for the salt tolerance of cowpea. Similarly, in the present study, we also found a slight decrease in *C. gynandra* under drought stress as compared to control groups, but this decrease did not affect the scavenging efficiency of *C. gynandra* against drought stress induced-ROS formation as evidenced by the unchanged levels of MDA (Fig. 6B). On the other hand, at 10 d of exposure, the SOD activity in *C. spinosa* was increased by drought stress. This increase of SOD activity might reflect enhanced $O_2^{\bullet -}$ production, probably as a result of electron leakage from the electron transport chain to molecular oxygen.

Constitutive levels of SOD in *C. gynandra* were higher than in *C. spinosa*, indicating that C_4 has a higher dismutating capacity than C_3. Similarly, Stepien and Klobus [14] also found that maize (C_4) had a higher constitutive level of SOD activity. In contrast to these results, Nayyar and Gupta [15] and Zhang and Kirkham [5] showed that the SOD activity levels varied insignificantly between maize/wheat, and sorghum/sunflower, respectively.

Zhang and Kirkham [5] reported that alterations in the relative contributions of different SOD isoenzymes to the total activity could differ between C_3 and C_4 and such a difference in isoenzymes could be related to drought resistance. In accordance with this suggestion, in the present study, the individual responses of SOD isoenzymes in both species under drought stress were identified. Although SOD activity was decreased by drought stress, the activities of Fe-SOD2 and Cu/Zn SOD2 were significantly increased in *C. gynandra* at the 5 d and 10 d levels of drought stress. We suggest that the chloroplast and cytosol compartments might be more important in scavenging $O_2^{\bullet -}$ in the stressed leaves of *C. gynandra*, owing to the higher relative contribution of Fe-SOD2 and Cu/Zn-SOD2 isoenzymes more than Mn-SOD isoenzyme. In contrast to *C. gynandra*, the Mn-SOD activities of *C. spinosa* were increased under both drought and control conditions which indicates mitochondrial based compartmentalization of $O_2^{\bullet -}$. These results suggest that an increase in photorespiratory NADH might cause over-reduction of the electron transport chain in mitochondria and $O_2^{\bullet -}$ radical production due to transfer of electrons to molecular oxygen.

POXs are the major scavengers of H_2O_2 in chloroplasts and cytosols. They also have important roles in growth, development, lignifications and subarization processes [49]. Increasing POX activities as a result of stress have been reported in C_3 tomato and C_4 maize [50,51]. In agreement with these results, the POX activity of *C. gynandra* and *C. spinosa* were enhanced by drought stress. Different POX isoenzyme patterns of *C. gynandra* and *C. spinosa* had been identified in this study. In *C. gynandra*, POX2 and POX5 played major roles in the effect of short term (5 d) stress, while POX8 and POX10 played major roles in the effect of long term (10 d) stress. On the other hand, in *C. spinosa*, POX7 and POX9 were enhanced by long term stress (10 d) indicating that they play a crucial part in the scavenging of H_2O_2.

CAT has a significant role in plant defence against drought-induced oxidative stress. The main site of CAT action is known to be the peroxisomes where the H_2O_2 evolving step of photorespiration occurs. The remarkable increase in CAT activity in particularly seedlings of *C. spinosa* at 5 d of drought suggests a strong induction in the H_2O_2 concentration which might be resulting from increased photorespiration and/or diffusing from other cell compartments as also reported by Abogadallah et al. [52] in banyard grass. The increase in CAT activity helps to overcome the damage of tissue metabolism by reducing the toxic level of H_2O_2. However, Harinasut et al. [53] showed that CAT activity did not change by increasing stress. Similarly, in the present study, at 5 d of drought, CAT activity in *C. gynandra* did not respond to drought stress. This unchanged



C.gynandra				
	5C	5S	10C	10S
NOX1	100 a	191.8 ± 6.4 b	100 a	253.8 ± 7.1 b
NOX2	nd	100.0	nd	100.0
NOX5	100 a	191.2 ± 5.6 b	100 a	137.9 ± 3.7 b
C.spinosa				
	5C	5S	10C	10S
NOX1	100 a	136.6 ± 2.4 b	100 a	226.3 ± 7.4 b
NOX3	100 a	120.2 ± 3.5 b	100 a	224.7 ± 7.2 b
NOX4	100 a	121.5 ± 2.7 b	100 a	174.8 ± 6.9 b

Fig. 5. Activity staining and % induction of NOX isoenzymes in the crude extract of seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d are shown. Samples applied to the gels contained 30 μg of protein. Lanes; 5C: 5 d control; 5S: 5 d stress; 10C: 10 d control; 10S: 10 d stress. The different letters are significantly different ($p < 0.05$) % inductions within the same species.

CAT activity reflects the importance of POX in H_2O_2 scavenging system in *C. gynandra* as evident by increased POX activity at the same time. CAT1 gene expression in *C. spinosa* was correlated with CAT activity but CAT1 gene expression in *C. gynandra* at 10 d did not show this correlation. This discrepancy may be due to redundant enzymes (different isoenzymes) of the CAT family in *C. gynandra*.

APX plays a vital role in scavenging H_2O_2 in water–water and ASC–GSH cycles [54]. Compared to the affinity of CAT and POX for H_2O_2, APX plays a more crucial role in the management of ROS in higher plants during stress due to its higher affinity for H_2O_2. A number of different reports have shown enhanced activities of APX in plants in response to different abiotic stresses [55–57]. In the present study, the activities of APX in both species were increased by drought stress. In *C. gynandra*, although APX levels increased remarkably, CAT activity was maintained at 5 d as compared to the control group. This suggests that APX and POX may be more important than CAT in scavenging H_2O_2 in the case of short term stress. On the other hand, the stronger induction of CAT as compared with APX showed that CAT might play a key role in scavenging H_2O_2 during long term stress (10 d). Since *C. spinosa* has higher activities of CAT and APX enzymes (both inherent and drought-induced), we suggest that APX and CAT enzymes have equal importance in the detoxification of H_2O_2 in the seedlings of *C. spinosa*. On the other hand, these increases in APX and CAT activities under drought stress did not prevent the accumulation of H_2O_2 in *C. spinosa*.

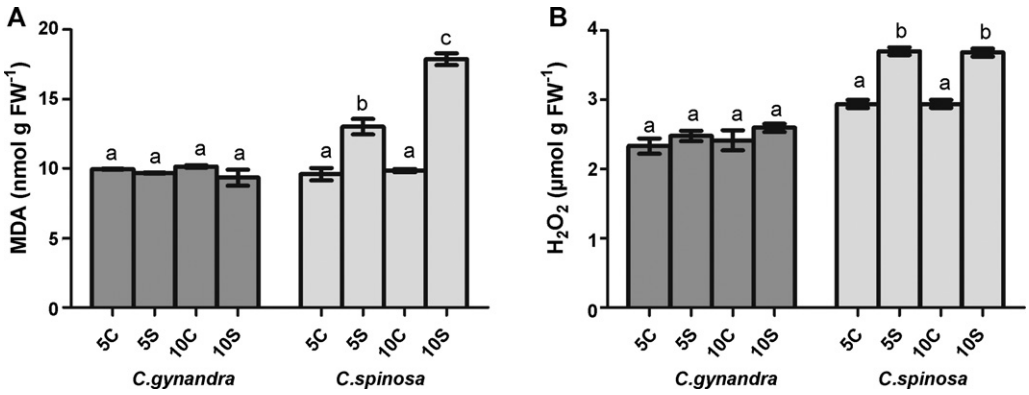


Fig. 6. The effects of drought treatment on MDA (A) and H_2O_2 (B) contents in seedlings of *C. gynandra* and *C. spinosa* on 5 and 10 d are shown. Data represents the average of two experiments with three replicates. Vertical bars indicate ± SE and values sharing a common letter are not significantly different at $p < 0.05$.

GR is a potential enzyme of the ASC–GSH cycle which plays an essential role in the plant's defence against ROS by maintaining the GSH level [58]. GR activity was generally higher in *C. spinosa* than in *C. gynandra* under control and stress conditions. Our results agree with those of Sharma and Dubey [59], who observed increases in GR activity for *Oryza sativa* seedlings subjected to drought stress. Increases in GR activity under stresses of salt, Cd and Cu^{+2} were also observed in *Capsicum annuum* and *C. orientinum* [60,61]. The enhanced activity of GR might assist the regeneration of NADPH to NADP, thereby ensuring the availability of NADP to accept electrons from the photosynthetic electron transport chain [62].

NADPH oxidases are membrane bound $O_2^{\bullet -}$ producing enzymes playing key roles in plant growth, development, and in biotic and abiotic stress responses [63]. NOX acts as a supplier of ROS which is needed for signal transduction and stress perception in plants [64]. Many studies have shown that NOX activity increases under drought stress [34,65]. In agreement with these studies, we also observed a strong increase in NOX activity in both *Cleome* species. However, we did not observe any significant differences in the constitutive levels of NOX activity between C_3 and C_4. But, at 10 d of stress treatment, the induced level of NOX activity of *C. gynandra* was higher than that of *C. spinosa*. This increasing pattern may cause the acclimation to stress before it gets more severe [66].

Lipid peroxidation, which refers to the oxidative degradation of lipids, is a widely used marker of oxidative damage. In this study, the level of lipid peroxidation was determined by measuring MDA content, which is a product of lipid peroxidation. Maintenance of membrane functionality due to unchanged MDA content in *C. gynandra* under drought stress may be attributed to the adequate response of the antioxidant system to drought-induced oxidative damage, as also observed in some other stress-tolerant plants such as maize [34], sorghum [5] and *Plantago maritima* [67]. On the other hand, in *C. spinosa,* increased MDA depending on time of drought treatment showed that the effect of antioxidant defence was insufficient under stress conditions to suppress the increasing ROS production.

H_2O_2 accumulation in cells may have two different effects. Its presence may cause the formation of $OH^{-\bullet}$ by the Fenton reaction which may act as a secondary messenger to induce gene expression or other responses [9]. In this study, we found that the H_2O_2 content of *C. gynandra* did not change with stress treatments, which may be caused by the efficient scavenging of H_2O_2 by antioxidant defence enzymes such as CAT, APX and POX. In contrast to this, there was an increase in the H_2O_2 content at 5 d and 10 d of drought stress in *C. spinosa*. This might be due to the dismutation of $O_2^{\bullet -}$ that has been produced in the mitochondrial respiration electron transport chain or during chloroplast photosynthetic electron transport or during photorespiratory flux. In *C. spinosa*, the increase in H_2O_2 formation and concentration and thus potential for damage, depends on the capacity of both production and scavenging of H_2O_2. On the other hand, despite the decrease of SOD activity in *C. gynandra* under drought stress, the levels of H_2O_2 scavenging enzymes were enhanced indicating an increase in H_2O_2 production. In this case, the increased production of H_2O_2 dismutated from $O_2^{\bullet -}$ might be caused by (1) an increased capacity of non-enzymatic antioxidant scavenging activity (ascorbate, glutathione, β carotene, α-tocopherol etc.) or (2) strongly expressed SOD under control conditions and thus both prevent oxidative damage to *C. gynandra* under stress as evidenced by unchanged MDA level.

5. Conclusions

To our knowledge, this is the first report on comparison of ROS formation and antioxidant enzymes in the C3 and C4 plants belonging to same genus, *Cleome*. (i) Under control conditions, constitutive levels of all antioxidant enzymes (except SOD) were consistently higher in *C. spinosa* (C_3) than in *C. gynandra* (C_4). (ii) Under drought stress, the activities of antioxidant enzymes generally increased, decreased and remained unchanged in *C. spinosa* and *C. gynandra* depending on duration of stress treatment (iii) C_3 plants produce H_2O_2 by increasing glycolate oxidase activity through photorespiration, whereas C_4 plants usually do not photorespire as reported by Zhang and Kirkham [5]. Similarly, in present study, the highest H_2O_2 content was observed in *C. spinosa* (C_3). As shown by an increase in MDA content for this C_3 plant, in response to stress conditions, changes in the amount of antioxidant enzymes was not sufficient to prevent peroxidation of membrane lipids.

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