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COMPARATIVE PHENOTYPING ASSESSMENT OF FOUR DIFFERENT OIL SEED CULTIVARS USING STRESS-INDUCED PHYSIOLOGICAL TRAITS IN WATER DEFICIT ENVIRONMENT

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Abstract

Comparative phenotyping assessment of four oilseed cultivars, two soybean (RAWAL and AJMERI) and sunflower (SF0054 and SF0024) in a water deficit environment, was studied. Phenotyping was performed using stress-induced physiological traits. Results depict that drought stress decreased the stomatal conductance, relative water content (RWC), and photosystem II quantum efficiencies of considered plants. Among the tested cultivars, SF0054 (sunflower) and AJMERI (soybean) have higher RWC, stomatal conductance, performance index (PI_{ABS}), the quantum yield of PSII (Fv-Fm ratio), and photochemical quenching (qP) comparing SF0024 and RAWAL under drought stress environment. Furthermore, SF0054 and AJMERI exhibited greater antioxidant enzyme activities and lesser H_2O_2 production under a drought stress environment. Therefore, these two cultivars could be used in crop improvement via plant breeding and genomics.

Key words: Crop breeding, Drought, Fluorescence imaging, Phenotyping, Phenomenological energy fluxes.

Introduction

Drought is a major threat and problem to agriculture and is considered the most disastrous environmental stress, that disturb not only crop growth but also cause a reduction in productivity (Lambers *et al*., 2008). Drought causes damages to plants' growth and productivity in various ways and to various extents. Therefore, in the current era, food production relies increasingly on the production of those cultivars which have more resistance to environmental stresses conditions and produce substantial yield (Luo, 2010). Thus, there is a crucial need to improve crops to drought stress so that more yield and good quality can be obtained. Improving crop species to environmental stress is the main goal for crop breeding. In this regard, the selection and identification of stress-tolerant physiotype need to supply candidate plants to crop breeders.

The Plant's phenotype is reflected by its genome, environmental conditions, and complex interactions among them (Chen *et al*., 2014). Phenomics is the characterization of phenotypes using extremely diverse phenotypic data (Houle *et al*., 2010). Discovery of non-destructive and non-invasive procedures for plant phenotyping and application automated the phenotypic studies (Finkel, 2009). Phenomics advancements are utilized as a vital plant research tool in plant selection and breeding procedures, expected to diminish the barrier in plant breeding (Furbank & Tester, 2011). Phenotyping via stress-induced physiological traits such as stomatal response, PS II efficiency, heat dissipation and energy loss, antioxidant efficiency and hydrogen peroxide production, and MDA content is subject of exploration in most of the stress-related studies (Siddiqui *et al*., 2014). Drought rigorously inhibits plant growth and development, are often associated with heat stress which suppresses the photochemical efficiency of PS II, inhibiting electron transport, releasing of membrane proteins, and discharging of bonded calcium and magnesium ions (Zlatev & Lidon, 2012). Similarly, drought stress causes a reduction in photosynthesis by limiting CO_2 availability through stomata (Flexas *et al.*, 2007) and photosynthetic efficiency via non-stomatal limitation (Lawlor & Cornic, 2002). Reduction in the absorption of photosynthetically active radiations, relegated light-harvesting index, and down-regulated radiation use efficiency are major factors responsible for the reduction in plant photosynthesis and productivity under drought stress (Earl & Davis., 2003).

Stress-induced fluorescence imaging is an effective and definitive tool to study plant stress. Chlorophyll fluorescence is related to photosynthetic apparatus; thus, such investigations could be beneficial to examine the plant's response against biotic and abiotic stress. Therefore, chlorophyll fluorescence can be put on to assess photosystem (PSII) activity (Gorbe & Calatayud, 2013) and be used to monitor plants' responses against drought stress. (Jansen *et al*., 2009).

Sunflower (*Helianthus annus* L.) and soybean (*Glycine max* L.) are major oil crops grown worldwide for edible oil. Sunflower is a low to medium drought drought-sensitive crop it has been observed that drought stress has a significant effect on its achene and yield (Iqbal *et al*., 2005). Similarly, drought affects the soybean protein and oil quantity and quality hence physiological traits along with breeding and molecular work can be done to improve drought tolerance in Soybean (Manavalan *et al*., 2009).

As drought is an emerging and increasing threat to crop growth and productivity, the new cultivars are coming to market to overcome the loss of drought stress. So, there is a need to evaluate those cultivars that have greater tolerance or resistance against drought stress environments. The comparative phenotyping of oil seed crops using physiological traits has been performed in the current research. This approach may be useful for selecting the best phenotype in drought drought-affected areas and future plant breeding/improvement programs.

Material and Methods

Seed sowing and plant establishment: The studies were performed at the Stress physiology Phenomic center, Department of Botany, University of Karachi. Two varieties of sunflower (SF0054 and SF0024) and soybean (RAWAL and AJMERI) were screened for their tolerance to drought stress in an experiment that provided identical environmental conditions and treatments. Seeds of sunflower and soybean were obtained from seed certification department Government of Pakistan. Surface sterilization of seeds were done using 1% sodium hypochlorite for five minutes and then rinsed with distilled water. Seeds were allowed for germination in a seedling tray. After one week of germination, the healthy and identical sized seedlings were transplanted into separate pots (45 × 15 cm) having sandy loam soil of about 4 Kg. Eight seedlings per pot were transplanted, and 14 days after germination, the seedlings were subjected to drought stress for subsequent 12-14 days, and control plants were watered regularly. The experiment was performed in three replicates for each treatment and control.

Relative water content: Youngest and fully-grown leaves were selected for relative water content (RWC) from each treatment (control and drought). Four discs from each treatment were cropped using cork-borer and immediately weighed to get fresh weight (FW), then the discs were placed in a 90 mm airtight plastic Petri-plate having distilled water for about 24 hours in the dark to get turgid weight (TW). Finally, the discs were oven-dried for 48 hours at 80°C and weighed to get dry weight (DW). RWC (%) was calculated using the formula by Barrs & Weatherley, 1962:

$$\text{RWC (\%)} = \{(\text{FW} - \text{DW}) \div (\text{TW} - \text{DW})\} \times 100$$

Stomatal conductance and chlorophyll fluorescence: Stomatal conductance (gs) was recorded using a Steady-state diffusion porometer, Model SC-1 (Decagon devices), while chlorophyll fluorescence was obtained using chlorophyll fluorescence meter (OS-30p+, Opti-science, USA). The tests were conducted on the youngest and fully expanded leaves between 9: 00 AM – 11: 00 AM. The JIP test protocol was conducted on dark-adapted leaves using leaf clips for 30 minutes and weak modulated red light (0.5 μ mol m^{-1} s^{-1}) with pulses of saturating light 1.6 s (6.8 μ mol m^{-1} s^{-1} PAR). Essential parameters of chlorophyll fluorescence were estimated, such as the ratio of variable to maximum fluorescence (Fv/Fm), performance index (PI), photochemical quenching (qP), non-photochemical quenching (qN). Specific energy fluxes of photosystem II including absorption per reaction center (ABS/RC), trapping per reaction center (TRo/RC), electron transport per reaction center (ETo/RC), dissipation per reaction center (DIo/RC) as well as per absorption basis (ABS/ABS, TRo/ABS, DIo/ABS, DIo/ABS) by the procedure of Maxwell & Johnson (2000).

Chlorophyll content: Chlorophyll content was estimated using chlorophyll content meter (Model CL-01, Hansatech, UK) on youngest fully expanded leaves of the plants.

Malondialdehyde content (MDA): MDA content was estimated by the by the scheme of Dhindsa *et al.*, (1981). 0.1 g of frozen plant material was homogenized with 0.5 % TCA [(0.5g TCA (trichloro-acetic acid), dissolved in 100ml distilled water]. This homogenate is then centrifuged at 5000rpm for 15 minutes. 1 ml supernatant was added to 3 ml of a mixture containing 20 g TCA and 0.5 g TBA 3 ml (thiobarbituric acid) in 100 ml distilled water. Contents were incubated for 30 minutes on a boiling water bath maintained at 100°C. After that cool, the contents at room temperature and record optical density (O.D) at 532nm and 600nm.

The extinction coefficient of this MDA -TBA is 155 mM^{-1} cm^{-1}.

$$\text{Concentration of MDA (μM)} = \frac{\text{Absorbance at 532 nm (A 532)} - \text{Absorbance at 600 nm (A 600)}}{155} \text{ x } 100$$

Hydrogen peroxide content (H_2O_2): Total H_2O_2 content was estimated by Velikova *et al*., (2000) scheme. In an ice bath, the fresh harvested 0.1g plant material was homogenized with 3 ml of 0.1 g % TCA (0.1 gm in TCA in 100 ml distilled water). This homogenate was then centrifuged at 12000rpm up to 15 minutes. 0.5 ml of supernatant was taken and added to 0.5 ml of 10 Mm Potassium phosphate buffer (pH 7.0), along with 1 ml of 1 molar Potassium iodide (KI). Absorbance was recorded at 390nm against TCA blank.

$$\text{Concentration of sample (μmol/ml)} = \frac{\text{(O.D from standard curve)}}{\text{(Conc. From standard curve)}} \times \text{O.D of sample}$$

$$\text{Amount of } H_2O_2 \text{ in μmol/gm} = \frac{\text{(Conc. of sample x Volume of extract x Dilution factor}}{\text{(Weight of sample) x 100}}$$

Antioxidant enzyme activity: 0.5 gm plant material was crushed in liquid nitrogen at 4°C along with 10 ml protein extraction buffer containing Tris-HCL (pH 6.8), 50 mg poly vinyl pyrrol iodine (PVP), 0.05 mM ethylene di-amine-tetra acetic-acid (EDTA) and the contents were centrifuged for 10 minutes at 10,000 rpm in a refrigerated micro centrifuge (Smart R-17, Hanil). The supernatant was collected as enzyme extract.

Catalase activity (enzyme number by NC IUBMB: EC 1.11.1.6) (enzyme number by NC IUBMB: EC 1.11.1.6) was estimated by the technique of Patterson *et al.*, (1984). 1 ml of enzyme extract and add 3 ml of reaction mixture containing H_2O_2 (10.5 mM) in 0.05 M Potassium phosphate buffer was taken at 25°C. Decrease in absorbance was recorded at 240 nm.

$$\text{Enzyme activity (unit ml}^{-1}) = \frac{\text{(O.D at 240 nm x Volume of reaction mixture x Dilution factor)}}{\text{(Extension coefficient x Volume of enzyme extract)}}$$

Superoxide dismutase (SOD) activity (enzyme number by NC IUBMB: EC 1.15.1.1) was estimated by the technique of Beyer & Fridovich (1987). 20 µl of enzyme extract, 10 µl riboflavin (4.4 mg per 100 ml distilled water) and 1.0 ml of reaction mixture [consisting of 27.0 ml of 0.05 M potassium phosphate buffer (Ph 7.8), 1.5 ml of L-methionine (300mg/2.7 ml), 1.0 ml of nitro-blue-tetrazolium (14.4 mg /10 ml distilled water) and 0.75 of triton X-100] were taken and then the mixture was illuminated up to 15 minutes. Blank was prepared by replacing enzyme extract with 20 µl buffer. A decrease in absorbance was recorded at 560 nm.

$$\text{Enzyme activity} = \frac{\text{(A 560 blank – A 560 sample)}}{\text{A 560 blank}} \text{ x 100 x 3.75}$$

Statistical analysis

The data were statistically analyzed using personal computer software package SPSS version 20.0. The values are denoted with ± standard errors. All the graphs were made using Microsoft Office Excel version 2016, with similar letters on bars indicating non-significance at $p \leq 0.05$.

Results

Relative water content (RWC) describes the sensitivity of plants against drought stress. RWC was found to be decreased in drought stress. However, sunflower SF0054 maintained a higher RWC than SF0024 and maintained up to 75.15%, while SF0024 maintained up to 68.95% compared to the respective control (Fig. 1). In drought stress soybean cultivar AJMERI maintained higher RWC than RAWAL showed 64.81%, while RAWAL showed lesser RWC and displayed 59.00% compared to control.

Stomatal conductance was decreased under drought stress showing variable patterns among the cultivars. Stomatal conductance in sunflower variety SF0054 was maintained at lesser decline than SF0024 against drought stress related to control (Fig. 1). Under drought stress, soybean varieties AJMERI showed lesser decline than RAWAL compared to control.

In our findings, chlorophyll content was shown to be increased under drought stress. In sunflower, SF0024 showed a much higher chlorophyll content than SF0054, while in soybean, RAWAL showed a much increase in chlorophyll content than AJMERI (Fig. 1).

Drought stress causes a decline in chlorophyll fluorescence. The maximum quantum yield of PS-II (Fv/Fm ratio) was observed to be declined under drought stress, in sunflower cultivars SF0054 Fv/Fm ratio was slightly higher than SF0024 and maintained up to 98.63% but in SF0024 was up to 96.97% compared to control, while in soybean cultivars AJMERI had slightly higher Fv/Fm ratio and was maintained up to 95.61% but RAWAL had 90.56% compare to control (Fig. 2).

Performance index indicated by PI_{ABS} was found to be declined under drought stress, in sunflower cultivar SF0054 it was higher than SF0024 and was maintained up to 81.87% compared to control, but in SF0024 it was much declined and reached up to 50.47%, while in soybean AJMERI had much higher PI_{ABS} than RAWAL and was maintained up to 27.36% but in RAWAL it was much declined and reached up to 12.36% compared to control (Fig. 2).

Photochemical quenching (qP) was shown to be declined under drought stress, in sunflower cultivar SF0054 qP was maintained up to 94.30% and in SF0024 decline was slightly higher and maintained up to 93.30% compared to control. In comparison, in soybean AJMERI had 93.37% and RAWAL had 87.62% maintenance of qP compared to control (Fig. 2).

The OJIP chlorophyll fluorescence yield curves obtained under drought stress shows that in sunflower cultivar SF0054, there is no change from O to I step of chlorophyll fluorescence yield, but the change was observed at P step where it was declined compared to control while in SF0024 there is an increment from O to I step but suddenly declined at P step of chlorophyll fluorescence yield compare to control, also SF0054 had higher chlorophyll fluorescence yield under drought stress compared to SF0024. In soybean OJIP chlorophyll fluorescence yield curves showed that in both RAWAL and AJMERI there is rise from O to J step but sudden declined was observed at I and P step of chlorophyll fluorescence yield compared to control, also AJMERI had higher chlorophyll fluorescence yield at I and P step than RAWAL under drought stress (Fig. 3).

The phenomenological energy fluxes such as light absorption (ABS), trapping (TR) of excitation energy, dissipation (DI) and electron transport (ET) per reaction center (RC) and per absorption (ABS) are presented in the form of spider plot (Fig. 4). In sunflower, SF0054 is much closer to control than SF0024. Still, dissipation increases and electron transport decreases compared to control, while electron transport was more in SF0054 than SF0024 and dissipation was more in SF0024 than SF0054 under drought stress. In soybean, both AJMERI and RAWAL had higher absorption, trapping, and dissipation than control and much higher in RAWAL than AJMERI while lower electron transport in both varieties compared to control and much lower in RAWAL than AJMERI.

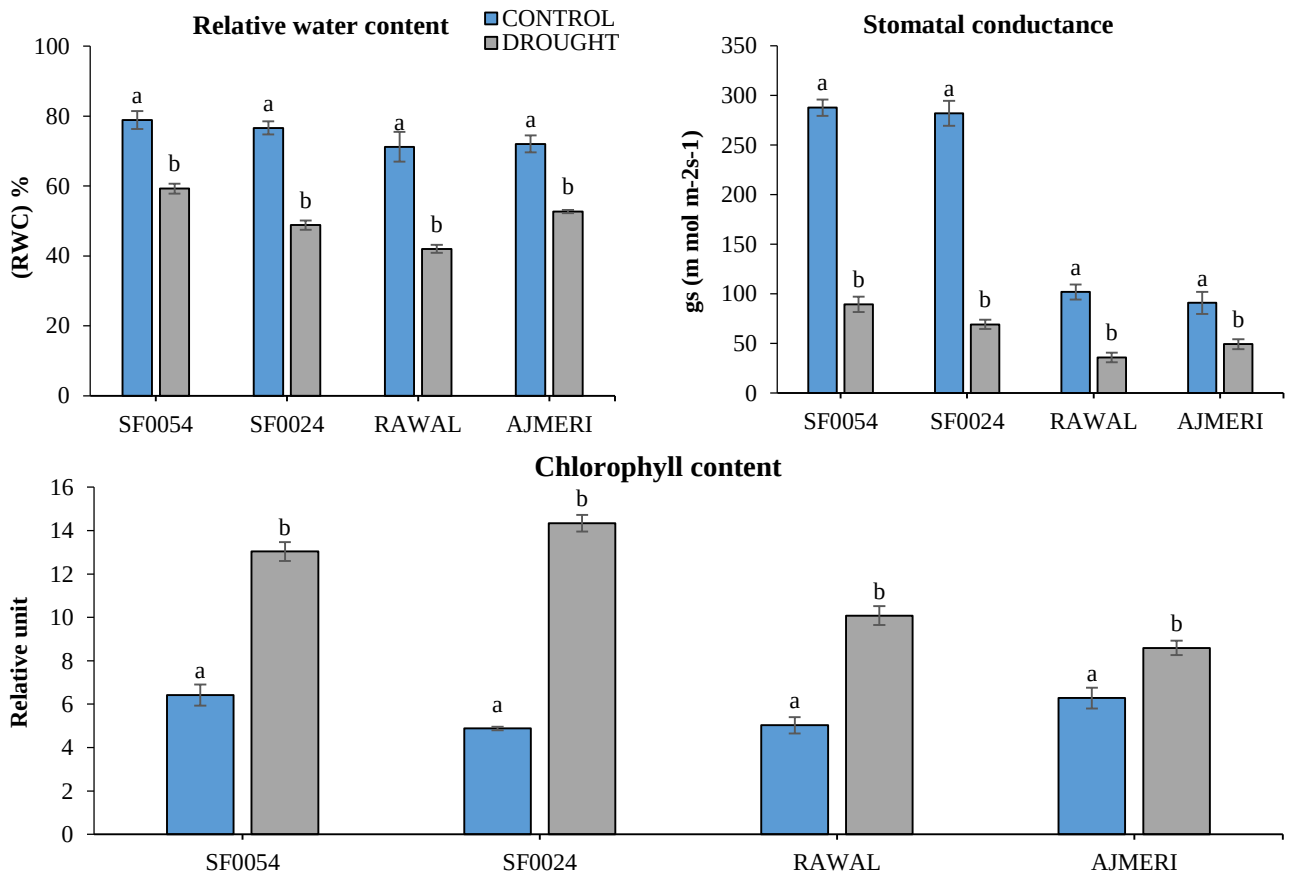

Fig. 1. Effect of drought stress on the RWC %, Stomatal conductance and chlorophyll content of various cultivars of oil seed crops; sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI). Upright lines on bars denote standard errors (±) while similar letters indicate non-significance at $p \leq 0.05$.

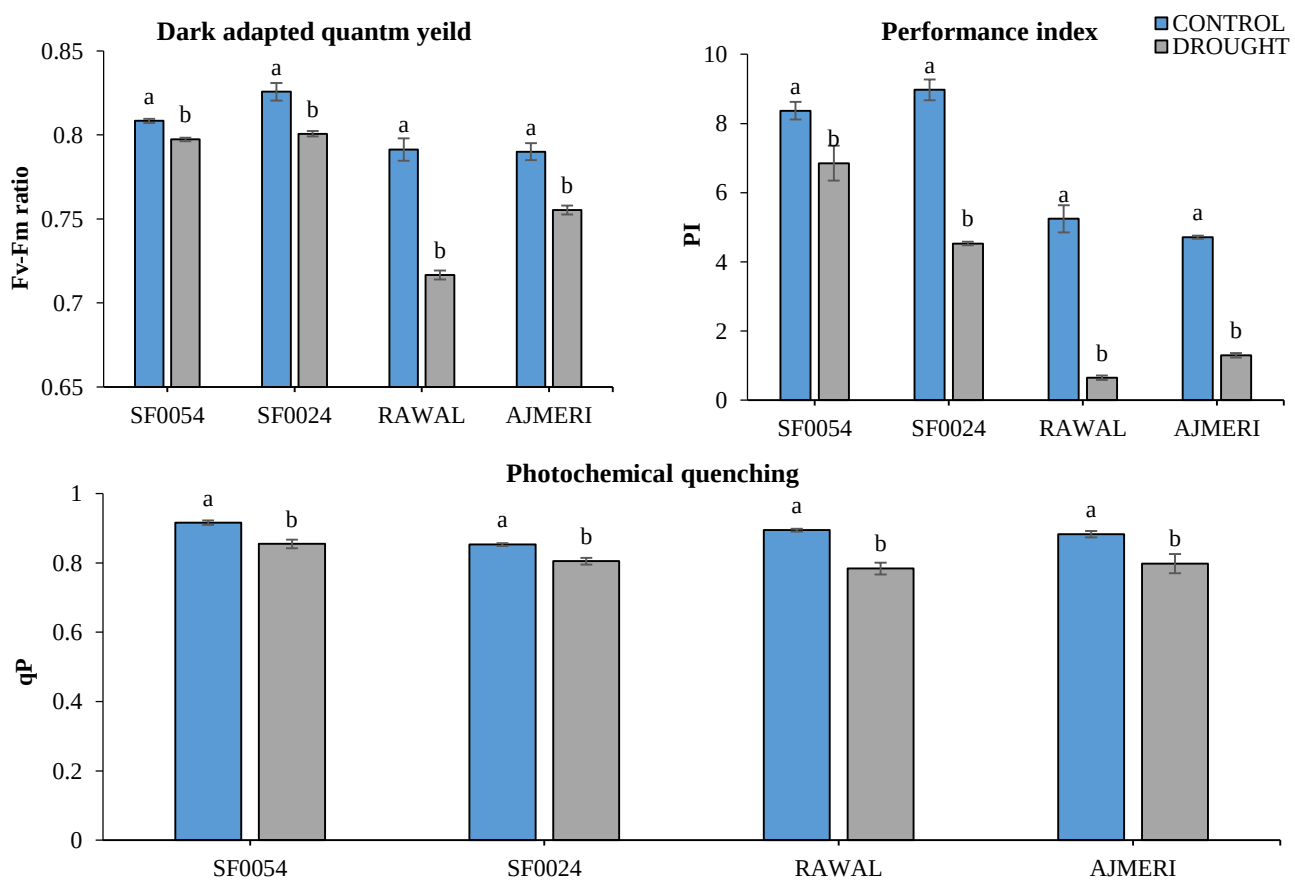

Fig. 2. Effect of drought stress on the PI_{ABS}, Fv-Fm ratio and qP of various cultivars of oil seed crops; sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI). Upright lines on bars denote standard errors (±) while similar letters indicate non-significance at $p \leq 0.05$.

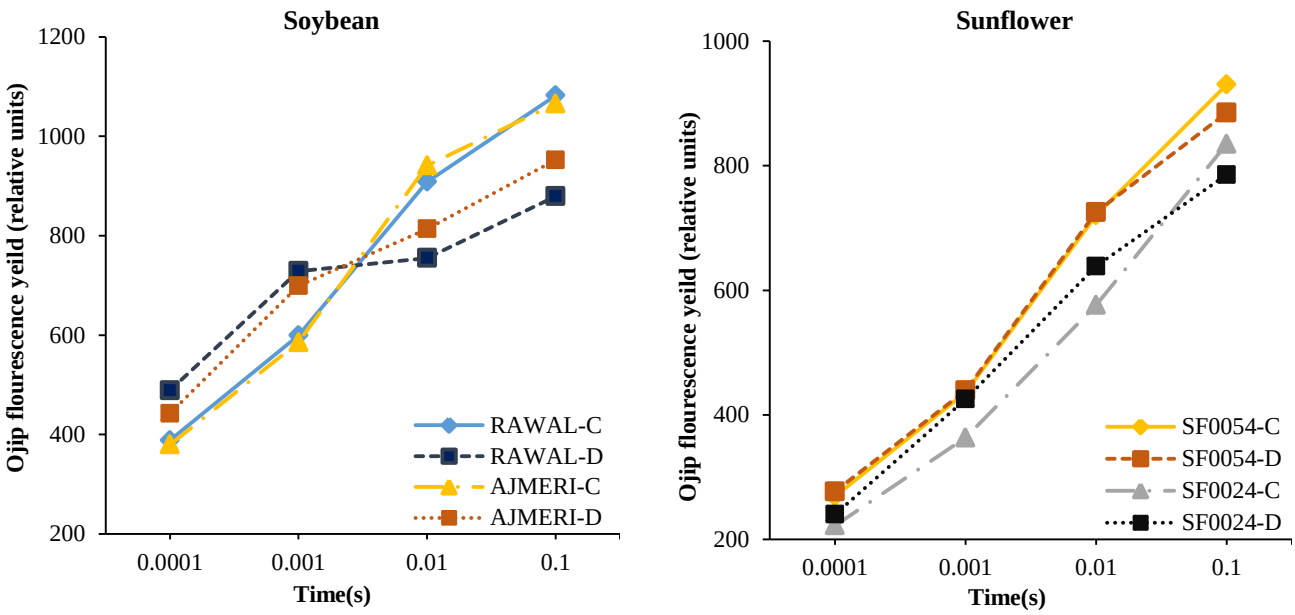

Fig. 3. Showing OJIP Chlorophyll fluorescence yield curves of studied cultivars of sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI) under drought stress. "C" stands for control and "D" for drought.

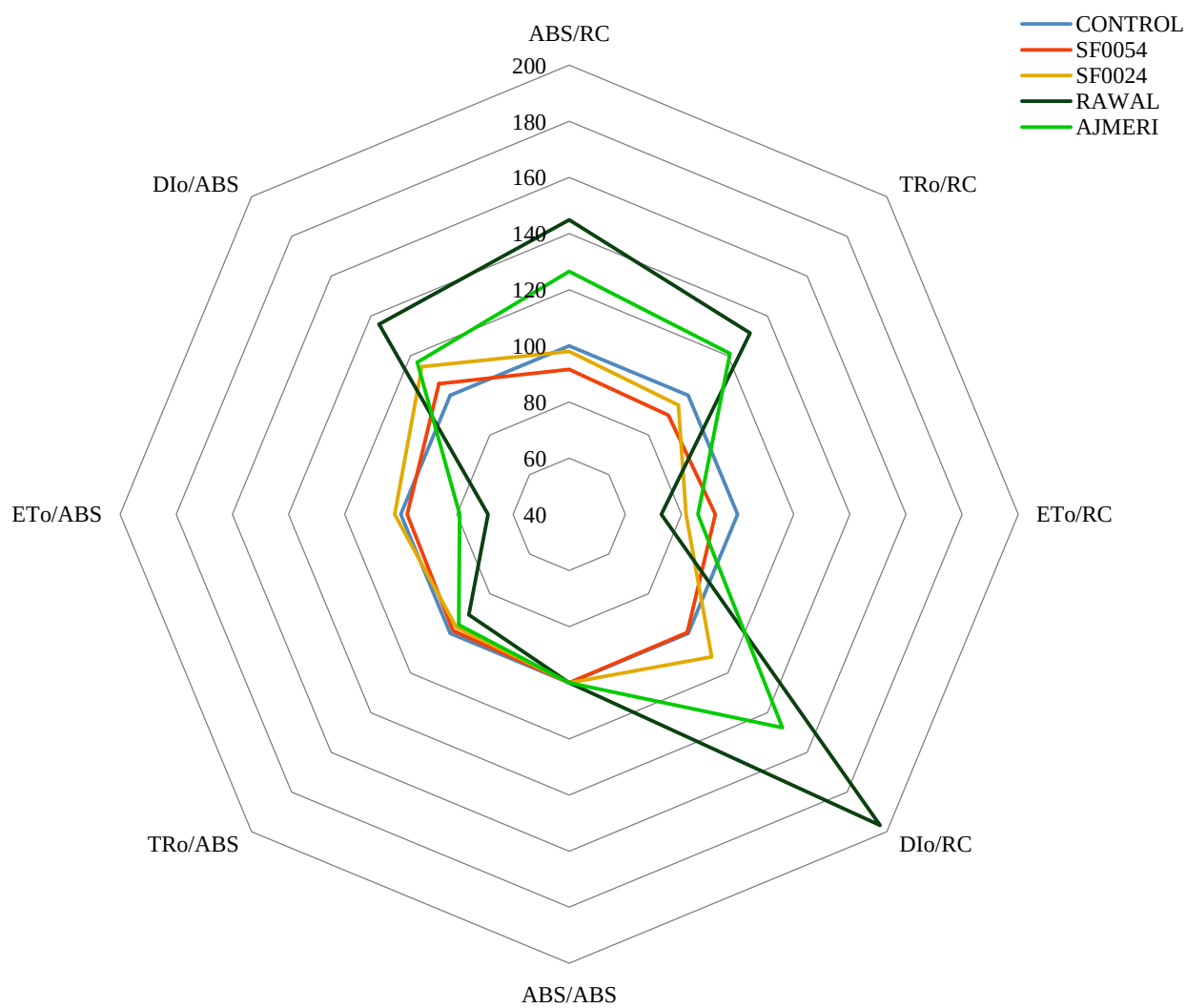

Fig. 4. A Spider plot showing phenomenological energy fluxes of Photosystem II for sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI) cultivars under drought stress. ABS = absorption, TR_o = trapping, ET_o= electron transport and DI_o = dissipation on reaction center (RC) and absorption (ABS) basis.

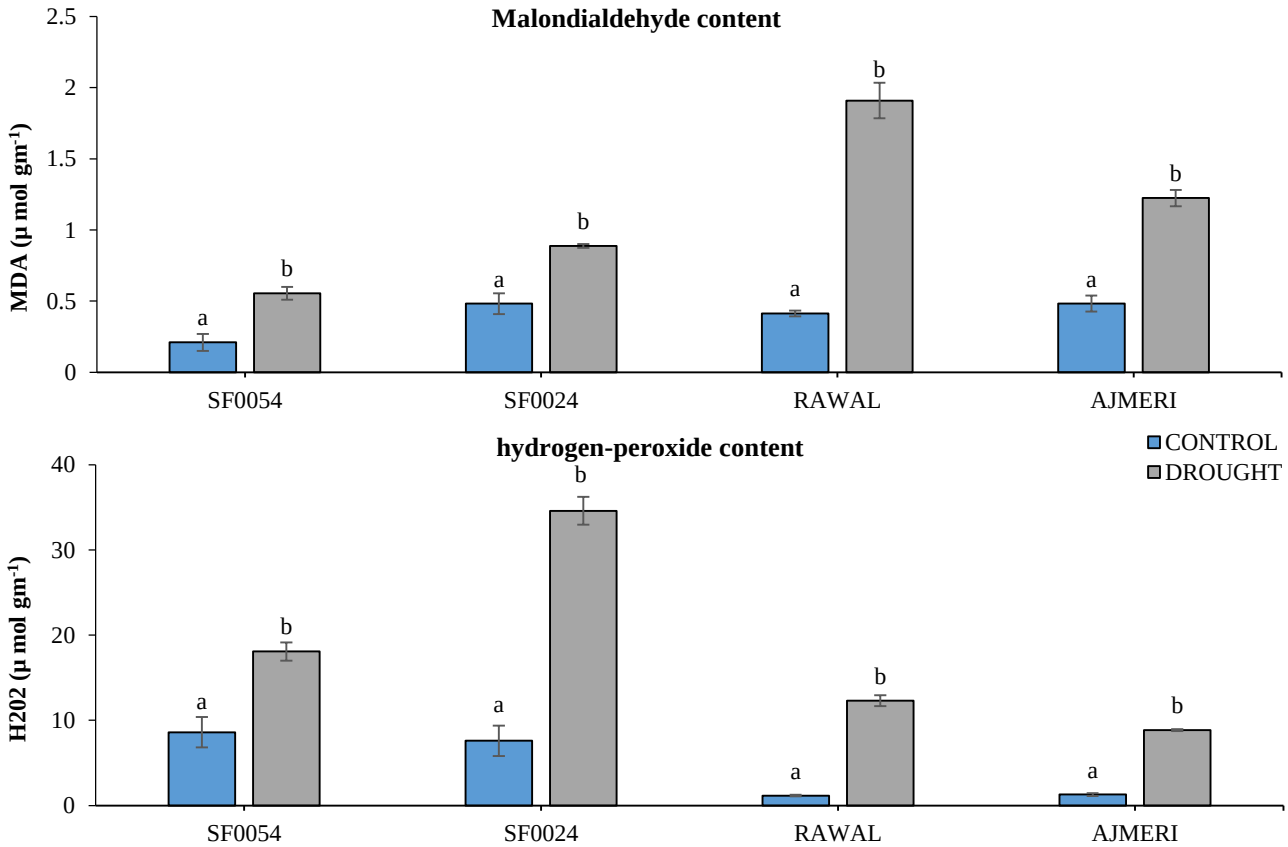

Fig. 5. Effect of drought stress on the H_2O_2 and MDA content of various cultivars of oil seed crops; sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI). Upright lines on bars denote standard errors (±) while similar letters indicate non-significance at $p \leq 0.05$.

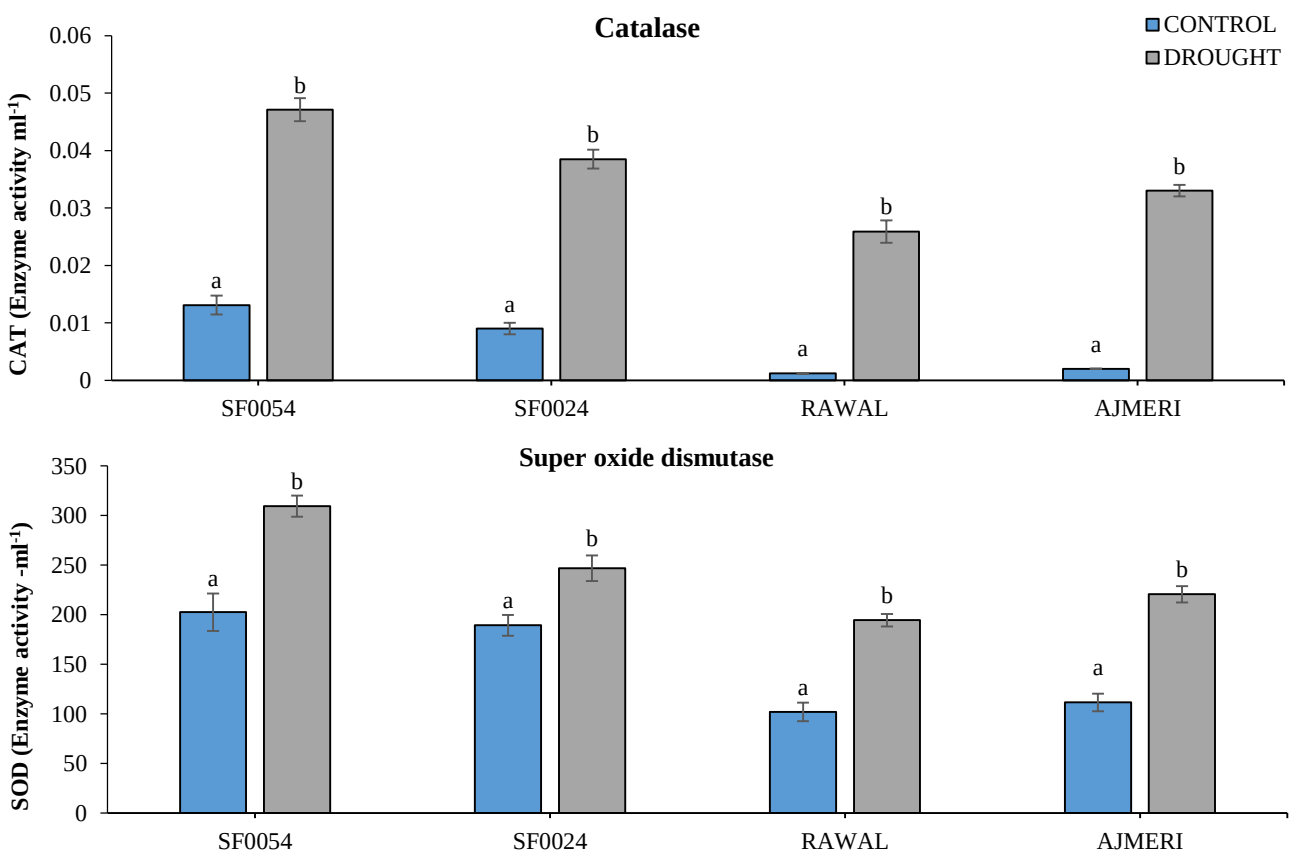

Fig. 6. Effect of drought stress on the antioxidant enzyme activity catalase and SOD (Super oxide dismutase) of various cultivars of oil seed crops; sunflower (SF0054, SF0024) and soybean (RAWAL, AJMERI). Upright lines on bars denote standard errors (±) while similar letters indicate non-significance at $p \leq 0.05$.

Hydrogen-per-oxide (H_2O_2) and malondialdehyde (MDA) are both important stress markers that are shown to be elevated under drought stress. In sunflower, SF0024 showed more enhanced H2O2, and MDA content than SF0054 compared to control, while in soybean RAWAL showed more H_2O_2 and MDA content than AJMERI (Fig. 5).

The antioxidant enzyme activity of catalase was enhanced under drought stress. In sunflower, SF0054 showed much more enhanced catalase activity than SF0024 under drought stress compared to control. In soybean, AJMERI showed much more catalase activity than RAWAL under drought stress compared to control (Fig. 6).

Superoxide dismutase activity under drought stress was found more in sunflower SF0054 showed much more enhanced activity than SF0024 under drought stress, while in soybean AJMERI showed much-enhanced activity under drought stress than RAWAL (Fig. 6).

Discussion

Drought is a major risk for the growth and productivity of crop plants. Under drought stress stomatal closure is one of the first responses that result in the decline of the rate of photosynthesis primarily (Mahajan & Tuteja, 2005). Under abiotic and abiotic stresses, the measurement of chlorophyll fluorescence was found to be a reliable intact, non-invasive technique to assess the physiological status. Phenotyping approaches such as fluorescence imaging and physiological stress markers in combination can be utilized as a strong and impactful tool for screening plants under drought stress tolerance for crop improvement and plant breeding purposes.

In our finding drought adversely affected the physiological and chlorophyll fluorescence traits of plants; furthermore, the effect was more pronounced in sensitive than tolerant plants. During comparative phenotyping assessment, SF0054 and AJMERI are found to be more tolerant than others. The tolerance was attributed to the maintenance of RWC, Stomatal conductance, and photosynthetic efficiency and chlorophyll fluorescence near to control plants as well as high antioxidant enzymes activity under stress. In contrast, -sensitive plants showed reductions in all above parameters and elevation of oxidants such as H2O2 and, ultimately, lipid peroxidation (MDA) under drought stress.

RWC is an important physiological strategy to retain more water in leaves under stress conditions. SF0054 and AJMERI have a higher ability to hold water under stress than others thus supposed to be more tolerant to drought stress than other varieties. Under drought stress, the accumulation of water-soluble compounds was recorded more in resistant than sensitive genotypes similar are the findings by Martin *et al*., 2002; Siddiqui & Khan, 2011; therefore, water holding capacity of plants can play a vital role in stress tolerance.

Stomatal conductance was found to decrease under stress, SF0054 and AJMERI had maintained high stomatal conductance than other cultivars. Drought stress elevates bio synthesis of abscisic acid (ABA) that results in reduction of stomatal conductance to overcome transpiration losses (Yamaguchi-Shinozaki & Shinozaki, 2006). Two different strategies have been observed to be adapted by drought tolerant plants under stress. Annuals and perennials reduce their leaf size and stomatal conductance (Querejeta *et al.*, 2003), while short lived seasonal avoid drought stress by elevating stomatal conductance to rise carbon gain. The later technique physiological approach helps the plants to grow rapidly, flower early, and gain more yield before the onset of drought (McKay *et al*., 2003). Hence, this strategy also seems to be adapted by the tolerant SF0054 and AJMERI.

The chlorophyll content was increased in all the assessed cultivars, but SF0024 and RAWAL showed much higher increment than others. The chlorophyll content was found to be decreased and this decline is associated with the less water content of the leaves (Munne-Bosch & Alegre, 2000). A contrary trend has been observed by some researchers with increased chlorophyll content under drought (Alaei, 2011; Khayatnezhad, 2011). This is maybe due to the reason that the exact influence of water deficit vary accordingly to the magnitude of the drought stress applied (Cameron, 1999) or maybe due to the reason that increased water deficit induces transpiration rate, so remove water from chloroplast of the leaves as a result chlorophyll molecule concentrated in the chloroplast hence chlorophyll content increases as found in our results but this effect should not be implicated at high and prolonged water stress where chlorophyll degradation reduces chlorophyll content, but this effect should be more studied, and further research should be carried out in this aspect.

Under drought Fv-Fm ratio, PI_{ABS} and qP were found more in SF0054 and AJMERI than other cultivars. Down-regulation of photochemical efficiency of PSII results under drought stress that has been reported by many scientists (Marija, 2013; Bertamini *et al*., 2007). PI can sense stress even before the visual symptoms on leaves and is a more sensitive photosynthetic parameter (Christen *et al*., 2007). Under drought stress, the Performance index was decreased of investigated genotypes (Marija, 2013). Photochemical quenching decreased under drought stress, indicating down regulation of PS II reactioncenters (Guo *et al.*, 2016). So Fv/Fm ratio, PIABS, and Photochemical quenching (qP) decreased in all cultivars but was maintained at higher levels in some such as SF0054 and AJMERI than others found to be tolerant.

In terms of phenomenological energy fluxes decrease in electron transport (ET_o/ABS) was observed in all cultivars under drought stress and maintained at a higher level in some, such as SF0054 and AJMERI, than others found to be tolerant. Dissipation was ecreased under drought stress and more in RAWAL and SF0024 than others and hence found to be sensitive. An increase in dissipation is mainly due to enhanced absorption (ABS) (Marija, 2013). In the studied cultivars the phenomenological fluxes such under drought stress. As absorption (ABS), trapping (TR_o), and dissipation (DI_o) per reaction center (RC) were found relatively high. ABS/RC and TRo/RC represent inactivation of some RCs, in addition the ratio of DI_o/RC was due to high dissipation of inactive reaction centers. Similar results have been reported by Faghire *et al*., (2015). Under drought stress ET_o/RC which represents the energy flow through PS II was inhibited that ultimately caused a

reduction in CO_2 fixation and photosynthesis (Krall & Edwards, 1992). Dissipation (DI_o/RC) and trapping (TR_o/RC) per reaction centre was less in SF0054 and AJMERI while ET_o/RC, was found to be more compared to others and hence found to be tolerant than others.

H_2O_2 and MDA content was found to be more in SF0024 and RAWAL comparing other varieties, while the antioxidant enzymes activity such as catalase and SOD was found to be more in SF0054and AJMERI than other cultivars under drought stress. In plants ROS (reactive oxygen species) accumulate within cells during abiotic stress as toxic products of aerobic metabolism (Huang *et al*., 2012). In biological membranes, lipid peroxidation increases once the level of ROS exceeds the capacity of plant to scavenge, affecting the cell's physiological processes. Malondialdehyde (MDA) evidences the oxidative damage done to the lipids of the cell as it is the one of the final products of lipid peroxidation therefore, antioxidant enzymes including superoxide dismutase and catalase etc. have been evolved in many higher plants to cope with ROS (Gill & Tuteja, 2010). Hence same interpretations are found in our results showing elevated antioxidant enzyme activity with less amount of H_2O_2 and MDA content in resistant than sensitive cultivars hence were found more tolerant to drought stress than others.

Conclusion

Comparative phenotyping of oilseed crops non-invasive phenotyping approaches such as chlorophyll fluorescence and physiological traits is a powerful tool for crop improvement and plant breeding. Drought stress negatively affects the physiology and photosystem II efficiency in crop plants. The cultivars SF0054 and AJMERI performed better under drought stress in terms of better RWC, Stomatal conductance, PI_{ABS}, qP, Fv/Fm ratio, and phenomenological energy fluxes such as increased ET_o/RC, decreased TR_o/RC and DI_o/RC hence found to be drought tolerant than other cultivars, therefore, are recommended for the drought affected areas to improve the productivity and for plant breeding purposes.

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