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Drought tolerance studies in *in vitro* grown *Camelina sativa* (L.) Crantz by exogenous application of polyethylene glycol.

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Abstract

Drought is one of the most common abiotic stress affecting plant growth and productivity. Plant cell and tissue culture has been a useful tool to study stress tolerance mechanisms under *in vitro* conditions largely because more number of plants can be screened in small space and in less time. The present study was aimed to investigate the drought-induced growth, physiological and biochemical changes in *Camelina sativa* by adding different concentrations (1%, 2%, 3%, 4%, 5%, 6%, and 7%) of polyethylene glycol (PEG 6000 MW) in Murashige and Skoog (MS) basal medium. Germination capacity, cotyledon unfolding and true leaf emergence increased significantly with increase in PEG concentration up to 2%. Thereafter increase in PEG concentration resulted in the decline in these parameters as compared to the control. Electrical Conductivity (EC) increased by 0.91 fold while Relative Water Content (RWC) and protein content decreased by 0.16 fold and 0.57 fold as compared to the control. Guiacol peroxidase activity (GPX) increased significantly by 1.97 folds. However, the change in malondialdehyde (MDA) content was not significant in *in vitro* grown plants. Pigments contents viz. chlorophyll (a, b) and carotenoid content increased up to 2% PEG treatment, after which further it declined as compared to the control. This study shows that *Camelina sativa* can tolerate PEG (2%) induced drought stress without any negative effect on growth and physiological parameters.

Keywords: Camelina. Drought. Osmotic adjustment. Oxidative damage. Antioxidant enzymes

Introduction

Camelina (*Camelina sativa*), belongs to the family Brassicaceae popularly known as Siberian mustard, False Flax and Gold of Pleasure, [36] native to Europe and Central Asia, has been traditionally cultivated as an oilseed crop and animal feed. With the rise in global fuel demand, depleting oil resources and rising oil prices, it has emerged out to be one of the potential sources for biofuel production. Like any other plants, growth and yield productivity of Camelina is also affected due to various environmental stress factors like heat, salinity, cold and drought. Of the different abiotic stresses, drought is one of the most important environmental factors affecting growth and yield of crops [18]. According to the World Water Council approximately 66% of consumed water is used for irrigation (in arid regions up to 90%) [28]. In future such a large consumption of water will create a serious problem for crop cultivation. This problem, to some

extent, could be resolved by cultivating such plant species which are able to withstand abiotic stresses without any significant yield decline.

Drought is one of the most important factors limiting plant growth, and is claimed to reduce production on about 25% of arable land throughout the World [13]. It causes morphological, physiological and biochemical changes in plants inhibiting plant growth and development. Seed germination, usually the most critical stage in seedling establishment, determines the success of crop yield and productivity [3]. Sensitivity to drought stress adversely affects seed germination in terms of root growth and shoot elongation [31], [8]. When plants are subjected to stress condition there is a change in biochemical processes of plants leading to the accumulation of reactive oxygen species (ROS) *viz*, hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and superoxide ions which are the inevitable by-products of normal cells [26]. A large number of reports deal with the deleterious effects of ROS, the production of which is stimulated under water stress conditions [9], [14], [23].

Scavenging of ROS in plant cells occurs by endogenous protective mechanism involving antioxidant molecules and enzymes. These enzymes include superoxide dismutase, ascorbate peroxidase, guaicol peroxidase, glutathione reductase and catalase. Enzymes either quench toxic compounds or regenerate antioxidants with the help of reducing power provided by the process of photosynthesis [32]. Plant also accumulates osmolytes such as proline under stressed condition which act as osmoprotectant. Hence, dehydration process during drought is characterized by changes in morphological and growth parameters (seed germination, emergence of cotyledon, shoot length and plant biomass), physiological processes (Electrical conductivity and relative water content) and biochemical processes (photosynthetic pigments, lipid peroxidation, guiacol peroxidase activity and protein content). Drought affects stomatal closure leading to a progressive suppression of photosynthesis. The decline in photosynthetic rate is not only due to increased stomatal function but also can be due to non-stomatal parameters (nutritional, biochemical, metabolic, and/or diffusional) [34].

Camelina grows mainly in winter as short duration annual crop (80-100 days). Since this crop was introduced in India in 2010 [1] there is very little information available on its ability to withstand different biotic and abiotic stress conditions prevailing in India. Years of research has proved the usefulness of *in vitro* culture system to study the response of plants to abiotic stress [27]. *In vitro* culture technique minimizes environmental variations as plants are cultured in defined nutrient media, homogeneity of stress application and controlled growth conditions viz. temperature, light and humidity. In addition, large plant population could be studied in limited space and in short time period. Polyethylene glycols (PEG) of high molecular weights have been in use since long to simulate drought stress in plants. PEG being a non-penetrating osmotic agent lowers the water potential in a way similar to soil drying. This neutral polymer is used to modify the osmotic potential of *in vitro* culture conditions. Though Camelina is known to exhibit drought tolerance but insufficient studies have been done to understand the mechanism of plant tolerance and oxidative stress in response to water deficit. Hence, the present study was conducted with the objective to find the tolerance of *Camelina sativa* (cv Calena) to different concentrations of poly ethylene glycol (PEG –6000), by studying its effect on growth, physiological and biochemical parameters under *in vitro* growth conditions.

Materials and Methods

Plant material, its treatment and germination

Camelina sativa cv. Calena (EC-643910) introduced in India for the first time in 2010 was used in the present study. The chemicals used were of analytical grade procured from Sigma Aldrich, USA. Camelina seeds were washed with distilled water under aseptic conditions under the laminar air flow hood. To sterilize, seeds were first treated for 5 min with 0.1% $HgCl_2$ (mercuric chloride) followed by 30 sec treatment with 70% ethanol and 3-4 washes of sterile distilled water. Seeds were blot dried and germinated in MS media [25] containing different concentrations (1%, 2%, 3%, 4%, 5%, 6%, and 7%) of Poly ethylene glycol. The cultures were maintained at 24 ± 2°C in 16 h light and 8 h dark cycle (cool white florescent light, 30 µmol $m^{-2}s^{-1}$) and 50- 60 % relative humidity (RH) in culture room.

Data on *in vitro* seed germination was recorded every day in terms of radicle emergence, cotyledon unfolding and true leaf emergence. Each treatment had 10 replicates of 20 seeds each. The germination capacity was estimated in terms of Timson's index of germination velocity using the formula:

Timson's index = Σ (G/t);

where G is germination percentage; t = total period of germination (days).

Germination percentage and vigour was also recorded for each treatment. The plants growing in different treatments were uprooted carefully and washed for recording fresh and dry weight of the whole plant. The samples were oven dried at 94°C for 24 h and dry weight was recorded. Moisture content was calculated using the formula:

Moisture content = (Fresh weight-dry weight /fresh weight) *100

Estimation of EC and RWC

Leaf disc of 1 cm^2 were cut and washed with distilled water, placed in test tube containing 10 ml distilled water and incubated at 25 °C on shaker (100 rpm) for 24 h. At the end of incubation the EC (EC I) of bathing solution was recorded by Electrical Conductivity Meter (WTW 315I/SET Germany). The samples were autoclaved at 121°C for 20 min to completely kill the tissues and release electrolytes. The second EC (EC II) was recorded after cooling the solution to room temperature (RT). The ionic concentration in the sap was determined by measuring the electrolytic conductivity by using the formula:

EC %= ECI/ ECII * 100

For RWC the fresh weight (FW) of leaf was recorded and then turgid weight (TW) was taken by sinking the leaf in water for 24 h. Dry weight (DW) was determined after drying the leaves for 24 h in the hot air oven at 90°C [6]. The RWC was calculated as:

RWC %= [(FW-DW) / (TW-DW)] *100

Estimation of the malondialdhyde (MDA) level

The level of lipid peroxidation in plant tissues was measured by determination of MDA. MDA content was determined with thiobarbituric acid (TBA) reaction. Tissue sample was homogenized in 2 ml of 10 % trichloroacetic acid (TCA) and 0.25 % TBA solution and centrifuged at 15,000 rpm for 15 min. The supernatant was heated at 95°C for 30 min and cooled immediately on an ice bath. The non-specific absorbance of the supernatant at 600 nm was subtracted from the absorbance at 532 nm for MDA measurement. The level of lipid peroxidation was expressed as mM/gm fresh weight of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1} [15].

Total MDA (mM/ml) = (Absorbance at 532- Absorbance at 600/ extinction coefficient * path length) * dilution factor

Estimation of chlorophyll pigments and chlorophyll stability index

Chlorophyll (*a*, *b*, and total) concentrations were determined from leaf sample ground in a pre-chilled mortar in 2 ml acetone (80% v/v). The homogenate was centrifuged at 3000 g for 10 min. After complete extraction, the mixture was filtered and supernatant was collected. The absorbance of the extract was measured at 663 and 645 nm using the spectrophotometer [5]. For pigment content measurement, the equations used are as follows:

Chlorophyll *a* = 12.25 A_{663}- 2.79A_{645}

Chlorophyll *b* = 21.50 A_{645}- 5.10 A_{663}

Total chlorophyll = 7.15 A_{663}+ 18.71 A_{645}

The chlorophyll stability indices (CSI) were measured using the formula [21]:

CSI = (Total chlorophyll content in stressed leaves/Total chlorophyll content in control leaves) * 100

Estimation of guaiacol peroxidase (GPX) activity

Guaiacol peroxidase activity was determined by monitoring the increase in absorbance at 470 nm as guaiacol was oxidized. The assay mixture contained 50 mM phosphate buffer (pH7.0), 0.1mM EDTA, 10 mM guiacol, 10 mM H_2O_2 and 50 µL of enzyme extract. Absorbance of reaction mixture at 470 nm was determined after every 15 s interval with the spectrophotometer [20]. Guiacol peroxidise activity was measured using the formula:

Guiacol peroxidise activity = final absorbance * extinction coefficient * 20 / leaf weight

Where, final absorbance = (first absorbance recorded– last absorbance recorded) at 470 nm

Protein content

Protein content was determined by Bradford method using bovine serum albumin (BSA) as a standard [10].

Statistical analysis

The program Crop Stat for Windows (7.2.2007.2 module), developed by the Biometrics unit, IRRI, Philippines was used for analysis of variance (ANOVA). The treatment means were compared by using least significant difference test (LSD) at a significance level of $P \leq 0.05$.

Results and Discussion

Effect of PEG stress on morphological parameters

Plant growth and vigour increased significantly with the increasing concentration of PEG up to 2% (Fig. 1) but further increase in PEG concentration lead to significant decline in growth and vigour of the plant. Though, the germination was 100% under all the conditions, but the rate of germination differed, as measured by Timson's index. Increase in the concentration of PEG upto 2% in the media significantly enhanced the rate of germination, cotyledon opening and true leaf emergence. Maximum germination percentage, cotyledon unfolding and true leaf emergence was recorded in 2% (94.01 %, 91.23 %, and 85.99 %, respectively) whereas minimum was recorded in 7% PEG concentration (55.34%, 55.06 %, and 55.13%, respectively). ANOVA showed significant difference in different treatments at $P<0.05$ significance level (Fig.2). Plant height also increased (8.02 cm) significantly ($P<0.05$) up to 2% PEG treatment compared to the control (3.05 cm). Thereafter, there was significant decrease in plant height with increased (>2 %) PEG concentration (Table 1). There was also a significant ($P<0.05$) increase in fresh and dry weights of the plants upto 2% PEG concentration, which further declined at increased concentration of PEG. The maximum fresh (0.384 mg) and dry weight (0.0333 mg) was recorded for 2% PEG treatment whereas minimum fresh (0.034 mg) and dry weight (0.0073 mg) was observed in 7% PEG treated plants (Fig. 3). Water stress causes changes in morphological and growth parameters amongst which germination is the most affected stage. Under water stress low water potential inhibits seed germination or delayed seed germination and causes poor seedling growth and establishment. Though 100% germination was observed in all the concentrations of PEG but growth vigour was significantly affected. Not all seedlings which germinated grew further. In some seedlings only radical emergence was observed but it did not grow further. Some seedlings had both radical and shoot emergence but there was no further growth and development. Such seedlings died eventually. The finding are in agreement with that previously found in *Brassica napus* and *Brassica juncea* [30][29]. Seedlings could withstand the adverse effect till 2% PEG treatment after which it started showing signs of reduced growth and vigour.

Fig. 1 Effect of different concentration (0% (control), 1%, 2%, 3%, 4%, 5%, 6%, 7%) of PEG treatment on growth and vigour of *Camelina sativa* (1 month old).

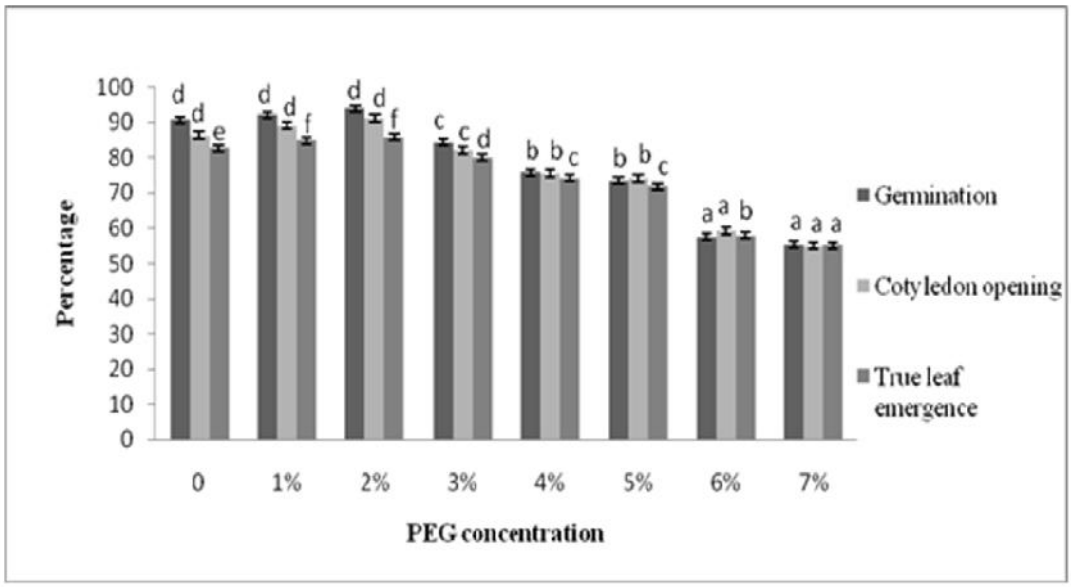

Fig. 2 Effect of PEG stress in *Camelina sativa* on rate of germination, cotyledon opening and true leaf emergence (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Table1 Effect of PEG stress on Moisture content, Plant height and Chlorophyll stability index in *Camelina sativa.*

Treatment	Moisture content (%)	Height (cm)	Chlorophyll stability index
Control	87.1	3.05	100
1% (PEG)	90	6.075	110.18
2%	91.2	8.025	171.2
3%	88	5.075	156.75
4%	84.8	4.2	103.811
5%	80.4	1.7	91.47
6%	79.5	1.3	80.66
7%	78.7	1.075	78.58
SE	1.61	0.176	4.199
LSD ***(P≤0.05)***	4.90	0.5191	12.73

Values indicated by the same letter are not significantly different at $P \leq 0.05$.

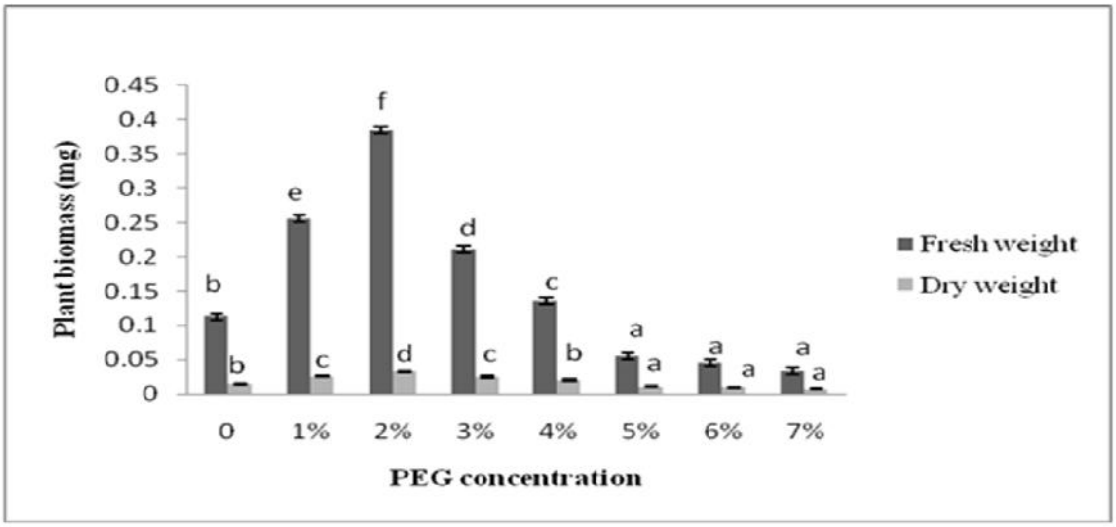

Fig. 3 Effect of PEG stress in *Camelina sativa* on fresh weight and dry weight biomass (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Effect of PEG stress on physiological parameters

Osmotic adjustment

Osmotic adjustment measured as RWC in leaves was significantly (P<0.05) low in 7% PEG treatment (81.092 %) as compared to the control (97.164%). This relate with higher EC of the leaf sap found in 7% PEG treatment (93.23%) as compared to the control (48.8%) (Fig. 4). The first physiological disorder, which takes place during germination, is reduction in the imbibition of water by seeds which leads to a series of metabolic changes, including change in enzyme activities and general reduction in hydrolysis and utilization of the seed reserve [2]. RWC is one of the parameters widely used to select high yielding genotypes under water stress and is attributed to the ability to absorb more water from the soil or the ability to check water loss through stomata [7]. It reflects the metabolic activity of plant tissue and is used as an index of dehydration tolerance. A non-significant decrease in the RWC was observed in stress condition indicating that Camelina seedlings have the ability to sustain their water content under stress, whereas this ability is lost under severe stress treatments. Similar type of observation has also been reported in rice [17] and in Tomatoes [33].

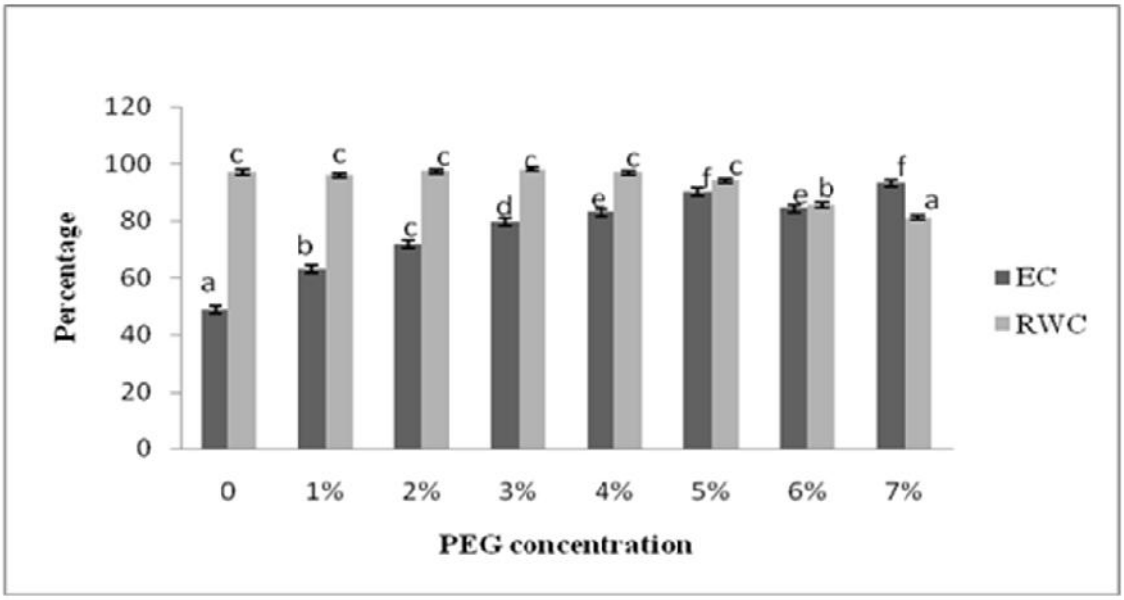

Fig. 4 Effect of PEG stress in *Camelina sativa* on Relative water content and Electrical conductivity (Values indicated by the same letter are not significantly different at P≤0.05) (1 month old).

Effect of PEG stress on biochemical parameters

Lipid peroxidation

The enhanced enzyme activities in Camelina under stress condition, shows a well organized defense system against ROS, under stress condition. Induced drought conditions increases the production of ROS which in turn increases the MDA content of the plant. Hence, MDA is considered to be a suitable marker for membrane lipid peroxidation [4]. The ROS generated by drought stress-induced oxidative stress can directly attack membrane lipids and increase lipid peroxidation [34]. In the present study MDA concentration increased but there was no significant difference among different treatments. Low concentration of MDA has been associated with drought tolerance in tomato cowpea, maize and chickpea [24]. PEG stress increased the level of lipid peroxidation in plants but statistically non significant MDA accumulation was observed in different PEG treatments (Fig. 5).

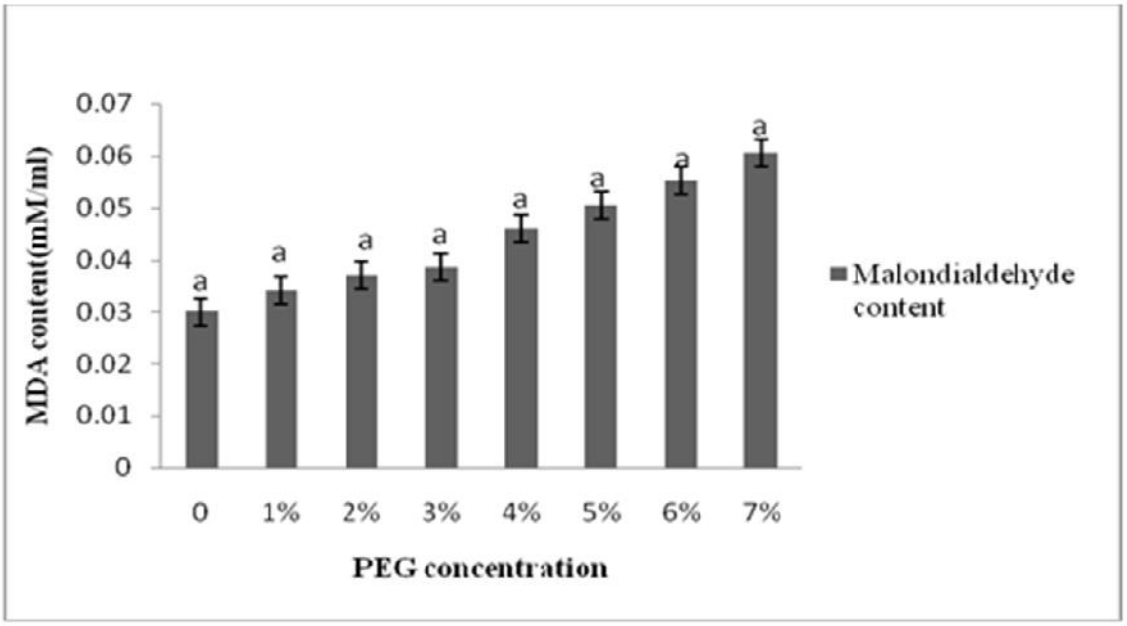

Fig. 5 Effect of PEG stress on Malondialdehyde content in *Camelina sativa* (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Guiacol peroxidase activity

Guiacol peroxidase plays a significant role in drought stress tolerance. in this study guiacol peroxidase activity showed a gradual increase in response to increased PEG treatment. GPX activity significantly increased ($P<0.05$) by 66.4 % in 7% PEG concentration with respect to the control (Fig. 6). Activity of guaiacol peroxidase enhances by increasing the duration of stress because of the decomposition of H_2O_2 [11]. Guaiacol peroxidase is widely accepted as stress "enzyme." GPOX decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and ethylene acting as a defence against various stresses by consuming H_2O_2.

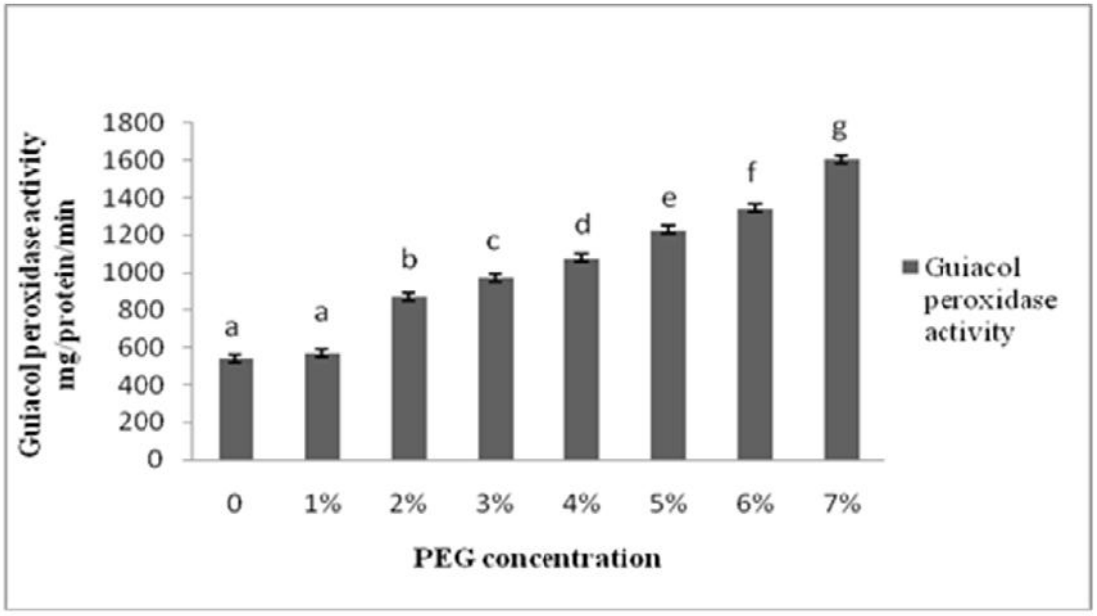

Fig. 6 Effect of PEG stress on Guiacol peroxidase activity in *Camelina sativa* (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Photosynthetic pigments

Photosynthetic pigments in plants were determined in the form of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content. It was found that there was significant difference in chlorophyll a content in 7% PEG in comparison to control. However, the difference was not significant in other concentrations studied. Chlorophyll b content increased significantly upto 2 % PEG treatment beyond which the chlorophyll b content decreased by 66.67 % in 7% PEG treatment compared to 2% PEG concentration. Total chlorophyll and carotenoid content was found to be highest in 2% PEG concentration compared to the control (Fig. 7). A decrease in total chlorophyll with drought stress implies a lowered capacity of the plant for light harvesting. The production of reactive oxygen species is mainly due to the absorption of excess energy in the photosynthetic apparatus, however degrading the absorbing pigments will cause the decrease in reactive oxygen species which plays a significant role under stress conditions. Chlorophyll content was affected in the present study which showed that long progressive stress along with some other environmental factors affect photosynthetic ability of the plant system. In our present investigation it was observed that chlorophyll pigments were not decreased proportionate to the increase in PEG induced water stress. Decreased or unchanged chlorophyll level during drought stress has been reported in many species like *Oryzae sativa* and *Phlomis fruticosa* [16, 22] depending on duration and severity of stress [35].

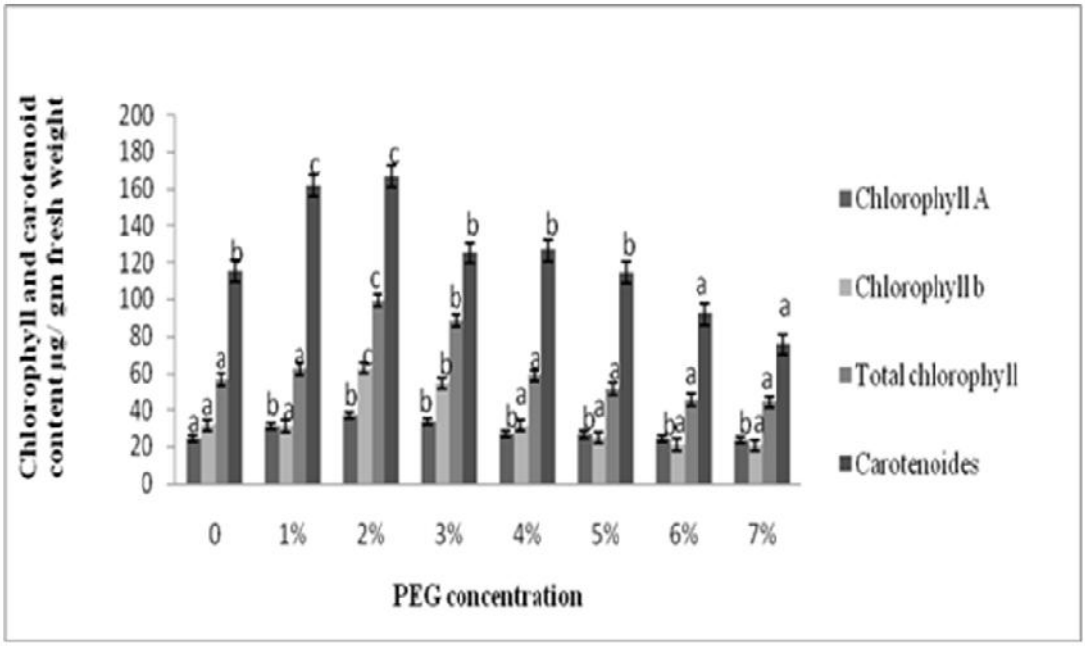

Fig. 7 Effect of PEG stress in *Camelina sativa* on Chlorophyll (a, b, a+b) and carotenoid content (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Protein content

The change in the protein content was measured in both control and stress induced plant. The results depicted that the protein content was found to increase in PEG stress up to 2 % but it decreased gradually with further increase in PEG concentration. In 2% PEG treatment, protein content was found to be highest which decreased by 64.82 % in 7% PEG concentration (Fig. 8). Proteins are assumed to protect the plant under stress by supporting the leaf structure during wilting process. Our results depicting increase in protein level to some extent is in congruent with the findings in *Eleusine coracana* [19]. However with increasing the concentration of PEG there was decrease in protein content. The possible reason for decreased protein content under water stress may be due to increased activity of protease leading to proteolysis or decreased synthesis or both. The other possible reason might be that leaf proteins undergo accelerated hydrolysis with severe water stress.

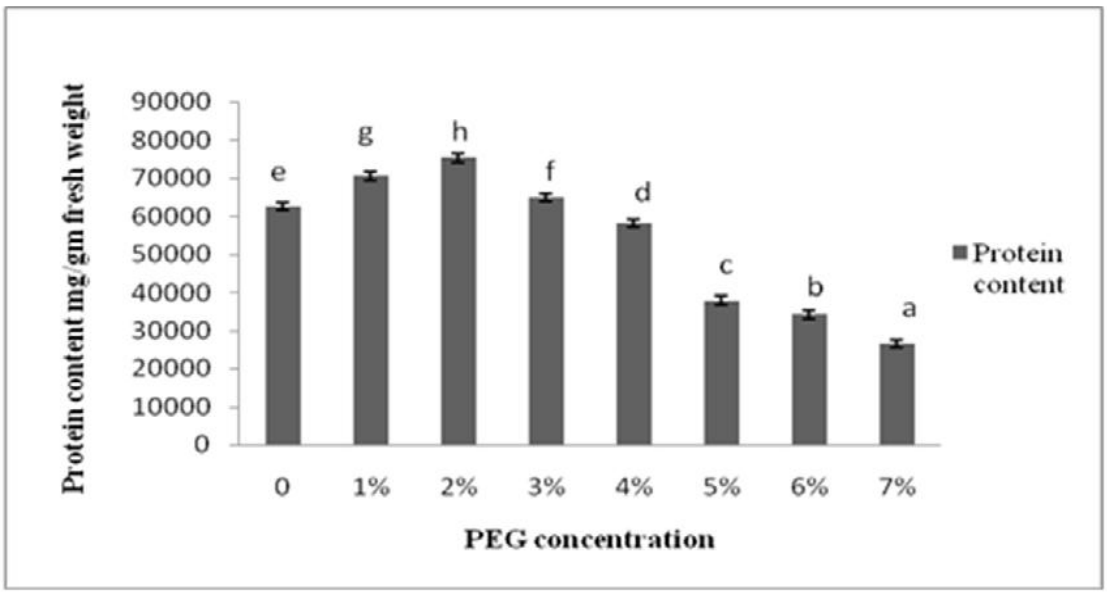

Fig. 8 Effect of PEG stress on Protein content in *Camelina sativa* (Values indicated by the same letter are not significantly different at $P \leq 0.05$) (1 month old).

Conclusion

Drought is a major restraint to agriculture production worldwide. Due to drought stress fertile lands are continuously rendered uncultivable. It limit crop productivity in multifold manner i.e causes serious affect on crop growth, gene expression, distribution, yield and quality depending on the plant growth stage, stress duration and severity. Several water stress inducing chemicals are polyethylene glycol, mannitol etc. Polyethylene glycol mimics as that caused by withdrawal of water without causing any toxic effect on plants [12]. *In vitro* screening is used as an efficient tool to screen plants for their drought tolerance. In current study, growth attributes of Camelina were adversely affected by drought stress leading to significant physiological and biochemical changes in Camelina. Seedlings were more susceptible to water-deficit stress to upto 2% PEG concentration as compared to drought stress induced with further increased concentration. The growth reduction under drought stress could be due to osmotic and as well as ionic pressure. This result suggested that the changes in *Camelina sativa* to its growth parameters, non-enzymatic and enzymatic antioxidant activities under *in vitro* conditions can be considered as factors of adaptive value conditions which led to differential responses in plants especially with respect to growth, osmotic adjustment, and antioxidant enzyme activities against drought stress.

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Declaration: The authors declare that there is no conflict of interest.

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