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Tolerance Response of Sunflower (*Helianthus annuus* L.) Cultivar NSSH-1084 to Water Logging Stress

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A B S T R A C T

Water logging remains a significant constraint to crop production across the globe. It affects plant growth, development and cause severe damage to plants from the early stage of growth. Sunflower is an oil seed cash crop favoured all over the world but the crop yield is influenced by water logging. The present study aims to evaluate the tolerance response of a sunflower (*Helianthus annuus* L.) cultivar NSSH-1084 to water logging stress. Here the effects of five water logging durations (0, 3, 6, 9, 12 days) were assessed during the early vegetative stage. The extent of influence on photosynthetic parameters, biochemical parameters and anti-oxidative enzymes, catalase (CAT) and Guaicol Peroxidase (GPX) were investigated during the water logging period. A decline in chl-a, chl-b and total chlorophyll was perceptible after 3days of treatment. Sequential increase in soluble sugar and protein content was found insignificant for tolerance response above 3 days of treatment. Malondialdehde (MDA) and proline content enhanced for all the durations of water logging. A parallel increase in CAT and GPX activity was observed till 3 days of water logging. The outcome of the investigation indicates remarkable tolerance response of NSSH- 1084 up to duration of 3 days for water logging stress.

Introduction

Water logging remains a significant constraint to crop production across the globe in areas with high rainfall and poor drainage particularly in tropical and subtropical regions. Water logging is one of the focal abiotic stresses, which affects crop growth (1)(2)(3). It is also a matter of worldwide concern affecting 16% of the soils in the United States, 10% of the agricultural lands of Russia and irrigated crop production areas of India, Pakistan, Bangladesh, and China (4)(5). Water logging causes yield losses in various crops such as wheat, barley, canola, lupins, field peas (6)(7), lentils and chickpeas (8). Water logging impedes the ability of soil to provide an optimum medium for plant growth and

alters its physical, chemical, electro-chemical and biological characteristics (9)(10)(11). Water logging hinders the growth of plants by reducing the dispersal of oxygen through the pore spaces in the soil around the root zone (12)(13)(14). Water logging decreases O2 diffusion capacity which leads to hypoxic or anoxic environments (15) (16).

Moreover, decreasing molecular oxygen prompts a sequence of changes in the physio-chemical properties of the soil (17). Many of these also change soil chemical and electro chemicals by decreasing redox potential and excess electron changes (17)(18)(19). This brings about the imbalance in metal availability near root region and rise in solubility of some selected metals to toxic levels, which are potentially damaging to plant roots (20) (21) (22) (23). Even though the accumulation of phytotoxic compounds requires time, the absence of oxygen alone is enough to change the plant metabolic activities to critical levels (24) (25). So the change in physio-chemical properties, redox potential, pH, level of toxicity of metals and oxygen deprivation impose a cascade of events to amplify abiotic stress upon the plants.

Under abiotic stress conditions, reactive oxygen species (ROS) levels are always elevated compared to pre-stress levels (26). Excessive production of various ROS such as superoxide radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen found in hypoxia-stressed leaf and root tissues can also cause severe damage to plants (27) (28) (29) (30). All of these lead to restricted root growth, reduced tiller number, premature leaf senescence and production of sterile florets thus affecting the grain yield (31)(32)(33).

When plants are subjected to environmental stresses, reactive oxygen species (ROS) are generated in response to stress conditions. ROS can cause oxidative damage to many cellular components, including membrane lipids, proteins, nucleic acids, and chlorophyll. Some osmolytes namely proline (Pro), glycine betaine (GB), trehalose (Tre) are also expressed under environmental stress to get rid of the adverse condition (34). Plants also possess several anti-oxidant enzymes systems that protect their cells from negative effects of ROS. As water submergence stress occurs frequently and can affect most habitats, plants have developed several strategies to cope with these challenges. One of the stress defence mechanism is the antioxidant defence system, which includes antioxidants and antioxidant enzymes (35). These include ascorbate(AA), reduced glutathione(GSH) and enzymes including superoxide dismutase(SOD), peroxidise (POD), ascorbate peroxidise(APX), catalase (CAT). Moreever, ROS are inevitable by-products of normal cell metabolism (34). But under normal conditions productions and destruction of ROS is well regulated in cell metabolism (36). When a plant faces harsh conditions, ROS production will overcome scavenging systems and oxidative stress will burst. In these conditions, ROS attack vital bio molecules and disturb the cell metabolism and ultimately the cell causes its own death (37).

The effects of water logging on plants includes inhibition of growth of roots, shoots and new leaves and in turn causing decreased growth in the entire plant; reductions in the net photosynthetic rate, photosynthetic electron transport rate and photo-system II (PS II), photochemical efficiency (38)(39); reactive oxygen species (ROS) metabolism disorders (40), reductions in element uptake; and inhibition of transport from roots to leaves (41). However, a wide variety of plants are known for the tolerance to water stress and oxygen deficiency during the adult stages of their life cycle (42)(43). All plants have tolerance to water stress, but the extent varies from species to species (42). For example,

flood sensitive plants like *Lycopersicum esculentum*, *Glycine max*, and *Helianthus annuus* show stunted growth and loss of yield potential in the waterlogged conditions, while plants like *Oryza sativa* can withstand water-logging for a considerable time. However, continuous submergence of *Oryza sativa* is also deleterious resulting in death and decay of the plant. So cultivation of tolerant crop varieties can be considered as one of the solution for lands prone to water logging and optimum yield of the crop species.

Sunflower is a prominent cash crop favoured by most of the farmers worldwide. In india the cultivation of sunflower is on high demand as an oil yielding crop besides its other uses as cosmetics, biodiesel, agrichemicals, surfactants, adhesives, softeners, lubricants etc. Sunflower oil is a great source of linoleic acid, omega-6 polyunsaturated fatty acid. It is favoured as one of the best industrial crop for its short duration, less demand for irrigation. It is mostly cultivated in agricultural land as a replacement to Rabi crops. But water logging in such lands has become the major obstacle for its optimum productivity reasoned by the erratic precipitation, irregular cyclonic depression and flood. So the aim of our objective is to examine the potential of a salt tolerant sunflower variety NSSH-1084 towards water logging and to study the different parameters of physiological and biochemical significance (44).

Materials and Methods

Seeds of sunflower (*Helianthus annuus* L.) of the variety NSSH-1084 were chosen for the study. The seeds were surface sterilised with 0.1 % HgCl2 for 2-3 minutes. Approximately 3-4 seeds were planted onto the cemented pot filled with 8 kg of soil in the ratio of soil: vermi-compost: sand(2:1:1/2). One healthy seedling out of 3 was allowed to grow after 10 days of germination. The selected seedlings were subjected to water logging treatment of different duration for 3 days, 6 days, 9 days and 12 days. One of the seedlings was treated normally with 500ml application of water. The total experimental design was carried out in pot culture. A single pot holds soil of 5 kg. All the pots were saturated with water up to a height of 1cm except control with a hole at the bottom of the pot for easy leak out of excess water. The whole experimental design was done in triplicate.

Estimation of Chlorophyll

The second leaf of the healthy plant of *Helianthus annuus* from the top was sampled for the experimental purpose. 0.1g of leaf sample (finely cut leaf tissue) was grinded to fine pulp with addition of chilled 80% acetone. Absorbance was taken at 645nm and 663nm for chlorophyll estimation. Total chlorophyll content in the leaves was estimated as per Arnon, 1949 (45).

Chlorophyll a = ((12.7×OD 663-2.69×OD 645) ×V/FW×1000)

Chlorophyll b = ((22.9×OD 645-4.68×OD 663) ×V/FW×1000)

Total Chlorophyll = ((20.2×OD 645-8.02×OD 663) ×V/FW×1000)

Soluble Protein Estimation

Soluble protein from healthy leaf was estimated using Bovine Serum Albumin (BSA) as standard as per Lowry method (46). The absorbance of each sample was recorded at 750nm after 30 min incubation. The concentration of protein content was determined with reference to standard curve made by using standard BSA (Bovine Serum Albumin). Finally the absorbance of protein extract and BSA was recorded at 750 nm.

Soluble Sugar Estimation

Carbohydrate of leaf sample was estimated and the content of the sample was quantified by using a standard curve of glucose with OD at 620nm as per Homme1992 (47).

Proline Estimation

Proline content of leaf estimated and further modified based on proline's reaction with ninhydrin as per Bates 1973(49). For proline colorimetric determinations, a 1:1:1 solution of proline, ninhydrin and glacial acetic acid was incubated at 100ºC for 1 hour. The reaction was arrested in an iced bath and the cromophore was extracted with 4 ml toluene and its absorbance was visualized 520 nm.

Lipid Peroxidation Estimation

Lipid peroxidation was carried out as per the standard procedure by measuring the amount of Malonodialdehyde (MDA) generated due to thiobarbituric acid reaction as per Heath and Packer 1968 (48). Leaves were grounded with a pestle and mortar in 1% TCA and centrifuged at 10,000 rpm for 5 min. To 1.0 ml of supernatant in a separate test tube, 4.0 ml of 0.55 TBA was added followed by heating at 95oC for 30 min and cooling in ice-cold water with further centrifugation at 5,000 rpm for 5 min. Absorbance was measured at 532nm and corrected for unspecific turbidity by subtracting the value at 600nm. The blank contained 1 % TBA in 20% TCA. MDA content was calculated using an extinction coefficient of 155mM-1cm-1 and the results expressed as µmol MDAg-1F.W.

Antioxidant Enzyme Extraction and Assay

Fresh leaves (0.5g) of *Helianthus annuus* L. were homogenised with a mortar and pestle under chilled conditions with phosphate buffer (0.1M, pH 7.5) and EDTA (0.5mm).The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The resulting supernatant was used for assay of different enzymes.

Catalase (CAT)

Catalase activity of control and stressed plants of *Helianthus annuus* was estimated (50). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H2O2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction started by adding H2O2 and decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H2O2 decomposed.

Guaiacol Peroxidase (GPX)

GPX was assayed and the reaction mixture comprises of phosphate buffer (pH= 6.0, 50 mM), H2O2 (10 mM), guaiacol (2.25 mM) and 50 µl of enzyme extract (51). The subsequent increase in absorbance of oxiguaiacol was measured at 470 nm and was defined as µmol of H2O2 per min.

Statistical Analysis

All results are presented as the mean values ± standard errors. The statistical significances of differences between mean values were assessed by analysis of variance and Duncan's multiple range tests. $P < 0.05$ was considered significant.

Results and Discussion

Chlorophyll content (Chl-a, Chl-b and total Chlorophyll)

Chl-a, Chl-b and total Chlorophyll content of leaf in mgg-1 FW were determined in plants subjected to different days of water logging at vegetative stage (Table-1).

During vegetative stage Chl a content

increases significantly only up to 3 days of water logging as compared to control.

The content varied with a value of 1.0020 mgg^{-1} FW at control to a highest value of 1.2620 mgg^{-1} FW at 3 days among the treatments but still higher 1.2091 mgg^{-1} FW at 6 days of treatment than the control.

The Chl a content decreases significantly at 9 days of water logging and continues up to 12 days of water logging with values of 0.7425 mgg^{-1} FW and 0.6147 mgg^{-1} FW respectively. Chlorophyll a level was seen to be lower than the control plants when plants were subjected to both 9 days and 12 days of water logging. The trend shows a gradual decrease of chlorophyll content from control to 12 days of water logging treatment except at the 3 days of submergence.

Chl-b content of the leaf showed a similar trend like Chl-a. During vegetative stage the Concentration of Chl b showed a magnitude of 0.4017 mgg^{-1} FW at control and increased significantly higher to 0.4970 mgg^{-1} FW at 3 days of water logging. There onwards it showed a deceasing trend of 0.4333, 0.3126, 0.1837 mgg^{-1} FW at 6 days, 9 days & 12 days of water logging respectively. The decreasing trend of Chl b from 9 days onwards indicated a lower level of Chl b than the control.

The total Chlorophyll content showed a similar trend that of Chl a and Chl b. During the vegetative stage the magnitude of total chlorophyll content was found to be 1.4098, 1.8590, 1.7428, 1.0551, 0.8984 mgg^{-1} FW at control, 3 days, 6 days, 9 days and 12 days of water logging respectively.

Following the trend shown by Chl-a & Chl-b the total Chlorophyll level was seen to be lower both at 9 and 12 days of water logging than control plants.

Protein Content

Total protein content results were given in Figure-1. The result showed an increasing trend of protein content up to 9 days of water logging during the vegetative stage. There was an increase of protein concentration from control to 3 days, 6 days and 9 days recorded at a value of 5.8928, 10.1785, 12.8000, 14.6640 µmol g^{-1} FW respectively. However the protein content during vegetative stage of 12 days of water logged plants was seen to be 5.343 µmol g^{-1} FW that stands at a value lower than the controlled plants at 5.898 µmol mg^{-1} FW.

Soluble Sugar Content

An increase in carbohydrate content from control to 12 days of water logging was evident from the outcome (Figure-2). The control plants showed a value of 105.4 mg/l FW followed by 124.1 mg/l FW at 3 days, 185.5 mg/l FW at 6 days, 239.8 mg/l FW at 9days and 242.7 mg/l FW at 12 days of water submergent plants.

Proline content

Water logging induced increase in proline content was observed in the leaves of all plants subjected to water logging (Figure-4) ranging from 3 days to 12 days as compared to control. The results were found to be 0.0715 µmol /g FW, 0.0981 µmol /g FW, 0.2565 µmol /g FW, 0.4925 µmol /g FW and 0.6927 µmol /g FW during vegetative stage from control to 12 days of water treatment.

Lipid peroxidation

Lipid peroxidation was estimated in terms of malondialdehyde (MDA) content of treated plants for water logging stress. Water logging induced increase in lipid peroxidation as MDA content was found to be ascending (Figure-3)

from control to 12 days treated plants at values of 0.6951 µmol /g FW, 1.1842 µmol /g FW, 1.6217 µmol /g FW, 2.3267 µmol /g FW and 2.8322 µmol /g FW for control, 3days, 6days, 9days and 12 days water logged plants respectively under the vegetative stage of growth.

Antioxidant Enzymes Activities

The GPX and CAT activities were recorded for all the treated plants under different range of water logged conditions. An increase in catalase activity was found from control to 3 days of water logged condition after which it deteriorates for more days of water logging.

Catalase Activity

During vegetative stage the activity of catalase was found to be highest in 3 days water logged plants with a value of 1.494 U mg^{-1} protein FW and decreased from 6 days to 12 days at values of 0.951, 0.724 & 0.535 U mg^{-1} protein FW at 6 days, 9 days & 12 days water logged conditions respectively than control with a value of 1.298 U mg-1 protein (Figure-5).

Guaicol Peroxidase Activity

The peroxidase activity in each treatment under different water logged conditions was evaluated statistically. A significant increase in GPX activity was seen to occur from control to 3 days water logged condition (Figure-6). But subsequent decrease was seen from 6 days to 12 days of water logging treatments. The range of peroxidase activity was recorded at values of 0.162, 0.200, 0.147, 0.146, 0.131 U mg^{-1} protein FW during vegetative period from control to 3 days, 6 days, 9 days and 12 days respectively. However the activity of GPX was found much lower than the activity of catalase for the respective level of water logging treatment.

Under natural conditions plants are frequently exposed to transient or permanent water logging. Flood drastically influences the soil physio-chemical properties of the soil. A deprivation of oxygen concentration particularly imparts hypoxic to anoxic condition, which in other hand influences plant growth, development and survival. One of the best characteristic of plant response to soil water logging is the metabolic transformation from aerobic respiration to anaerobic fermentation (52,53). Plants also show adaptations with regards to metabolic, physiological and morphological features. Our present study aims to establish a relationship among the photosynthetic parameters, adaptive molecules expressed during such stress and the activities of the anti oxidant enzymes namely catalase and peroxidise (GPX) during the vegetative stage of growth.

Starting with the preliminary focus towards the chlorophyll content, there has been seen a significant increase for chl-a, chl-b and total chlorophyll concentrations up to 3 days of water logging as compared to the control, whereas the concentrations decrease thereafter up to the last treatment of 12 days. However, all the photoshynthetic pigments stood at a higher value than the control till 6 days of water logging. Previous studies also found decline of photosynthetic pigments in several crop species eg tomato, mungbean, cucumber, maize, wheat etc when subjectd to water logging(54,55,56,57,58,59). The result of our experiment was in agreement with the above literature only after 3 days of treatment. But the time of application and the variety used for examining the abiotic stress is equally important for establishing any conclusion (60). Ozcubukcu and Ergun 2013 suggested the superior pigment content of a tolerant wheat cultivar Ducula-4 over a sensitive cultivar Dogankent for the similar degree of water logging stress and also found the significant improvement of the negative effects of the

stress in former than later. The protein content of different duration of water logging was observed to increase during the vegetative stage. While many of the literature confirmed decline of protein content with increase of submergence stress, Ashraf and Mehmood (1990) reported that there was an increase in soluble protein in *Brassica juncea* and a significant increase in total amino acids in *B. carinata*. compared to control (61).

Carbohydrate content: During water submergence there is a considerable shift from aerobic to anaerobic respiration as an adaptive mechanism. Our present study of investigation showed an increase in soluble sugar content under varying water logging conditions. Due to shifting of energy metabolism from aerobic mode to anaerobic mode under hypoxia or anoxia the energy requirements of tissue is greatly restricted as very few ATPs are generated per molecule of glucose. A high level of anaerobic metabolism in hypoxic or anoxic roots is therefore very important to supply the energy charge high enough which can sustain metabolism in roots for the survival of plants (62). Thus maintaining adequate level of readily metabolizable sugar in hypoxic or anoxic roots is one of the adaptive mechanism to water logging or oxygen deficient environments (63)(64)(65)(66). So a significant increase in root sugar level was established in many plants, mostly in adaptive plants.

Many of the authors has conformed decrease in content of carbohydrate or soluble sugar in *vigna radiate*, ragi and rice (66)(68), but Pravin and Karmoker *(2013*) reported an increase in reducing sugar level in root, stem and leaf of jute(67). Similarly three of the tolerant maize genotypes(CM 121, CM 122 and CM 132) showing better tolerance against the stress maintaining better soluble sugar and starch accumulation ability was reported by Baranwal & Singh 2002(58). So the ascending soluble sugar content in our result supports the tolerance response to the stress for prolong period.

The content of proline had shown an increasing trend for varying water logging periods during the vegetative stage. The proline shows the same mechanism of defence as under other abiotic stresses. It acts as osmoticum during stress and prevents membranes disintegration and maintains membrane stability. A wide range of report has observed such outcome in sunflower, *Brassica*, wheat and jute (69)(70)(71)(67).

Lipid peroxidation measured as MDA content had shown an increasing value from control to 12 days of water logging. Hence the oxidative damage obviously has enhanced the rate of lipid peroxidation under this prolonged period of treatment and shown a higher magnitude of membrane disintegration. Arbona *et al.*, 2008 while working on *citrus* reported that there exist a direct relationship stress sensitivity and accumulation of MDA(72).Water logging induced increase in MDA content in two hybrids of maize(DH605, ZD958)suggested an impact of water logging on membrane integrity and thus membrane deterioration (73).

Antioxidant activities: Exposure of plants to most adverse conditions like hypoxia or anoxia possess oxidative stress, which affects plant growth due to production of ROS such as superoxide radicals and H2O2 (74).

The activity of catalase has shown a similar trend for all the respective treatments. The catalase activity increases up to the level of 3 days of water logging and then decreases onwards as compared to control.

Table.1 Effect of water logging on chlorophyll content a, b & total chlorophyll (mg/g FW) of leaf during vegetative stage

Treatment	Chlorophyll content-a	Chlorophyll content-b	Total chlorophyll content
Control	1.0028±0.02	0.4017±0.01	1.4098±0.08
3 Days	1.2620±0.04	0.4970±0.04	1.8590±0.01
6 Days	1.2091±0.01	0.4333±0.01	1.7428±0.01
9 Days	0.7425±0.08	0.3126±0.07	1.0551±0.03
12 Days	0.6147±0.06	0.1837±0.09	0.8984±0.03

Fig.1 Effect of water logging on protein content (µmol/ g FW) of leaf during vegetative stage.

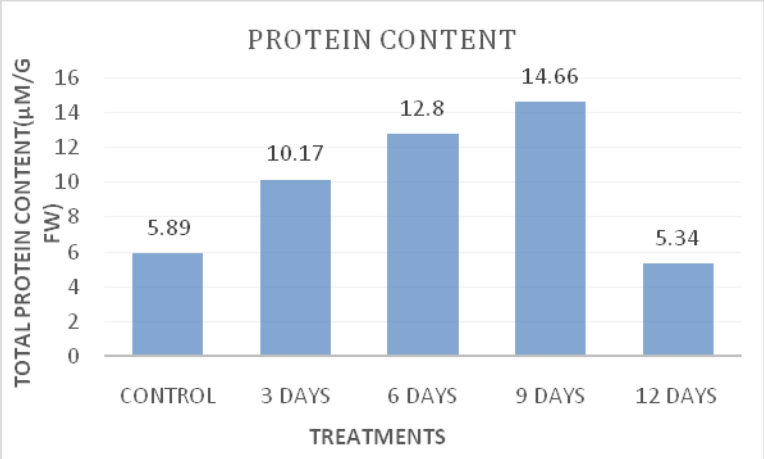


Fig.2 Effect of water logging on soluble sugar content (mg/l FW) of leaf during vegetative stage

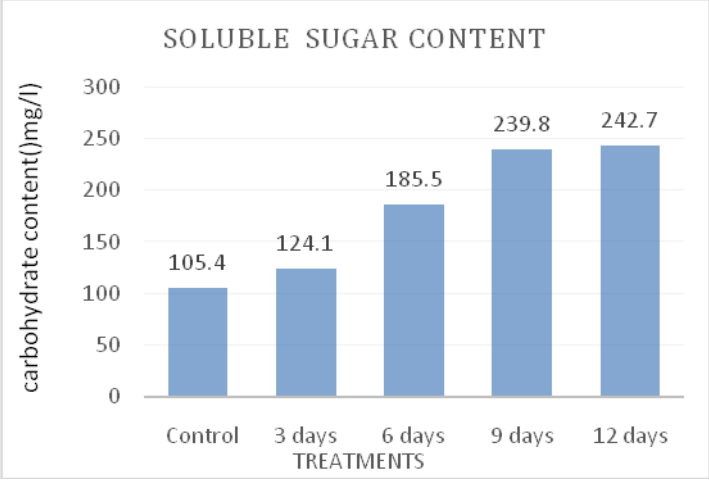


Fig.3 Effect of water logging on lipid peroxidation(µmol MDA/g FW) of leaf during vegetative stage

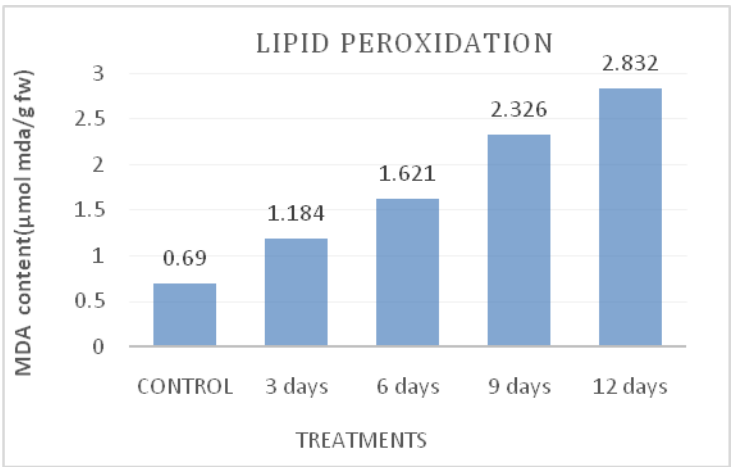


Fig.4 Effect of water logging on proline content (µmol proline/g FW) of leaf during vegetative stage

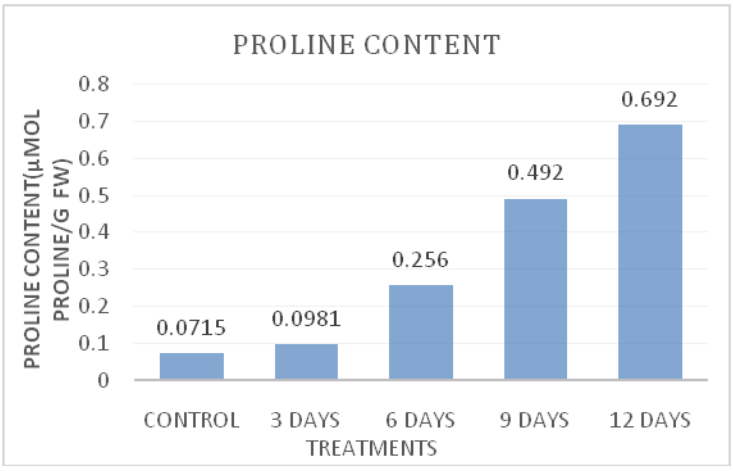


Fig.5 Effect of water logging on catalase activity (U/mg protein FW) of leaf during vegetative stage

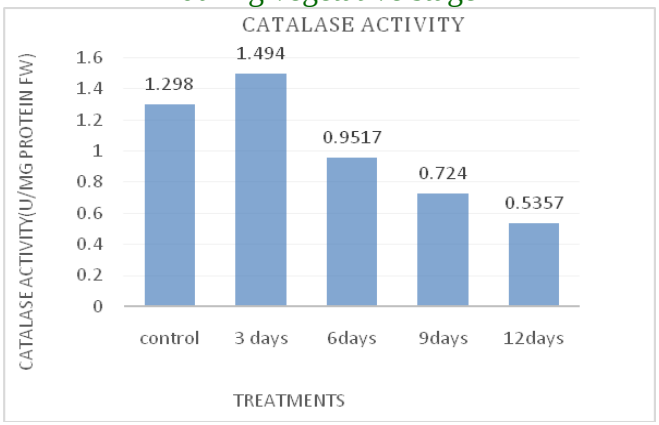
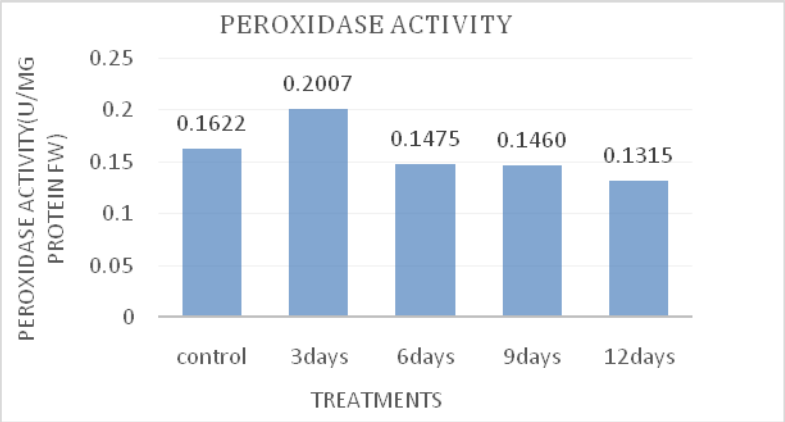


Fig.6 Effect of water logging on peroxidase activity(U/mg protein FW) of leaf during vegetative stage



An exact similar activity has been shown by peroxidase enzyme. However, the activity of peroxidase was significantly lower than the activity of catalase at their respective treatments.

The decrease in activity of catalase (CAT) and ascorbate peroxidise(APX) was marked during water logging conditions in *Mungbean* by Ahmed *et al.*, (57). APX served as a criteria for evaluating water logging in *tomato* and *egg* plant root (75). The varying degree of water logging stress generated oxidative stress and production of reactive oxygen species(ROS)and thus marked an increase in activities of enzymatic antioxidants in maize seedling (76)(77).Water logging for 72 hours lead to reduction in CAT, APX activity and reduction was more pronounced in susceptible genotype than in tolerant one in *zea mays* two tolerant variety (LMS,PRAKASH,) and three susceptibility variety (PMH2,JH-3459 and LM-14).

Moreover, the fact that the activities of catalase and peroxidise, the two H2O2 scavenging enzymes, were above the control levels on the 3rd day signified their contribution to the antioxidant defence system. The present study, which was concerned with the effect of water logging stress, indicated that the activities of CAT and Gua-dep peroxidise (PODs) increased with the increase in the severity of the stress that withstand up to 3days and the parallelism among these enzymes themselves might provide partial protection, especially until the 3rd day of the treatment period.

In our study, the observed increases in the catalase(CAT) and Gu-dep peroxidise (GPX) activity of *Helianthus annuus* L. variety NSSH-1084 leaves till 3 days of water logging constituted the first line of defence via detoxification of free oxygen radicals. Additionally, similar changes were observed in both activities. However the activity of CAT is significantly higher than GPX for the respective duration of treatment.

The defence system was observed to stand strong till 3days of water logging as indicated by all the parameters studied, but failed to withstand prolonged submergence stress more than 3 days evident from the decline of photosynthetic pigments, escalation of lipid proxidation and reduction in antioxidant enzyme's activity.

The outcome suggests tolerance of the sunflower (*H. annuus* L.) variety NSSH-1084 towards water logging stress well up to 3 days.

The tolerance response undergoes impairment of defence mechanism when subjected to prolonged period of water submergence.

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