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Research Article

Polyethylene Gycol (PEG) Induced Water Stress in Four Different Genotypes of Pea Seedlings and Evaluation of The Induced Defense Mechanism

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Abstract

The present work was undertaken on artificially induced water stress on pea seedlings under in vitro conditions in order to select the drought tolerant line. Artificial water stress was induced with PEG-6000 on 15-day old seedlings of four varieties for 4, 8 and 12 days. The activities of antioxidative enzymes like peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase were assayed in the stressed and control plants. POX activity was increased in the initial stages of stress, but its activity was decreased significantly on the 12th day in all the varieties. APOX also showed a similar trend but the maximum activity was noted in Var 3 on the 8th day. CAT activity decreased in var 1 and var 2 when compared with the control, which, however, increased significantly in var 3 and var 4. A slight increase in the GR activity was observed in var 1 and var 2 at the initial stages of the drought stress but its activity decreased significantly on the 12th day in both these varieties when compared with control plants. However, its activity also increased steadily in var 3 and var 4. Maximum SOD activities were noted on the 4th day of drought stress in all four varieties but its activities decreased steadily on the subsequent 8th and 12th days when compared with control. When antioxidative activities were compared among the four varieties, var 3 and var 4 showed maximum increase in antioxidant activity during the period of drought stress. Among the four varieties, var 3 and var 4 showed greater accumulation of H2O2 during the stress days and were maximum at 12th day. Lipid peroxidation also increased in the same varieties. Maximum proline content was noted in both the root and leaf of var 3, followed by var 4. It was further noted that the chlorophyll content decreased significantly in all four varieties in subsequent longer drought stresses. The accumulation of proline content was steadily higher with an increase in the stress length in all the four varieties. During the drought stress, all the varieties showed an increase in ascorbate content but, it was maximum in var 4 followed by var 3 and the least ascorbate was noted in var 1. The present findings indicate that water stress induces oxidative stress in all the four varieties. However, antioxidative mechanisms were found to be more pronounced in var 4 which, therefore, may be considered as the most tolerant to drought stress.

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Introduction

Plants have limited mechanisms of drought stress avoidance; therefore, they require flexible means of adaptation to change drought conditions (Zhang et al., 2004; Pradhan and Chakraborty, 2012). Tolerance to this abiotic stress is a complex phenomenon, comprising a number of physiochemical processes at both cellular and whole organism levels activated at different stages of plant development. Both enzymatic and non-enzymatic antioxidants provide protection against oxidative damage (Munne-Bosch and Algere, 2000). Water induces several physiological and biochemical and molecular responses in several crop plants, which

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would help them to adapt to such limiting environmental conditions (Bajaj et al., 1999; Arora et al., 2002; Lama and Chakraborty, 2012). Drought is a worldwide problem constraining plant production (Chinnusamy et al., 2004) and is prone to acute periods due to little rainfall in the growing season as the environment deteriorates. The lack of adequate moisture leading to water stress is a common occurrence in rain-fed areas, brought about by infrequent rain and poor irrigation (Wang et al., 2005).

Proline and quaternary ammonium compounds, eg. Glycinebetaine, prolinebetaine, etc. are key osmolytes contributing towards osmotic adjustment (Huang et al., 2000; Kavikishore et al., 2005). One of the most important responses of plants to drought and other stress is the overproduction of different types of compatible solutes (Ashraf and Harris, 2004; Serraj and Sincleair, 2002). Of these solutes, proline is widely distributed in plants and normally accumulates in larger quantities than other amino acids in drought-stressed plants (Ashraf, 2004; Irigoyen et al., 1992). Free proline and sugar contents significantly increased in Vigna radiata nodules under drought, but nodules normally contain more praline than leaves (Hooda et al., 1999). Great efforts have been made to decipher the molecular mechanisms of drought tolerance (Bartels and Nelson, 1994; Mahajan and Tuteja, 2005). It inhibits the photosynthesis of plants, causes changes of chlorophyll content and components and damages the photosynthetic apparatus (Escuredo et al., 1998). When plants are subjected to drought stress, a variety of active oxygen species are generated, such as superoxide, H_2O^2 and hydroxyl radicals, which cause damage in plants. They are toxic to living organisms and, unless removed rapidly, they destroy or inactivate various cellular components (Trippi et al., 1989).

Pea (*Pisum sativum*) is an important edible leguminous seed crop for human nutrition. Its seeds contain 18-20% dry matter, and 10-12% is carbohydrate and 5-8% is protein (Vural et al., 2000). Pea is used as a fresh vegetable, frozen or canned. According to FAO 2004 data, about 12.2 million tonnes of pea production were achieved in 6.3 million ha agricultural lands of the world with an average yield of 1.930 kg ha-1 (Anonymous, 2007). In Turkey, the pea production area was 1.568 ha with a total production of 4373 tones and an average yield of 2.79 kg ha-1 in 2006 (TUIK, 2007).

The pea is a cool-season vegetable crop of mild climate regions. Therefore, it gives a higher yield in cold-humid regions compared to warm-dry areas. Its minimum temperature range for germination is between 1-6°C and it can survive in low temperatures up to -5°C. Even though pea can grow in many soils, the best yield can be obtained in clay-loam, deep, productive, moist, slightly acid (pH 6.5-7.0) soils. When the soil is productive and moist, vegetative growth is advanced. On the other hand, pea seed yield decreases. In addition, owing to its taproots, the pea can use a plant's nutrients and water from different soil layers and increase the organic matter content of soil. As a legume crop, the pea is able to fixate 50-150 kg ha-1 nitrogen from air (Sehirali, 1988; Akdag, 2001). Thus, considering the importance of pea cultivation in the district of Darjeeling, the present study was undertaken to determine how four varieties respond to water stress in terms of over expression of antioxidant enzymes or other biomolecules.

Materials and methods

Induction of water stress

Four pea varieties were selected (Table 1) for this present experiment. For induction of water stress, initially seeds were soaked overnight and surface sterilized inside the laminar hood with 0.1% mercuric chloride ($HgCl_2$) (Hi-Media Pvt. Ltd, India) for two minutes and they were rinsed several times with sterile double distilled water to remove $HgCl_2$. The seeds were transferred to sterile petriplates containing sterile double distilled water. The seeds were kept in petriplates and grown in vitro aseptically for 15 days. After 15 days, the seedlings were subjected to drought stress by application of Polyethylene glycol (PEG) 6000 (Hi-Media, Pvt. Ltd, India) and various biochemical tests were performed on 0, 4th, 8th and 12 days of stress along with the control plants.

Table. 1 Varietal names and their respective code names

Sl. No.	Varietal name	Code
1	Palam Priya	Var 1
2	Arka Ajit	Var 2
3	Arka Karthik	Var 3
4	DPPM-65	Var 4

Preparation of the enzyme extract

For determination of enzyme activities, around 1 g of the leaves collected from the treated and control plants were ground to fine powder with a mortar and pestle under liquid nitrogen in 10 ml of cold 50mM sodium phosphate buffer, pH 7.5, containing 1% (w/v) polyvinylpolypyrrolidone (PVP). The

homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was directly used as a crude extract for enzyme assays.

Assay of activities

Peroxidase (POD: EC. 1.11.17)

Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisdin in the presence of H_2O_2 (Chakraborty et al., 1993). Specific activity was expressed as ΔA_{460} mg protein^{-1} min^{-1}.

Ascorbate peroxidase (APX: EC.1.11.1.11)

Activity of ascorbate peroxidase was assessed following the protocol of Nakano and Asada (1981) where its activity was determined by monitoring the decrease in absorbance at 290 nm. The enzyme activity was expressed as ΔA_{290} mg protein^{-1} min^{-1}.

Catalase (CAT: EC. 1.11.1.6)

Catalase activity was estimated by Upadhyaya and Panda (2004). It was calculated by estimating the breakdown of H_2O_2, which was measured at 240 nm. The enzyme activity was expressed as Δ A245 mg protein^{-1} min^{-1}.

Superoxide dismutase (SOD: EC. 1.15.1.1)

The enzyme activity was estimated by monitoring the inhibition of the photochemical reduction of NBT according to the method described by Dhindsa et al., (1981) with some minor modifications. The absorbance of samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzyme.

Glutathione reductase

Glutathione reductase activity was determined by following the protocol of Lee and Lee (2000). It was determined by the oxidation of NADPH at 340 nm. Enzyme activity was expressed as µM NADPH oxidized mg protein^{-1} min^{-1}.

Protein content

Protein content was determined by following Lowry's method (1951).

Lipid peroxidation

Lipid peroxidation was measured following the procedure of Heath and Packer, (1968) where MDA was determined by the thiobarbituric acid (TBA) reaction. The absorbance was measured at 532 nm and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1}.

Estimation of H_2O_2

H_2O_2 was extracted and quantified according to the method described by Jena and Chowdhuri (1981).

Ascorbate

Ascorbate was extracted and estimated by following the protocol of Mukherjee and Choudhuri (1983).

Determination of chlorophyll content

Total chlorophyll was estimated following the standard protocol (Harborne, 1973). Chlorophyll was extracted in 80% acetone and the extract was filtered. Absorbance of the filtrate was noted at 663 nm and 645 nm wavelengths and the chlorophyll content was calculated using a standard formula.

Statistical analysis

Experiments were set up in a completely randomized block design. Each experiment was carried out with 3 replicates. Data was analyzed by one-way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test at p\0.05 (Duncan 1955) on the statistical package of SPSS 10.

Result and Discussion

Four varieties of 15-day-old pea seedlings were subjected to drought stress in vitro by the application of PEG-6000. On the 4th, 8th and 12th days of drought stress plants were sampled for various biochemical assays along with the controls. No significant morphological changes were seen in the experimental plants during the initial stages of drought stress. However, slight wilting of leaves was observed on the 12th day of stress. An essays of antioxidative enzyme activities showed that the POX activity increased in the initial stages of stress (Fig 1a), but its activity was decreased significantly on the 12th day in all the varieties. APOX (Fig 1b) also showed a similar trend but the maximum activity was noted in Var 3 on the 8th day. CAT activity (Fig 1c) decreased in var 1 and var 2 when compared with

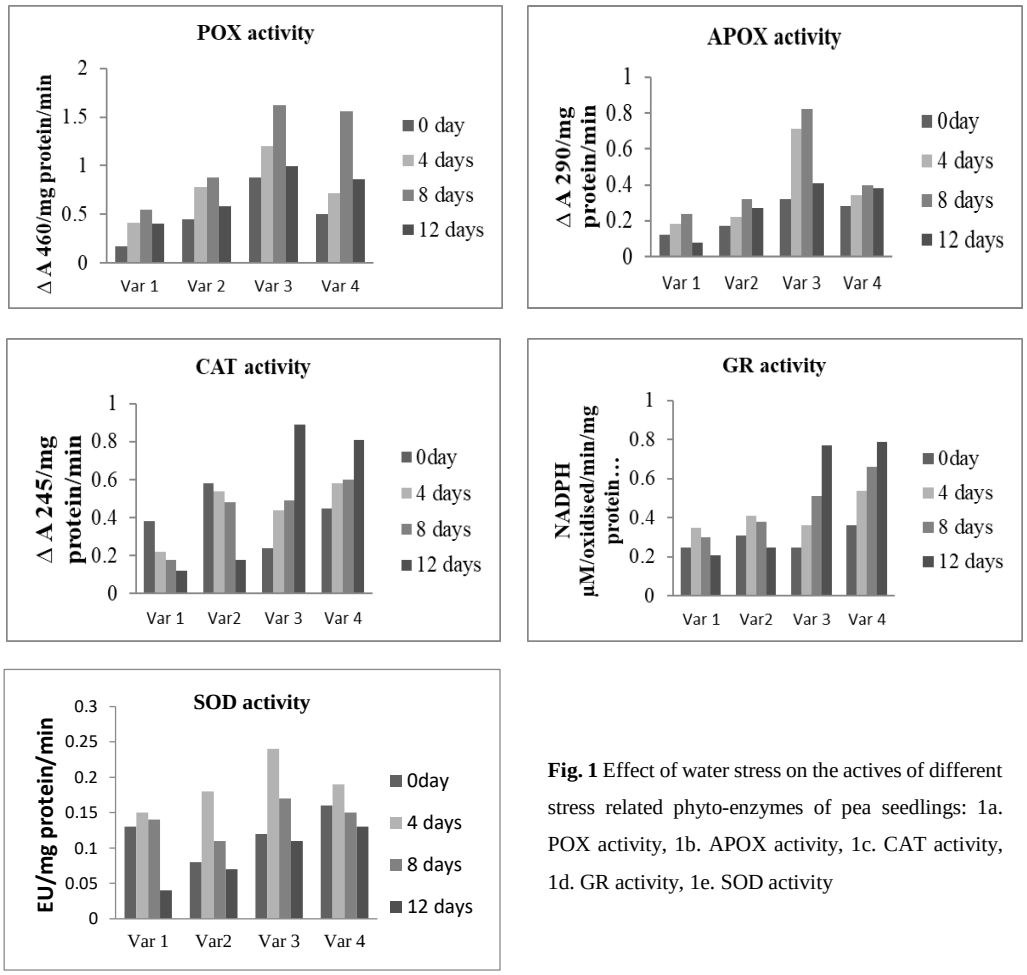

Fig. 1 Effect of water stress on the actives of different stress related phyto-enzymes of pea seedlings: 1a. POX activity, 1b. APOX activity, 1c. CAT activity, 1d. GR activity, 1e. SOD activity

the control, which, however, increased significantly in var 3 and var 4. Similar trends were noted in maize (Lama and Chakraborty, 2012). A slight increase in the GR activity was observed in var 1 and var2 at the initial stages of the drought stress but its activity decreased significantly on the 12th day in both these varieties when compared with the control plants. However, its activity increased steadily even in var 3 and var 4 (Fig 1d). Maximum SOD activities (Fig 1e) were noted on the 4th day of drought stress in all four varieties but its activities decreased steadily on the subsequent 8th and 12th days when compared with control. When antioxidative activities were compared among the four varieties, var 3 and var 4 showed maximum increase in antioxidant activity during the period of drought stress.

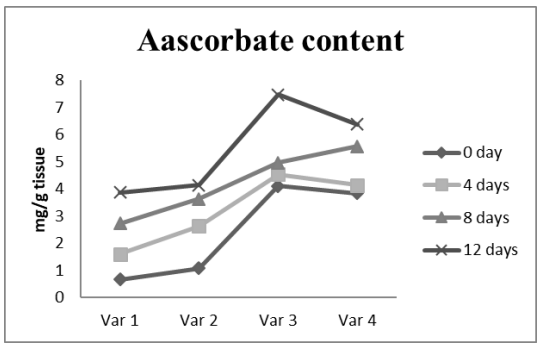

Fig. 2 Effect of water stress on Ascorbate content in pea seedlings

Table. 3 Proline content in leaf and root of four varieties of pea seedlings following water stress.

Varieties	Stress period (d)	Proline content	
		Leaf	*Root*
Var 1	0	0.07	0.19
	4	0.24	0.3
	8	0.42	0.5
	12	0.88	1.11
Var 2	0	0.19	0.3
	4	0.34	0.44
	8	0.66	0.75
	12	0.89	0.98
Var 3	0	0.71	0.98
	4	1.11	1.56
	8	1.98	2.22
	12	2.54	2.75
Var 4	0	0.66	1.11
	4	0.98	1.21
	8	1.45	1.63
	12	**2.45**	**2.11**

capacity of better protective mechanisms against oxidative damage. Among the four varieties, var 3 and var 4 showed greater accumulation of H_2O_2 It has been pointed out by many earlier workers (e.g. Sharma and Dubey, 2005; Tu´rkhan et al., 2005; Lama and Chakraborty, 2012) that maintaining a high level of antioxidative enzyme activities may contribute to drought tolerance by increasing the during the stress days and were maximum on the 12th day (**Table 2**). Lipid peroxidation also increased in the same varieties (**Table 2**).

Table. 2 H_2O_2 accumulation and lipid peroxidation in pea seedlings following water stress.

Varieties	Stress period (d)	H_2O_2 a	Lipid b
Var 1	0	1.55	0.009
	4	5.68	0.018
	8	8.69	0.028
	12	14.69	0.038
Var 2	0	2.22	0.005
	4	6.59	0.017
	8	9.45	0.022
	12	14.11	0.025
Var 3	0	2.05	0.008
	4	7.89	0.012
	8	11.44	0.024
	12	**15.9**	**0.035**
Var 4	0	1.89	0.016
	4	7.88	0.029
	8	12.77	**0.039**
	12	**18.51**	0.021

It was further noted that the chlorophyll content decreased significantly in all four varieties in subsequent longer drought stresses (**Fig. 3**). Similar observations were also made in maize (Lama and Chakraborty, 2012) and barley (Kuroda et al., 1990). The accumulation of proline content was steadily During the drought stress, all the varieties showed an increase in ascorbate content but, it was maximum in var 4 followed by var 3 and the least ascorbate was noted in var 1 (**Fig 2**). Ascorbate can directly act as a free radical scavenger (Bowler et al., 1992; Larson, 1998; Lama and Chakarborty, 2012).

higher with an increase in the stress length in all the four varieties (**Table 3**). Maximum proline content was noted in both the root and leaf of var 3, followed by var 4. Accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes and repressed activity of proline degradation (Delauney et al., 1993; Peng et al., 1996). Accumulation of proline is an important indicator of drought in plants including bacteria and algae (Lama and Charaborty, 2012). This important amino acid has been reported to play multiple physiological functions in plants subjected to drought (Lama and Charaborty, 2012).

Conclusion

The current data show that water stress causes oxidative damage in all four varieties of pea. However, antioxidative mechanisms were found to be more evident in var 4, which may be considered the most tolerant to drought. It is generally understood that laboratory circumstances may not always reflect the genuine behaviour of plants subjected to water stress in the field. On the other hand, such findings may aid in understanding the mechanism of drought stress management and the selection or development of drought-resistant pea genotypes.

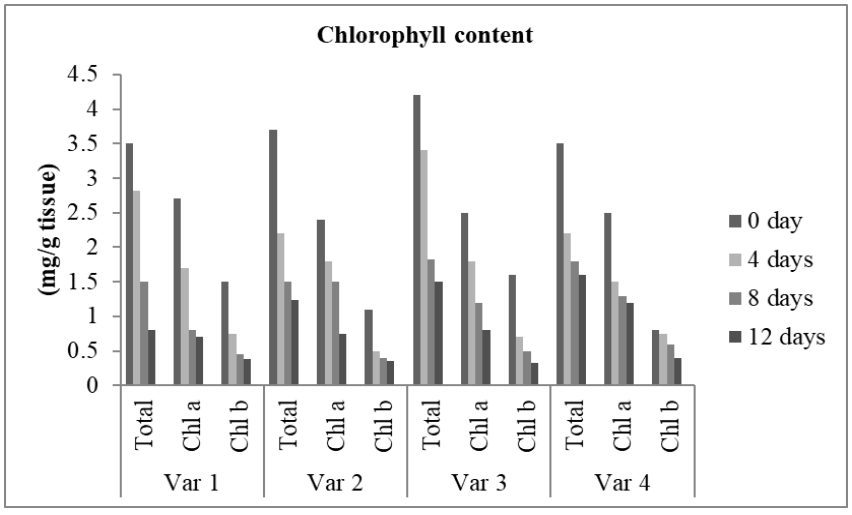

Fig. 3 Effect of water stress on chlorophyll content in pea seedlings

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