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INSIGHTS INTO PHYSIOLOGICAL AND BIOCHEMICAL BASIS OF WATERLOGGING TOLERANCE IN PIGEONPEA (*CAJANUS CAJAN* L. MILLSP.)

Seema Kumari, Preetam Kumar Roy, Vikash Kumar Jha and Dharamsheela Thakur*

Department of Molecular Biology and Genetic Engineering, Bihar Agricultural University, Sabour, Bhagalpur-813210, Bihar, India.

*Corresponding author E-mail: dscdri@gmail.com

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ABSTRACT

Waterlogging stress is major abiotic stress having detrimental effects on pigeonpea productivity worldwide. To identify waterlogging-tolerant pigeonpea genotypes and their adaptive mechanisms, 13 genotypes were subjected to six days of waterlogging at the 30-day early vegetative stage. Analysis of survival data revealed that ICPL 20241 and ICPL 20092 exhibited high level of tolerance, whereas ICPL 87 and ICP 7035 were highly susceptible. While waterlogging induced a general decline in chlorophyll content, tolerant genotypes maintained significantly greater photosynthetic integrity than susceptible ones. Tolerant genotypes demonstrated a robust adaptive response characterized by a significant increase in peroxidase (POX) and alcoholic dehydrogenase (ADH) activities, facilitating better antioxidant defense and anaerobic energy production. Furthermore, total soluble sugar (TSS) content increased in the roots of tolerant genotypes but declined in susceptible ones, suggesting that efficient sugar mobilization is a key survival strategy under hypoxic conditions. These findings identify specific resilient germplasm and establish chlorophyll and TSS retention along with increase in ADH and POX activity as vital selection criteria for breeding waterlogging-tolerant pigeonpea varieties.

***Key words*:** Pigeonpea, waterlogging, alcohol dehydrogenase, peroxidase, sucrose synthase

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is multipurpose legume crop, grown mainly in the semiarid tropical region of Asia, Africa, Latin America and the Caribbean. Globally, it is cultivated in 5.40 million hectares with production of 4.60 million tonnes (FAOSTAT 2024). India is largest producer with acreage of 4.13 milllion hectare and production of 3.42 million tonnes (Directorate of Economics & Statistics, DAC&FW, Government of India; DES-2024). Pigeonpea productivity is affected by several biotic and abiotic stress. Pigeonpea is primarily cultivated in rainfed ecosystems during the monsoon season and is prone to transient waterlogging due to continuous and heavy downpours during the early monsoon period. Waterlogging occurs due to prolonged accumulation of water on soil surface without infiltrating into the soil. Waterlogging leads to leaching of important minerals or essential intermediate metabolites from roots into water. It also leads to hypoxia (deficiency of oxygen) and if prolonged, anoxia (absence of oxygen) in the soil because of lower rate of diffusion of oxygen in water as compared to air. Prolonged period of waterlogging leads to plant senescence and death resulting in poor crop stand and productivity. Consequently, waterlogging has emerged as a critical production constraint, particularly when it occurs during the initial crop growth stages and causes 30% to 40% economic yield loss globally (Basavaraj *et al.*, 2023).

Agronomic intervention such as use of raised beds, ridge sowing can prevent waterlogging but these options are not economically viable. Use of tolerant genotypes is the most economical and effective way to prevent losses.

Waterlogging stress induces various morphological, physiological and biochemical alterations in plant cell for adaptation which can be used as marker for identification of stress tolerant genotypes. There is need to identify waterlogging stress tolerant genotypes and morpho-physiological traits linked to waterlogging tolerance. The present study was done to identify genotypes capable of withstanding waterlogging stress and to identify physiological and biochemical markers for waterlogging stress tolerance.

Material and Methods

Plant material and growth conditions

An experiment was conducted with 13 pigeonpea genotypes; ICPL 87, Malviya-13, Bahar, ICP 5028, ICPL 20092, AK 13B, Pusa 151, ICPL 149, ICPL 87119**,** ICPL 20241, ICPH 2431, ICP 7035 and ICPL 84023. Five seeds were sown in 25cm×25cm (h×dia) plastic pots filled with mixture of clay loam soil, vermicompost and sand during Kharif season 2023. Waterlogging treatment was given by placing pots with 30 days old plants in cemented tank filled with water (5 cm above soil). Waterlogging treatment was done for 6 days. Plant survival was recorded at 2, 4 and 6 days of waterlogging treatment and after 5 and 10 days of water withdrawal. Chlorophyll content in leaf was estimated at 2, 4 and 6 days of waterlogging treatment. Root and leaf samples were collected before and after 6 days of waterlogging treatment for estimation of total soluble sugar and enzymatic activity.

Plant Survival

Plant survival was calculated by counting the total number of plants and the number of live plants after 2, 4 and 6 days of waterlogging treatments and after 5 and 10 days of water withdrawal. Plant survival was expressed in the term of percentage survival.

$$\text{Plant survival percentage} = \frac{\text{No. of live plants}}{\text{Total no. of plants}} \times 100$$

Chlorophyll Estimation

Chlorophyll content was estimated as described by Hiscox and Israelstam (1979). Leaf sample (0.05 gm) was cut into small pieces and transferred to test tube containing 10 ml DMSO and incubated in water bath at 65 °C for 4 h. Tubes were cooled down to room temperature. Absorbance was recorded in spectrophotometer at 645 nm and 663 nm. Chlorophyll *a*, *b* and total chlorophyll content was calculated using the following Arnon (1949) formulae:

$$\text{mg chlorophyll } a\text{/g tissue} = \frac{12.7\,(A663) - 2.69\,(A645) \times V}{1000 \times W}$$

$$\text{mg chlorophyll } b\text{/g tissue} = \frac{22.9\,(A645) - 4.68\,(A663) \times V}{1000 \times W}$$

Total chlorophyll= chl *a*+chl *b*

Where,

A= absorbance at specific wavelength

V= final volume of chlorophyll extract in 80% acetone

W= fresh weight of tissue extracted

Peroxidase Assay

Peroxidase activity was assayed as described by Putter (2010). 250 mg of leaves were homogenized in 2 ml of 0.1 M phosphate buffer (pH 7.0). The extract was centrifuged at 10000 rpm for 10 min at 4 °C and supernatant was taken as enzyme source. The assay mixture consist of 3 ml of the buffer solution, 0.05 ml 20mM guaiacol solution, 0.1 ml enzyme extract and 0.03 ml of 12.3 mM hydrogen peroxide solution. The mixture was well shaken and placed in the spectrophotometer in a cuvette. Initial absorbance was recorded at 436 nm and then increase in the absorbance was noted at the interval of 30 sec on UV-Vis spectrophotometer. The time required for the mixture optical density to be increased by 0.1 (Δt) at 436 nm was recorded and used in calculations.

$$\text{The enzyme activity (unit/min/mg protein)} = \frac{\Delta OD}{T} + \frac{1}{\varepsilon} + \frac{1}{\text{Protein mg/mix V}}$$

Where,

ΔOD= Change in OD (0.1)

T = Time in minute

ε = Molar extinction coefficient of hydrogen peroxide solution (12.3 mM)

V = Volume of the enzyme used in ml

Alcohol Dehydrogenase (ADH) Assay

ADH activity was assayed by the reverse reaction, i.e. oxidation of ethanol by ADH with the help of NAD, resulting into the synthesis of acetaldehyde and NADH (Toyama, 1995). Enzyme was extracted in 50 mM Tris–HCl (pH8.9) containing 2 mM DTT. Plant tissue of 250 mg was first pulverized with liquid nitrogen and then homogenized with 2.0 ml of extraction buffer. The extract was centrifuged at 12,000 rpm for 15 min at 4 °C in a refrigerated centrifuge, and the supernatant was used as source of enzyme. The reaction mixture contained 50 mM Tris buffer, 15 mg/ml NAD, 96% ethanol and 0.1 ml enzyme. Reaction mixtures except NAD were prepared in test tubes, and used as blank to adjust zero. NAD was added to initiate the reaction and increase in absorbance due to NADH at 340 nm recorded for 5 min with 30 sec

interval in UV–vis spectrophotometer. Enzyme activity was calculated as follows:

$$\text{Enzyme activity (unit/mg protein)} = \frac{\Delta A340/\text{min}}{6.22 \times \text{mg protein/mlx V}}$$

Where,

ΔA340 = Change in absorbance at 340nm

V = Volume of enzyme used in reaction

Total soluble sugar estimation

Total soluble sugar was estimated as described by Richard *et al.*, (1998). For estimation of sugars, 0.05 mg sample was crushed with 80% ethanol in glass vials and preserved. For extraction of sugars the supernatant (alcohol in which the leaf material was stored) was decanted into a beaker. The extraction was repeated three to four times by boiling the sample with 80% (v/v) ethanol in water bath each time and centrifuge at 4000 rpm for 10 minutes, supernatant volume was made up to 10 ml with water. Soluble sugar was estimated in 0.1ml extract by adding 0.5 ml phenol and 2.5 ml H_2SO_4 followed by 5 min shaking and reaction mixture was cooled for 20 min and absorbance recorded at 490 nm. Soluble sugar was calculated using standard curve of Dextrose.

Protein estimation

Total soluble protein content in plant samples was determined according to the method of Lowry *et al.*, (1975), with bovine serum albumin as a calibration standard.

Statistical analysis

The design of the experiment was completely randomised design. Average mean values for at least three independent assays with three replicates each were recorded for each experiment. The data was subjected to ANOVA for completely randomized design. Differences at P<0.01 were considered statistically significant.

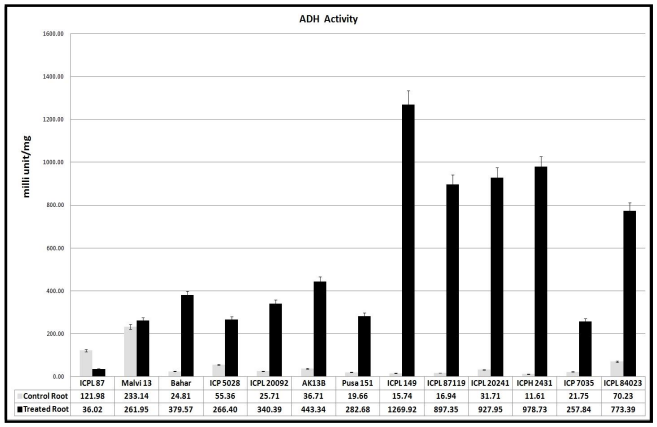

Fig. 1: Estimation of ADH Activity in pigeonpea genotypes (each bar represents Mean ± SD value).

Table 1: Plant survival percent during waterlogging treatment and recovery.

Genotype	2WL	4WL	6WL	5W	10W
ICPL 87	100.00	86.67	31.67	8.33	5.00
Malvi-13	100.00	100.00	86.67	60.00	60.00
Bahar	100.00	100.00	58.33	51.67	51.67
ICP 5028	100.00	100.00	56.67	48.33	48.33
ICPL 20092	100.00	100.00	90.00	83.33	83.33
AK13B	100.00	100.00	88.33	76.67	76.67
Pusa 151	100.00	100.00	35.00	50.00	50.00
ICPL 149	100.00	100.00	75.00	66.67	66.67
ICPL 87119	100.00	100.00	85.00	75.00	70.00
ICPL 20241	100.00	100.00	93.33	90.00	90.00
ICPH 2431	100.00	100.00	76.67	71.67	71.67
ICP 7035	100.00	85.00	25.00	11.67	11.67
ICPL 84023	100.00	100.00	80.00	75.00	75.00
Grouped mean	**100.00**	**97.82**	**67.82**	**59.10**	**58.85**
SEM±	**0.00**	**1.85**	**4.41**	**6.08**	**6.11**
CV%	**0.00**	**3.27**	**11.26**	**17.82**	**18.00**
CD at 5%	**0.00**	**5.37**	**12.82**	**17.67**	**17.78**

2WL: After 2 days of WL; 4WL: After 4 days of WL; 6WL: After 6 days of WL; 5W: After 5 days of water withdrawal; 10W: After 10 days of water withdrawal

Result and Discussion

Survival Percentage

Pigeonpea is a waterlogging sensitive crop. Pigeonpea genotypes suffered around 95-100% mortality during waterlogging stress and recovery period (Kumutha *et al.*, 2009). Due to waterlogging there is limited oxygen concentration around the root zone leading to restricted generation of ATPs per molecule of glucose as energy metabolism shift from aerobic to anaerobic mode under hypoxia or anoxia. A high level of anaerobic metabolism is therefore very important to supply the energy charge under hypoxic condition. Plants with adequate levels of readily metabolizable (fermentable) sugars in hypoxic or anoxic roots to sustain metabolism in roots can survive in oxygen deficient environment (Sairam *et al.*, 2009b; Irfan *et al.*, 2010; Singla and Inubushi, 2013).

In present investigation a significant reduction in survival of plants was recorded among all the genotypes except ICPL 20241 and ICPL 20092 (Table 1) during six days of waterlogging treatment and after 5 and 10 days of recovery. Survival percentage was least in ICPL 87 followed by ICP 7035. Thus genotypes, ICPL 20241 and ICPL 20092 showed highest level of tolerance whereas genotypes ICPL 87 and ICP 7035 showed susceptibility towards waterlogging stress.

Chlorophyll content

Waterlogging leads to destruction of chlorophyll which

Table 2: Chlorophyll content.

Genotypes	Control			After 2 days of WL			After 4 days of WL			After 6 days of WL		
	Chl a	Chl b b	Total Chl.	Chl a	Chl b b	Total Chl.	Chl a	Chl b b	Total Chl.	Chl a	Chl b b	Total Chl.
ICPL 87	**1.89**	**0.46**	**2.35**	**1.95**	**0.53**	**2.48**	**1.75**	**0.30**	**2.06**	**0.87**	**0.27**	**1.14**
Malvi 13	1.95	0.41	2.35	2.21	0.52	2.74	1.79	0.16	1.94	1.34	0.34	1.68
Bahar	2.31	0.78	3.09	2.23	0.65	2.89	1.98	0.42	2.41	1.38	0.41	1.79
ICP 5028	1.94	0.46	2.40	2.07	0.43	2.49	1.55	0.57	2.12	1.12	0.36	1.47
ICPL 20092	**1.83**	**0.53**	**2.35**	**2.02**	**0.46**	**2.47**	**1.64**	**0.56**	**2.10**	**1.56**	**0.46**	**2.02**
A K13B	2.04	0.43	2.47	2.12	0.37	2.49	1.85	0.35	2.20	1.45	0.43	1.89
Pusa 151	3.79	0.96	4.76	3.39	0.85	4.24	3.63	0.72	4.35	1.39	0.31	1.70
ICPL 149	3.05	0.62	3.67	2.55	0.76	3.31	1.75	0.52	2.27	1.94	0.62	2.57
ICPL 87119	3.29	0.66	3.96	2.74	0.85	3.59	2.20	0.60	2.80	1.27	0.44	1.71
ICPL 20241	**3.34**	**0.73**	**4.07**	**2.05**	**0.66**	**2.71**	**2.20**	**0.62**	**2.82**	**2.06**	**0.61**	**2.67**
ICPH 2431	3.58	0.79	4.37	2.41	0.77	3.18	0.97	0.29	1.26	2.84	1.09	3.93
ICP 7035	**4.04**	**0.87**	**4.91**	**3.37**	**0.82**	**4.19**	**1.87**	**0.56**	**2.43**	**1.82**	**0.55**	**2.37**
ICPL 84023	4.39	0.78	5.17	2.94	0.84	3.78	3.27	0.62	3.89	3.15	0.56	3.67
Grouped mean	2.88	0.65	3.53	2.46	0.67	3.13	1.94	0.47	2.40	1.73	0.53	2.24
SEM±	0.08	0.05	0.08	0.11	0.04	0.11	0.09	0.04	0.11	0.08	0.03	0.14
CV%	4.95	12.10	3.93	7.49	10.87	5.87	8.18	13.53	7.83	7.63	11.07	11.00
CD at 5%	0.24	0.13	0.23	0.31	0.12	0.31	0.27	0.11	0.32	0.22	0.10	0.41

affects the photosynthesis capacity of plant under waterlogged conditions (Ashraf *et al.*, 2011). With the advancement of waterlogging treatment duration the chlorophyll concentration was found to be decreased among the pigeonpea genotypes (Bansal and Srivastava, 2015; Sairam *et al.*, 2019a). In the present investigation decrease in chlorophyll content was observed after 4 days of waterlogging treatment and got further decreased after 6 days in all the genotype (Table 2). The decrease in the chlorophyll content is maximum in susceptible genotype (ICPL 87) and least in tolerant genotype (ICPL 20092). Less destruction of chlorophyll is associated with better survival of pigeonpea genotypes under waterlogging and thus can be considered as important trait for waterlogging tolerance study.

ADH Activity

Under anaerobic condition alcohol dehydrogenase (ADH) converts acetaldehyde to ethanol along with regeneration of NAD^+, which is foremost requirement of glycolytic pathway's continuation under oxygen deprivation. In present study ADH activity showed a significant increase under waterlogging condition among the genotypes except ICPL 87 (Fig. 1). Maximum ADH activity under control was observed in Malvi13 (233.14) and under waterlogged condition in ICPL 149 (1269.92). Maximum increase in ADH activity was observed among the tolerant genotypes ICPL 149, ICPL 20241, ICPL 87119, ICPH2431 and ICPL 84023. Similar increase in ADH activity in pigeonpea roots under waterlogged condition has been reported by Kumutha *et al.*, (2008) and Bansal and Srivastava (2015). ADH plays a very important role in detoxification of alcohol and facilitating glycolytic pathway which is the source of ATP under anaerobic condition of the treated root. Thus, it can be concluded that activation of ethanolic fermentation pathway is adaptive mechanism by which plants thrive in anaerobic environment.

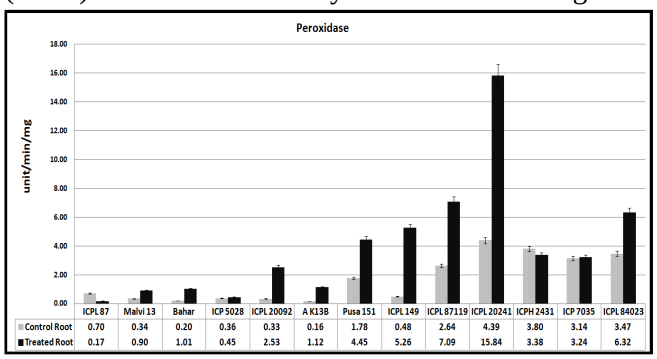

Fig. 2: Estimation of peroxidase activity in pigeonpea genotypes (each bar represents Mean ± SD value).

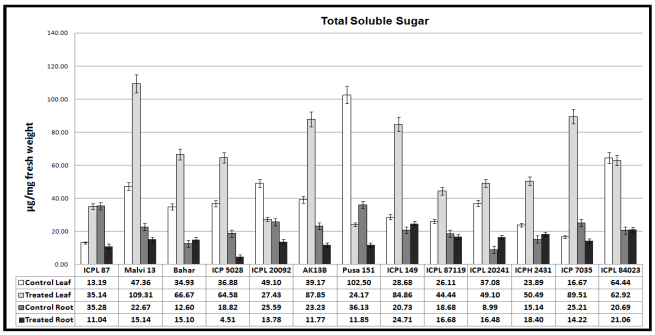

Fig. 3: Estimation of Total soluble sugar content in pigeonpea genotypes (each bar represents Mean ± SD value).

Peroxidase Activity

During hypoxia/anoxia, redox potential of cell got altered leading to the production of reactive oxygen species (ROS), which includes free radicals (O^{2-}, OH.), H_2O_2 and phenols. Plant possess antioxidant enzyme (peroxidase, catalase, superoxide dismutase, ascorbate peroxidase) and non-enzymatic antioxidants (ascorbate, glutathione etc) to scavenge the ROS (Singh *et al.*, 2017). H_2O_2 is the most stable ROS causing damage to the cell membrane. Peroxidases are major enzymatic scavenger of H_2O_2. Antioxidant response acts as an indicator of water logging tolerance or sensitivity. In the present investigation peroxidase activity increased in response to waterlogging stress in the root of all pigeon pea genotypes except ICPL 87 and ICPH 2431 (Fig. 2). Peroxidase activity was highest in ICPL-20241 among the studied genotypes. Fold change in peroxidase activity is more among tolerant genotypes ICPL 20092 (7.66), ICPL 20241 (3.6) and ICPL 84023 (1.821) as compared to susceptible genotypes ICP7035 (1.03) and ICPL87 (0.24). Our results are consistent with the other studies where antioxidant enzymes such as superoxide dismutase and peroxidase increased in pigeon pea roots at the seedling stage, when stress was imposed for 6 days (Bansal and Srivastava, 2012; Singh *et al.*, 2017). Increase in the peroxidase activity led to detoxification of H_2O_2 during water logging leading to water logging tolerance in the tolerant genotypes.

Total soluble sugar content

Waterlogging induced hypoxia/anoxia leads to limited generation of ATPs per molecule of glucose since energy metabolism shift from aerobic to anaerobic mode. A high level of anaerobic metabolism is therefore very important to supply the energy under hypoxic condition. Plants with adequate levels of readily available sugars to sustain metabolism in roots can survive in oxygen deficient environment (Irfan *et al.*, 2010; Singla and Inubushi, 2013). The amount of root sugar reserve determines the level of waterlogging tolerance. In present investigation increase in total soluble sugar was observed in leaf of almost all genotypes except genotype ICPL 20092, Pusa 151 and ICPL 84023. In root tissue decrease in total soluble sugar was observed in eight genotypes (Fig. 3). The decline in total soluble sugar content was less in tolerant genotypes and higher in sensitive genotype. Increase in total soluble sugar content was observed in root of highly tolerant genotypes ICPL 20241 and moderately tolerant genotype and ICPL 84023, ICPL 149 and ICPH 2431. Increased total sugar content and less decrease in soluble sugar content in the roots may be an adaptive mechanism for waterlogging stress tolerance. Genotypes, which could sustain adequate supply of readily usable sugars, have a better chance of survival than the ones, which are lacking on this count. Similar observations were reported by Kumutha *et al.*, (2008) in green gram roots, greater concentration of total soluble sugars under waterlogging stress suggesting a carbohydrate based tolerance mechanism.

Conclusion

In the present study, tolerant and susceptible genotypes were identified based on their survival under waterlogging stress condition. Tolerant genotypes exhibited higher chlorophyll retention than susceptible genotypes. Peroxidase and alcoholic dehydrogenase activity increased significantly as a consequence of waterlogged conditions, and were higher in tolerant than susceptible genotypes. Increase in total soluble sugars content was observed in response to water logging in root of tolerant genotypes whereas decline in total soluble sugar content was observed in susceptible genotype. Findings of present investigation establish chlorophyll and total soluble sugars retention along with increase in alcoholic dehydrogenase and peroxidase activity as vital selection criteria for identification and breeding of waterlogging-tolerant pigeonpea varieties.

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