

Photosynthetic, physiological and biochemical events associated with polyethylene glycol-mediated osmotic stress tolerance in taro (*Colocasia esculenta* L. Schott)

M.R. SAHOO^{*,**,#+}, M. DASGUPTA^{*,**,#}, P.C. KOLE^{***}, and A. MUKHERJEE^{****}

ICAR Research Complex for NEH Region, Lamphelpat, Imphal, Manipur, 795004, India*

University of Tennessee, Knoxville, TN, 37996, USA**

Department of Crop Improvement, Horticulture and Agricultural Botany, Institute of Agriculture, Visva Bharati, Sriniketan, West Bengal, 731236, India***

Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar, Odisha, 751019, India****

Abstract

Six genotypes of taro (*Colocasia esculenta* L. Schott) were evaluated under *in vitro* and *in vivo* polyethylene glycol (PEG-6000)-mediated osmotic stress conditions. A significant variation in growth response was observed among the taro genotypes under *in vitro*-induced stress conditions. *In vivo* results indicated a significant effect of osmotic stress on photosynthetic parameters, such as net photosynthetic rate, transpiration rate, stomatal conductance, stomatal resistance, internal CO₂ concentration, carboxylation efficiency, and transpiration efficiency on the tested genotypes at the tuberization stage. Lesser variations in photosynthesis and higher accumulation of proline, phenols, and antioxidative enzymes, namely, superoxide dismutase and guaiacol peroxidase, were associated with yield maintenance under osmotic stress conditions. The genotypes DP-89, IGCOL-4, and Ramhipur showed a higher degree of tolerance towards osmotic stress with a minimum variation in the studied parameters. These genotypes could be lines of interest for intensification of breeding strategies to develop drought-tolerant plants.

Additional key words: antioxidative enzymes; osmotic stress; photosynthesis; physiology; polyethylene glycol; taro.

Introduction

Osmotic stress triggers a broad range of plant responses, which affect many physiological and biochemical events (Flexas *et al.* 2002), cellular and molecular processes (Muscolo *et al.* 2015) and leads to a yield reduction (Ramírez *et al.* 2015). To mitigate the adverse effects of osmotic stress, plants undergo various changes involving a mixture of stress tolerance and avoidance mechanisms (Yue *et al.* 2006). The tolerance strategies involve immediate physiological and biochemical responses (Sourour *et al.* 2017), whereas the avoidance mechanisms are associated with the morphological adaptations (Blum 2005). A better understanding of the tolerance behavior at

physiological and biochemical level (Li and Liu 2016) leads to identification of genotypes which could well adapt to water deficit and maintain growth and development during stress period (Chandra *et al.* 2004). An earlier exposure to stress at tissue or cellular level enhances plant's tolerance to later abiotic insult (Chen and Arora 2013) which is considered as acclimatization (Chen *et al.* 2012) or plant priming (Li *et al.* 2015). Hence, *in vitro* stress induction studies offer a meaningful tool for understanding stress tolerance in tuber crops (Dasgupta *et al.* 2008). However, rigorous screening and evaluation under *in vivo*-induced stress conditions and understanding

Received 3 June 2017, accepted 19 October 2017, published as online-first 13 April 2018.

*Corresponding author; e-mail: manas.sahoo@icar.gov.in

Abbreviations: BA – 6-benzyladenine; C_i – intercellular CO₂ concentration; CE – carboxylation efficiency; Chl – chlorophyll; CSI – chlorophyll stability index; E – transpiration rate; EDTA – ethylene diamine tetraacetic acid; GA₃ – gibberellic acid; GPX – guaiacol peroxidase; g_s – stomatal conductance; NAA – α-naphthalene acetic acid; NBT – nitroblue tetrazolium; PAGE – polyacrylamide gel electrophoresis; P_N – photosynthetic rate; ROS – reactive oxygen species; RS – reducing sugar; R_s – stomatal resistance; RWC – relative water content; SOD – superoxide dismutase; TE – transpiration efficiency; TSS – total soluble sugar.

Acknowledgements: The work was supported by Indian Council of Agricultural Research, New Delhi, India. The authors are also thankful to the Director and Head, Central Tuber Crops Research Institute, Regional Centre, Bhubaneswar, India for infrastructure facilities.

#These authors contributed equally to this work.

induced-tolerance mechanisms confer long-term adaptation to harsh environments.

Taro [*Colocasia esculenta* (L.) Schott] is one of the important food crops in the developing world and considered to be a climate resilient crop. Its edible corms are a rich source of carbohydrates and minerals, adding immense commercial value to this crop. Production of taro often threatened by water scarcity leads to 90% of yield loss to total crop failure (Ravi and Chowdhury 1997). The major factor limiting taro growth is the amount of soil moisture available to the crop during the growing season. The unavailability of water at a tuberization stage results in a tuber yield reduction (Sunitha *et al.* 2013) as a cumulative effect of reduced leaf area, dry matter production, and some physiological parameters (Ravi and Chowdhury 1997, Mabhaudhi *et al.* 2013). An understanding of the genetic architecture of drought tolerance components would be an important milestone for breeding advancement (Obidiegwu *et al.* 2015).

Elevated leaf area, chlorophyll (Chl) content, relative water content (RWC), net photosynthetic rate, and subsequently an increase in proline concentrations are key indices for osmotic stress tolerance (Luo *et al.* 2016). Osmotic stress also triggers rapid accumulation of reactive

oxygen species (ROS), which could be toxic to plants (Wei *et al.* 2015). Consequently, plants deploy a ROS-scavenging system comprising of enzymatic and nonenzymatic antioxidants (Gill and Tuteja 2010). Information on photosynthetic changes, biochemical events and role of antioxidative enzymes in ROS scavenging is scanty for this nutrition-rich crop under polyethylene glycol (PEG)-mediated osmotic stress. Thus, there is a need to understand the physiological changes, photosynthetic and biochemical regulations, and ROS defense machineries in taro under induced stress conditions in order to develop a stress-tolerant line.

Keeping this in view, we studied the mechanisms adopted by six taro genotypes under *in vitro* and *in vivo* PEG-mediated osmotic stress conditions by investigating their morphological traits in terms of leaf area and physiological attributes [RWC, Chl stability index (CSI), and photosynthetic parameters]. We also reported the PEG-induced biochemical changes in taro leaves and the role of antioxidative enzymes, such as superoxide dismutase (SOD) and guaiacol peroxidase (GPX), and antioxidants, *i.e.*, phenol content, in PEG-induced ROS-scavenging mechanisms.

Materials and methods

Study site and plant materials: Six genotypes of taro comprising of three tolerant (DP-89, IGCOL-4, and Ramhipur), two moderately tolerant (DP-25 and Jhankri), and one susceptible (Hunger Local) were selected from a wide genetic base of 172 genotypes showing various degree of tolerance to osmotic stress (Sahoo *et al.* 2006a). This study was undertaken at the Regional Centre of Central Tuber Crops Research Institute (RCCTCRI), Bhubaneswar (20° 15' N latitude, 85° 52' E longitude, and altitude of 26 m a. s. l.), India. The climatic condition of the place is warm and moist, with hot summer and mild winter.

***In vitro* culture medium, culture conditions and observations:** Selected genotypes were raised *in vitro* through cormel tip cultures under MS (Murashige and Skoog 1962) media supplemented with plant growth regulators, *i.e.*, α -naphthalene acetic acid (NAA, 2.7 μ M), 6-benzyladenine (BA, 4.4 μ M), and gibberellic acid (GA₃, 1.45 μ M) (Dasgupta *et al.* 2008). The MS media with above mentioned growth regulators and 3% sucrose were used as the control. In order to induce osmotic stress (-0.2 MPa), the same media were supplemented with PEG-6000 (118.0 g L⁻¹) prior to pH adjustment (5.7 \pm 1) and dissolved in 0.8% Difco-Bacto agar (Hi-Media, India). The medium was sterilized at 105 kPa for 15 min in a steam autoclave (REMI, India). Cormel tips (5–10 mm) of the six selected taro genotypes were surface-sterilized with 0.1% sodium hypochlorite (Merck, India) solution for 5–7 min, followed by thorough rinsing in sterile distilled water thrice and inoculated in the test tubes (25 \times 100 mm)

containing MS medium with or without PEG-6000. The cultures were maintained at 25 \pm 2°C with 16/8-h light/dark cycle and 45 μ mol(photon) m⁻² s⁻¹ illumination provided by cool/white fluorescence tubes (Phillips, India) with 55–60% relative humidity for six weeks. Each treatment included six replicates of each genotype and the experiment was carried out in 6 \times 2 factors completely randomized design (CRD). *In vitro* growth responses, such as days to sprout, number of leaves, shoots and roots, the length of shoots and roots, were recorded after six weeks of growth.

***In vivo* experimental layout and observations:** Six-week-old plantlets were acclimatized and maintained in the earthen pots (30 cm in diameter) containing garden soil, sand, and farm yard manure (1:1:1) under greenhouse [16-h photoperiod, 350 μ mol(photon) m⁻² s⁻¹ light intensity, and temperature of 25/20°C day/night]. The pots were watered with PEG-6000 solutions (0 and -0.2 MPa) at 2-week interval to maintain the stress. This experiment was designed with the same six genotypes and two treatments of PEG stress, replicated thrice under 6 \times 2 factorial CRD. Morphological, physiological, and biochemical parameters associated with stress tolerance were investigated at the crucial period of tuberization (45 d after planting).

Leaf area: The leaf area was calculated according to the linear measurement method as described by Biradar *et al.* (1978). The length and breadth of the leaf was multiplied by 0.917 to calculate the leaf area (leaf area = length \times breadth \times 0.917).

Relative water content (RWC) of the leaf was measured as an indicator of a leaf hydration status according to Perez *et al.* (2002). Fresh leaves were collected randomly from each replication and weighed (FM) immediately followed by submerging in distilled water for 4 h at 22°C. The turgid mass (TM) was measured after blotting the submerged leaves and the dry mass (DM) was determined after the samples were dried for 72 h at 70°C in a hot air oven (Remi, India). The RWC was calculated as $RWC = [(FM - DM)/(TM - DM)] \times 100$.

Chl stability index (CSI) is based on pigment changes induced by heating. The leaf samples were subjected to heat treatment by immersing in distilled water bath at 56°C for 30 min. The Chl content in treated and control samples kept at room temperature was determined using a soil plant analytical development (SPAD-502) portable leaf chlorophyll meter (Minolta Corp., Romsey, NJ). Three SPAD readings were taken on each of the leaves to obtain the average Chl content under each treatment and replications. CSI was calculated by the formula as described by Mohan *et al.* (2000), *i.e.*, $CSI = [(Chl \text{ content of treated samples}/Chl \text{ content of control samples}) \times 100]$.

Photosynthetic measurements: All gas-exchange measurements, such as the net carbon assimilation rate/ photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), stomatal resistance (R_s), intercellular CO_2 concentration (C_i), carboxylation efficiency (CE, P_N/C_i), and transpiration efficiency (TE, P_N/E), were determined between 11:00–13:00 h on the first fully expanded leaf using a portable open-system infrared gas analyzer (LCA-4, Analytical Development Co. Ltd., UK). The measurement light was maintained at about 800 $\mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ at controlled CO_2 concentration of $350 \pm 5 \text{ cm}^3 \text{ m}^{-3}$. The chamber temperature ranged between $25 \pm 2^\circ\text{C}$ during the day time. Readings were logged until a stable reading was reached and each parameter was calculated from the average of three readings.

Biochemical estimations: The total leaf soluble protein content was measured by the protein dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as standard. Total soluble sugar (TSS) and reducing sugar (RS) were quantified following the anthrone method as described by Roe (1955) and Nelson (1944), respectively, using glucose as standard. Proline content was estimated from the leaf extracts by following the standard protocol of Bates *et al.* (1973) and L-proline as standard.

Antioxidative enzyme assays, preparation of enzyme extracts and assay conditions: For the preparation of antioxidative enzyme extracts, the newly emerged fully expanded leaves (fresh mass of 250 mg) of taro genotypes were ground in liquid nitrogen to fine powder and were homogenized with 50 mM $NaPO_4$ buffer (pH 7.8) containing 1 mM EDTA, 0.1% Triton X-100, 1 mM

ascorbate, and 10% sorbitol. The homogenate was centrifuged at 15,000 rpm at 4°C for 20 min and the supernatant was used for the following enzyme assays. The total soluble protein concentrations were also determined by the spectrophotometric method of Bradford using BSA as the standard (Bradford 1976).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined following Giannopolitis and Ries (1977), which is based on the ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT). The reaction mixture (4.0 ml) comprised of 50 mM phosphate buffer (pH 7.8), 0.1 μM EDTA, 13 mM methionine, 75 mM NBT, 2 mM riboflavin, and 0.2 μl of enzyme extract. Riboflavin was added last and tubes were shaken and illuminated with two 20-W fluorescent tubes. The reaction was allowed to proceed for 15 min after which the illuminating tubes were switched off and the tubes were covered with a black cloth. The absorbance of the reaction mixture was taken at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required for 50% inhibition of the NBT photoreduction rate and the results were expressed as unit per milligram of protein.

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was quantified as described by Urbanek *et al.* (1991) in a reaction mixture (2.0 ml) containing 100 mM phosphate buffer (pH 7.0), 0.1 μM EDTA, 5.0 mM guaiacol, 15.0 mM H_2O_2 , and 50 μl of enzyme extract. The increase in absorbance was recorded at an interval of 30 s for 3 min at 470 nm. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient ($26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). All the spectrophotometric assays were performed using a UV-Vis spectrophotometer (Thermo Fischer Scientific, USA) at room temperature as described by Dasgupta *et al.* (2008).

The isoform profiles of SOD and GPX were examined by discontinuous native polyacrylamide gel electrophoresis (native PAGE, Laemmli 1970). Native PAGE was carried out by using 7.5% separating gel containing 1.5 M Tris buffer (pH 8.8) and 4.5% stacking gel containing 0.5 M Tris buffer (pH 6.8). An equal amount of protein (60 μg) from each extract was loaded along with sample loading dye (Tris, pH 6.8, 0.1% bromophenol blue, and 15% glycerol) in the wells. The electrophoresis was carried out in the presence of Tris-glycine buffer (25 mM Tris, pH 8.3, and 192 mM glycine) at 20 mA constant current until the blue dye reached the bottom of the gel. Immediately after electrophoresis, staining activity was carried out by incubating the gel in substrate solution as described by Sahoo *et al.* (2007).

For the assay of phenol content, leaf samples of 0.5 g per replication per treatment were homogenized with 75% ethanol, shaken for 15 min at 70°C, and the extract was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge (REMI, India). The supernatant was collected and used for the estimation of phenolics using Folin-Ciocalteu's method (Zieslin and Ben-Zaken 1993). A standard curve was generated with different concentrations

of catechol (*Hi Media*, India) and was used to quantify the phenolics, which was expressed in milligrams of catechol produced per gram of leaf fresh mass.

Corm yield: Taro corms were harvested at 120 d after planting. The plant debris was removed from the corms and weighed to obtain the corm yield (g per plant). The pattern of stress tolerance was estimated based on the variations in the studied parameters under moisture stress as compared with control.

Statistical analysis: The experiments were conducted in

Results

Effect of PEG-mediated osmotic stress on growth responses of taro genotypes *in vitro*: The *ANOVA* (mean sum of square, MSS) for the growth parameters *in vitro* indicated a significant effect of PEG treatment on the tested genotypes (Table 1). The result revealed that the days to sprout decreased significantly under *in vitro* PEG-mediated osmotic stress as compared with control (Fig. 1A). Days to sprout were delayed under PEG (14.3 d) as compared to stress-free control (5.4 d). Under osmotic stress, Ramhipur exhibited early sprouting (11.7 d) followed by Jhankri (12.2 d), and IGCOL-4 (12.7 d) (Fig. 1A).

PEG-mediated osmotic stress had a significant detrimental effect on all the studied growth parameters, such as number of leaves, shoots and roots, length of shoots and roots. However, the significant genotypic variation was observed in all the parameters except of the number of leaves and roots (Table 1). The mean number of leaves varied from 2.0 to 2.3 in tested genotypes; and it was reduced to 1.0–2.0 under induced osmotic stress (Fig. 1B). Shoot emergence was also affected at -0.2 MPa stress; the mean number of shoots (Fig. 1C) and roots (Fig. 1D) per explant was less than 2 in all studied genotypes. After six weeks of cultivation, control taro plantlets attained the height of 4.5–6.25 cm; it was reduced significantly to 3.10–5.02 cm under osmotic stress media (Fig. 1E).

6×2 factorial CRD and the data were analysed statistically following the analysis of variance (*ANOVA*); significance was tested at probability level $P \leq 0.05$ and $P \leq 0.01$. Graphical representations are means of three replications with duplicate determinations. For the assay of antioxidant enzymes, repetition was performed twice within each replication. *Tukey's* test was performed to compare significant differences between the mean values (Tukey 1949). Yield reduction was correlated with the variations in photosynthesis, physiological, and biochemical properties under stress using *Pearson's* correlation coefficient (r values) at significance level $P \leq 0.05$ and $P \leq 0.01$.

The average root length also significantly decreased among the studied genotypes (Fig. 1F). The rate of decrease in length of the shoots and roots was lower in DP-25, IGCOL-4, Ramhipur, Jhankri, and DP-89, and higher in Hunger Local.

Effect of PEG on morphological and physiological properties of taro genotypes *in vivo*: The mean sum of squares (MSS) and their significance according to the two-way *ANOVA* for six taro genotypes under *in vivo* PEG-mediated osmotic stress are summarized in Table 2. The *ANOVA* for the studied parameters indicated significant effects of the genotypes, PEG stress, and genotypes \times PEG stress interaction for all the studied parameters with an exception of transpiration rate (E) and stomatal resistance (R_s). The result of the effect of PEG-6000-mediated osmotic stress on morphological and physiological properties of taro plants are represented in Fig. 2.

The leaf area ranged from 127.62 to 235.0 cm² among the genotypes under controlled conditions, which was significantly reduced to 125.02 to 217.0 cm² under stress (Fig. 2A). Although, IGCOL-4 had attained lower leaf area under both control and stress conditions, the rate of decrease in leaf area was lower in IGCOL-4 and higher in Hunger Local (Fig. 2A). RWC under control was ranged

Table 1. Two-way analysis of variance (mean sum of squares) for growth parameters of taro genotypes under *in vitro* PEG-mediated osmotic stress conditions in a 6×2 factorial experiment in a complete randomised design (CRD). *, ** and ^{NS} – $P < 0.05$, $P < 0.01$, and $P > 0.05$, respectively.

Source	<i>df</i>	Time to sprout [d]	No. of leaves	No. of shoots	No. of roots	Shoot length	Root length
Genotype	5	26.91**	0.78 ^{NS}	1.22**	0.36 ^{NS}	6.26**	0.20**
PEG	1	1395.68**	3.13*	2.00*	1.68**	38.28**	22.67**
Genotype \times PEG	5	15.25**	0.29 ^{NS}	0.10 ^{NS}	0.18 ^{NS}	2.49*	0.18**
Error	60	4.28	0.51	0.41	0.21	0.96	0.04

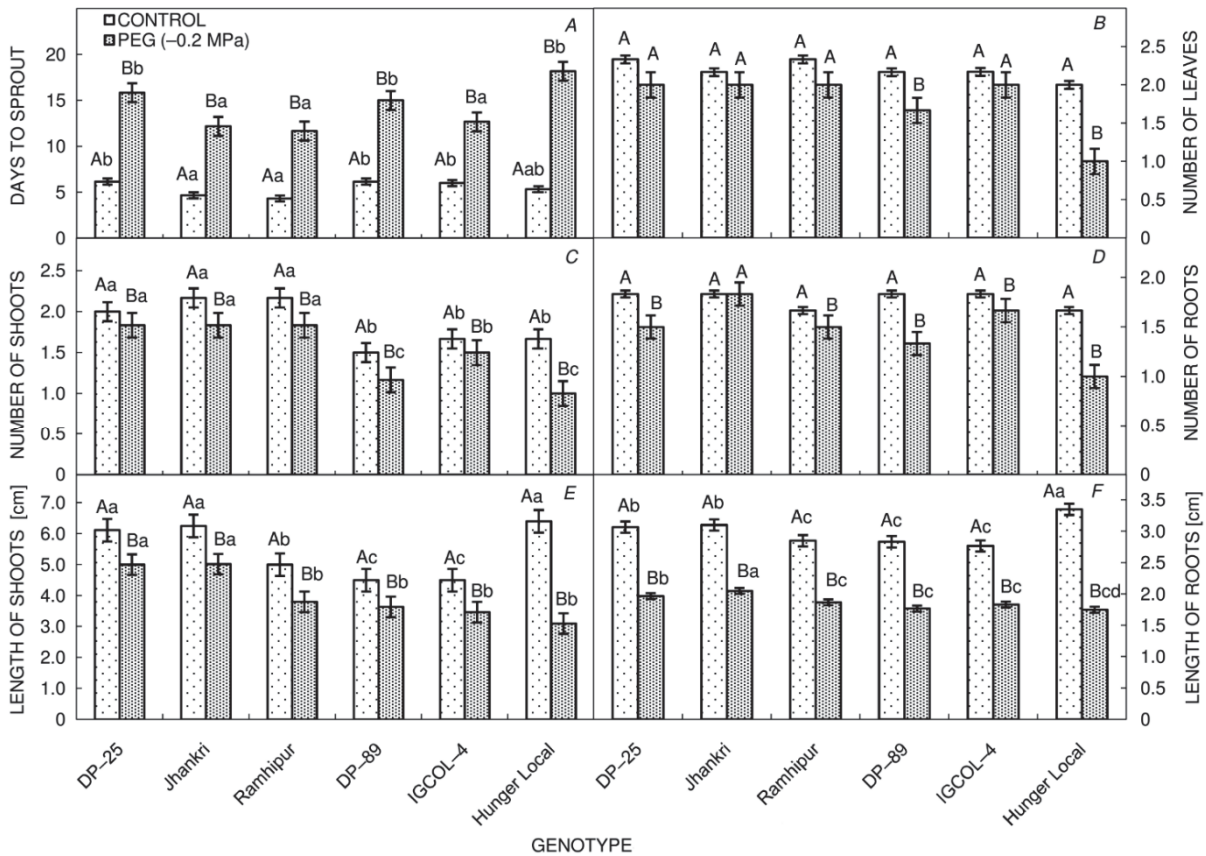


Fig. 1. Effect of *in vitro* PEG-mediated osmotic stress on a growth response of taro genotypes. *A* – days to sprout; *B* – number of leaves; *C* – number of shoots; *D* – number of roots; *E* – length of shoots, and *F* – length of roots. Values are the mean of six replicates and bars represent standard error of means. Different letters in uppercase represent significant differences between the treatments (control and -0.2 MPa) in the genotypes and lowercase represents significant difference between the genotypes under each treatment according to Tukey's test.

Table 2. Two-way analysis of variance (mean sum of squares) for morpho-physiological and biochemical properties of taro genotypes under *in vivo* PEG-mediated osmotic stress conditions in a 6×2 factorial experiment in a complete randomised design (CRD). *, ** and ^{NS} – $P \leq 0.05$, $P \leq 0.01$, and $P > 0.05$, respectively.

Source	Genotype	PEG	Genotype \times PEG	Error
<i>Df</i>	5	1	5	24
Leaf area	7792.27**	2414.55**	410.26**	84.87
Relative water content (RWC)	65.81**	657.60**	85.39**	5.10
Chlorophyll stability index (CSI)	89.22**	873.41**	57.14**	11.67
Photosynthetic rate (P_N)	10.34**	71.01**	5.00*	1.50
Transpiration rate (<i>E</i>)	1.88**	0.91*	0.03 ^{NS}	0.20
Stomatal conductance (g_s)	400.08**	929.03**	133.86**	27.28
Stomatal resistance (R_s)	0.0005**	0.0005*	0.0001 ^{NS}	0.0001
Intercellular CO ₂ concentration (C_i)	12075.86**	27914.61**	398.97*	149.99
Carboxylation efficiency (CE)	0.00037**	0.00038**	0.00012*	0.00004
Transpiration efficiency (TE)	12.82**	12.63**	5.73*	1.61
Total soluble protein content	1012.38**	468.36**	38.45**	1.36
Proline content	165.05**	354.90**	21.87**	2.12
Total soluble sugar (TSS)	59.85**	108.64**	22.44**	1.00
Reducing sugar (RS)	16.74**	22.58**	2.64**	0.32
Superoxide dismutase (SOD)	3806.19**	52227.48**	993.73**	0.52
Guaiacol peroxidase (GPX)	0.00341**	0.05549**	0.00138**	0.00003
Phenol content	0.06**	1.09**	0.04**	0.01
Yield	322.75**	5236.93**	186.50**	43.61

between 96.1 (IGCOL-4) to 98.5% (Jhankri), while under the PEG stress, it was between 78.0 (Hunger Local) to 94.5% (DP-89) (Fig. 2B). Percent decrease in RWC was more pronounced in leaves of Hunger Local (19.2%) and it was the lowest in DP-89 (1.8%). Similarly, CSI in leaves under control varied from 92.29 (IGCOL-4) to 99.05

(DP-25); it was reduced to 72.16 (Hunger Local)–88.98 (Ramhipur) under stress (Fig. 2C). Higher reduction in CSI was recorded in Hunger Local (22.4%) as compared to other genotypes IGCOL-4, Ramhipur, DP-89, Jhankri, and DP-25, where the rate of decrease in CSI was in the range of 4.6–11.8%.

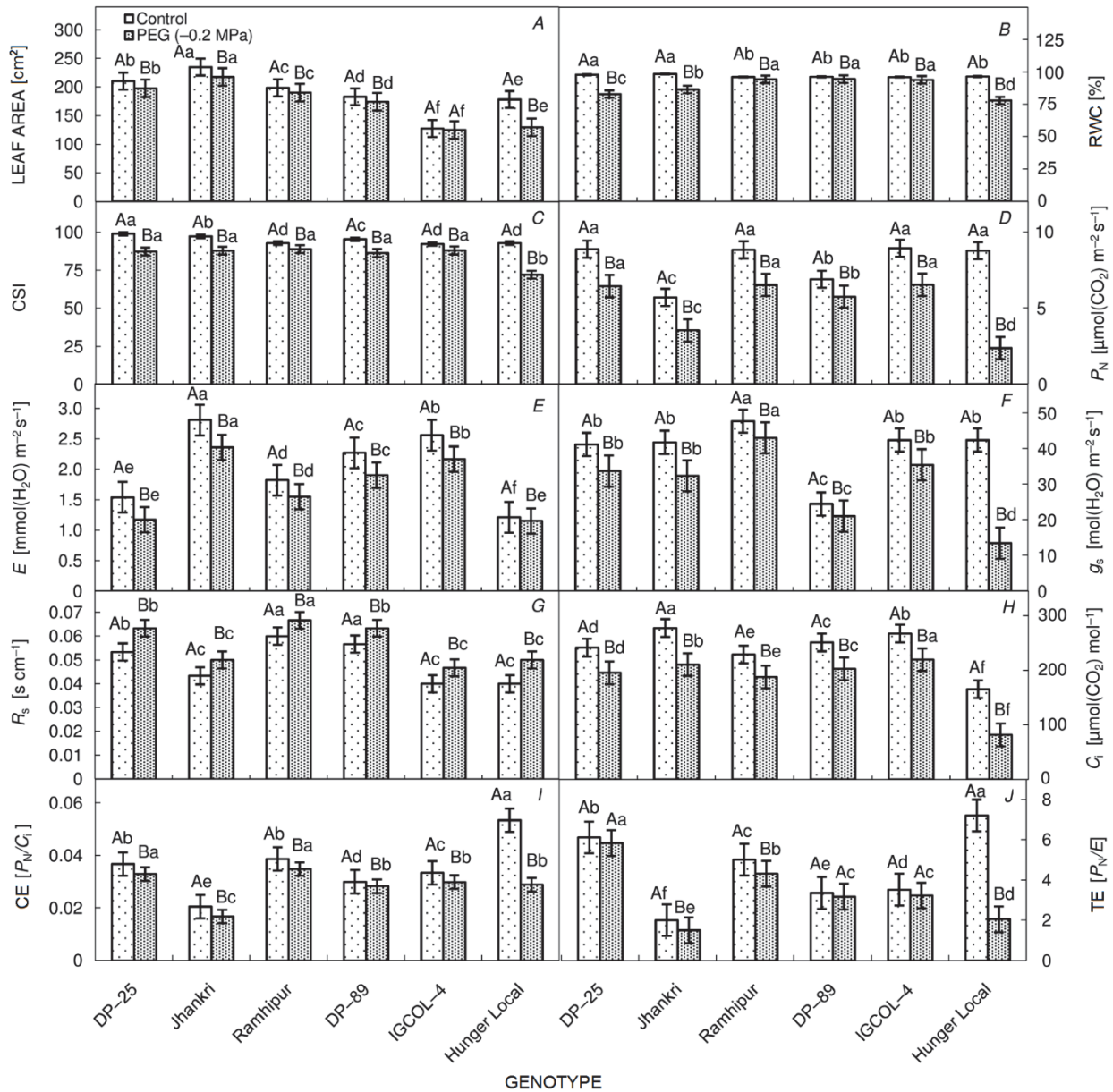


Fig. 2. Morphological and physiological properties of taro genotypes as influenced by PEG-mediated osmotic stress conditions. *A* – leaf area; *B* – relative water content (RWC); *C* – chlorophyll stability index (CSI); *D* – photosynthetic rate (P_N); *E* – transpiration rate (E); *F* – stomatal conductance (g_s); *G* – stomatal resistance (R_s); *H* – intercellular CO_2 concentration (C_i); *I* – carboxylation efficiency (CE, P_N/C_i); *J* – transpiration efficiency (TE, P_N/E). Values are the mean of three replicates and bars represent standard error of means. Different letters in uppercase represent significant differences between the treatments (control and -0.2 MPa) in the genotypes and lowercase represents significant difference between the genotypes under each treatment according to Tukey's test.

Effect of PEG on photosynthetic measurements of taro genotypes *in vivo*: The photosynthetic rate (P_N) significantly declined in all the studied genotypes under

moisture stress as compared with control (Fig. 2D). The extent of reduction in P_N under PEG stress in comparison to control was lower in DP-89 (16.6%), Ramhipur

(26.1%), IGCOL-4 (27.0%), DP-25 (27.4%), and Jhankri (38.2%), whereas, the same was more pronounced in Hunger Local (73.0%). The E was recorded as 1.21–2.81 mmol(H₂O) m⁻² s⁻¹ in control plants and reduced to 1.15–2.36 mmol(H₂O) m⁻² s⁻¹ under stress (Fig. 2E). Reduction in E was higher in DP-25 (23.8%) followed by DP-89 (16.2%), Jhankri (16.0%), IGCOL-4 (15.4%), Ramhipur (14.9%), and lower in Hunger Local (4.95%).

Similarly, PEG-mediated osmotic stress resulted in the reduction of stomatal conductance (g_s) in all six genotypes studied (Fig. 2F). However, percent reduction was more pronounced in Hunger Local (68.3%), whereas it was lesser in the genotypes Ramhipur (9.9%), DP-89 (14.0%), and IGCOL-4 (16.4%). The studied genotypes registered an increase in R_s under osmotic stress, while compared with control (Fig. 2G), the rate of increase in R_s was lower in Ramhipur (11.8%) and DP-89 (11.8%) and higher in Hunger Local (25.0%). A significant decrease in internal CO₂ concentration (C_i) was observed across the genotypes due to osmotic stress as compared with control (Fig. 2H). The rate of decrease was lower in resistant genotypes and higher in Hunger Local. CE and TE were adversely affected in the taro plants under PEG-mediated osmotic stress. Hunger Local showed a higher degree of reduction in the CE and TE (Fig. 2I,J).

Effect of PEG on biochemical properties of taro genotypes *in vivo*:

Changes in total soluble proteins, as influenced by osmotic stress, is presented in Fig. 3A. Total soluble protein content in leaf tissue of taro genotypes under stress-free control was 31.32 (Hunger Local) to 58.15 mg g⁻¹(FM) (DP-25), which decreased to 15.57 (Hunger Local)–50.13 mg g⁻¹(FM) (DP-25) under PEG-mediated osmotic stress (Fig. 3A). Percent reduction in protein content was remarkably higher in Hunger Local (50.3%) in comparison to the tolerant to moderately tolerant genotypes which ranged between 6.6 (DP-89)–16.8% (Jhankri).

On the contrary, the proline content increased under PEG stress in the genotypes investigated (Fig. 3B). The genotypes DP-89 and DP-25 accumulated more proline (70 and 77.8%, respectively) in their leaf tissue, whereas Hunger Local showed only 6.3% increase in the proline content under osmotic stress.

Total soluble sugar (TSS) and reducing sugar (RS) content in the leaf tissues of taro genotypes were reduced significantly under moisture stress. IGCOL-4 and DP-25 exhibited lower variations in TSS (Fig. 3C) and RS

(Fig. 3D) content, whereas Hunger Local showed higher reduction under moisture stress compared with control.

Effect of PEG on antioxidative enzyme activities of taro genotypes *in vivo*:

Leaf SOD activity was 69.77 (Hunger Local)–106.67 unit mg⁻¹(protein) (DP-89) under the stress-free conditions and 95.20 (Hunger Local)–202.27 unit mg⁻¹(protein) (DP-25) under osmotic stress (–0.2 MPa) [Fig. 3E]. The PEG-induced SOD activity was more conspicuous in IGCOL-4 (96.3%), DP-25 (93.7%), and Ramhipur (90.9%) than that in Hunger Local (36.5%). The isozyme profiles under PEG stress revealed that both the cathodal and anodal isoforms of SOD were visible in all the genotypes, however, the intensity of bands of DP-89 were more prominent compared to other genotypes (Fig. 3E). A similar trend was observed for GPX activity, which also increased significantly in all the tested genotypes under stress conditions (Fig. 3F). GPX under control condition was in the range of 0.10 (Jhankri and Hunger Local)–0.15 (IGCOL-4) unit mg⁻¹ (protein), while the activity was 0.15 (Hunger Local)–0.25 (DP-25) unit mg⁻¹(protein) under stress. Zymogram of GPX also exhibited a faint band, however, intensity of band was comparatively prominent in Ramhipur and DP-25 (Fig. 3F).

A significant accumulation of total phenols was observed in leaf tissues of taro genotypes under stress conditions (Fig. 3G). The increase in the phenol content was higher in DP-89 (43.1%) followed by Jhankri (35.7%), IGCOL-4 (32.8%), and DP-25 (26.5%). Hunger Local (9.0%) and Ramhipur (9.3%) showed the lower accumulation of phenols under moisture stress.

Yield:

Corm yield of taro genotypes decreased under moisture stress compared with control (Fig. 3H). The rate of decrease in corm yield was in the range of 15.0–16.2% in tolerant genotypes (IGCOL-4, Ramhipur, and DP-89), moderate (20.3% and 21.5%, in DP-25 and Jhankri, respectively), and higher in Hunger Local (41.4%). Yield reduction was significantly and strongly positively correlated with leaf area ($r = 0.994$), CSI ($r = 0.950$), P_N ($r = 0.968$), g_s ($r = 0.986$), C_i ($r = 0.987$), CE ($r = 0.978$), TE ($r = 0.960$), protein ($r = 0.984$), TSS ($r = 0.924$), RS ($r = 0.998$), and inversely correlated with SOD ($r = -0.919$) (Table 3). Based on the results of morphological and physiological changes, photosynthetic variations and biochemical properties, the pattern of osmotic stress tolerance in the tested genotypes of taro was in order of DP-89 > IGCOL-4 > Ramhipur > DP-25 > Jhankri > Hunger Local.

Discussion

We studied the growth responses, water relations, photosynthesis, accumulation of proline, and antioxidative systems in six genotypes of taro under *in vitro* and *in vivo* PEG-6000-mediated osmotic stress conditions. PEG of high molecular mass acts as a nonpenetrating osmotic

agent, lowering the water potential in the media, and thereby, it is widely used in osmotic-stress tolerance studies in plants (Albiski *et al.* 2012). The first and foremost detrimental effect of PEG in the shoot proliferation media was evident by delayed sprouting of the

cornel tip explants followed by gradual growth retardation in the tested genotypes. Under *in vitro* PEG stress, early bud break was observed in Ramhipur, followed by Jhankri and IGCOL-4. The shoot proliferation in term of a number of leaves, number of shoots, length of shoot and rooting was highly affected due to PEG as compared with the control. Under PEG-stress conditions, Hunger Local showed higher retardation up to 50% in shoot proliferation and rooting, which reflected its susceptibility towards PEG-mediated osmotic stress. IGCOL-4, DP-25, Jhankri, and Ramhipur showed an indication of tolerance towards PEG stress by maintaining the growth *in vitro*. Reports

have shown that limited water availability results in reduced plant growth due to impairment of cell division and expansion (Hussain *et al.* 2008). Early sprouting with higher growth maintenance under PEG medium showed early indication towards moisture stress (Sahoo *et al.* 2006b).

The inherent ability of a plant to tolerate drought stress conditions is reciprocated by its ability to acclimatize under *in vivo* conditions (Anjum *et al.* 2011). As a drought avoidance strategy, plants are associated with the long-term morphological adjustment under stress through a reduction in plant size and leaf area (Levitt 1980).

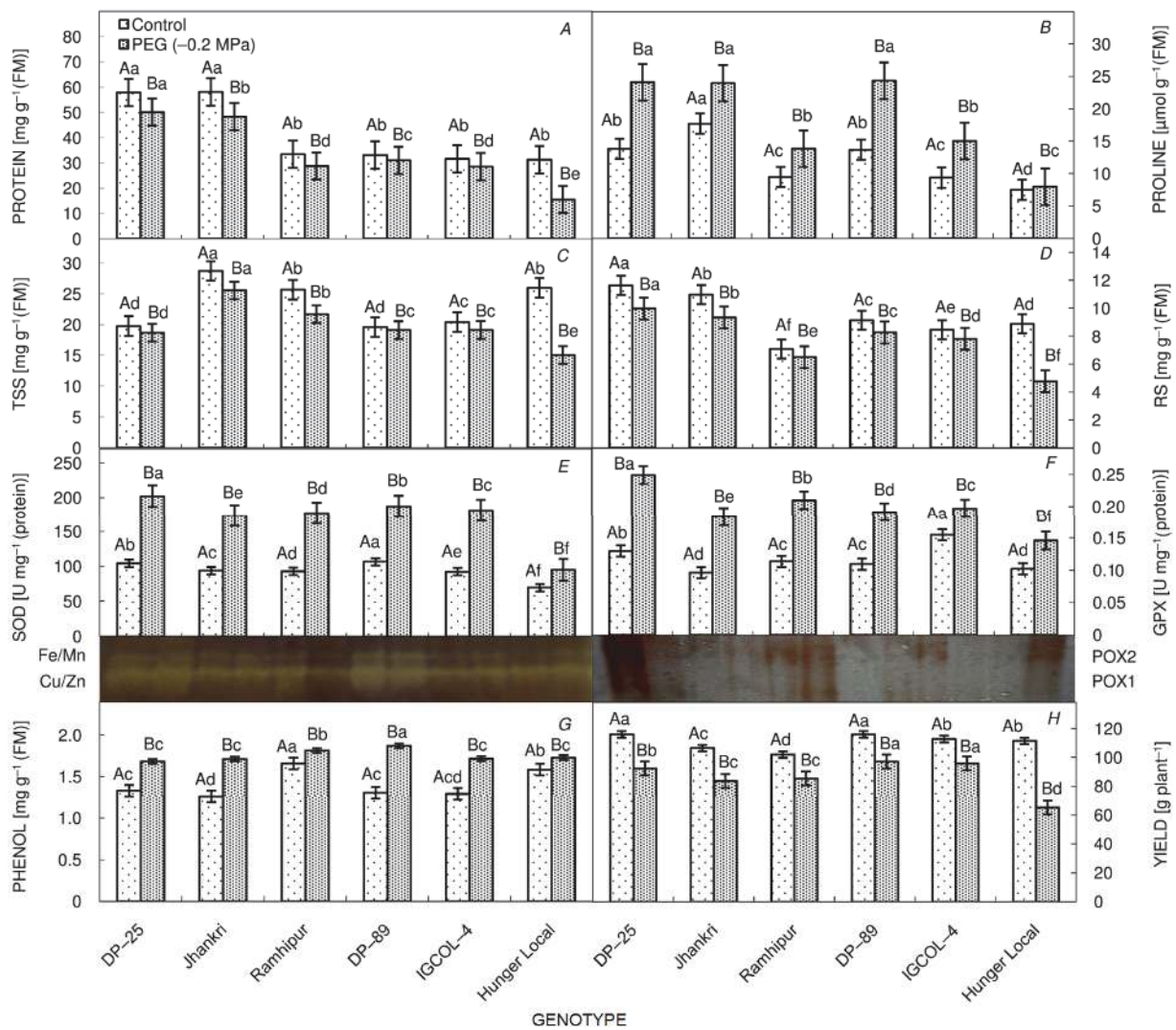


Fig. 3. Biochemical properties and yield of taro genotypes as influenced by PEG-mediated osmotic stress conditions. *A* – total soluble protein content; *B* – proline content; *C* – total soluble sugar (TSS); *D* – reducing sugar (RS); *E* – superoxide dismutase (SOD); *F* – guaiacol peroxidase (GPX); *G* – phenol content; *H* – yield. Values are the mean of three replicates and bars represent standard error of means. *Different letters in uppercase* represent significant differences between the treatments (control and -0.2 MPa) in the genotypes and *lowercase* represents significant difference between the genotypes under each treatment according to *Tukey's* test.

Table 3. Correlation coefficient (r value) between yield and photosynthetic, physiological, and biochemical properties of taro genotypes under *in vivo* PEG-mediated osmotic stress conditions. *, ** and ^{NS} – $P \leq 0.05$, $P \leq 0.01$, and $P > 0.05$, respectively.

Source	Yield
Leaf area	0.994**
Relative water content (RWC)	0.815*
Chlorophyll stability index (CSI)	0.950**
Photosynthetic rate (P_N)	0.968**
Transpiration rate (E)	-0.731*
Stomatal conductance (g_s)	0.986**
Stomatal resistance (R_s)	0.863*
Internal CO ₂ concentration (C_i)	0.987**
Carboxylation efficiency (CE)	0.978**
Transpiration efficiency (TE)	0.960**
Total soluble protein content	0.984**
Proline content	-0.814*
Total soluble sugar (TSS)	0.924**
Reducing sugar (RS)	0.998**
Superoxide dismutase (SOD)	-0.919**
Guaiacol peroxidase (GPX)	-0.286 ^{NS}
Phenol content	-0.546 ^{NS}

Among the morphological traits, leaf area is adversely affected by drought stress and correlated with biomass production and yield (Blum 2009). Reduction in leaf surface area for transpiration is one of the earliest visible impacts of water stress, which was also evident in our study. All the studied genotypes showed a reduction in leaf area under stress conditions. Interestingly, we observed that IGCOL-4 attained lesser leaf area under both control and stress conditions, and it managed to cope up the stress with lesser reduction in the leaf area. However, the percent reduction in the leaf area of Ramhipur, DP-89, and DP-25 were similar with IGCOL-4. Our results are in accordance with the findings of Mabhaudhi and Modi (2015) who had demonstrated a higher leaf area index (LAI) for a taro landrace, which managed to avoid drought stress through reduced canopy growth.

The RWC of the plants is considered as a measure of plant water status, reflecting its metabolic activity in tissues and used as an important index for dehydration tolerance (Anjum *et al.* 2011). In our study, RWC was significantly reduced by the PEG stress in all studied taro genotypes with the highest percent reduction in Hunger Local and lower reduction was found in DP-89. The genotypes Ramhipur and IGCOL-4 also showed lesser reduction in RWC, which is correlated with the reduced leaf area that might contribute towards reducing plant water use.

Lower reduction in the Chl content is a strategy employed by the plants to remove excess radiation energy under limited water availability. In our study, we observed a significant decrease in CSI of taro leaves exposed to PEG-mediated osmotic stress. The genotypes Ramhipur and IGCOL-4 showed the lower decrease (4.2 and 4.6%)

in leaf CSI in comparison to Hunger Local (22.4%). We have observed the similar results in a taro hybrid and its parents when imposed to PEG stress (Sahoo *et al.* 2006b). Mabhaudhi and Modi (2015) suggested that the Umbumbulu (UM) landrace of taro was able to down-regulate its photosynthesis by decreasing CO₂ availability and lowering CSI. Maintenance of lower variation in leaf area accompanied by minimum changes in RWC and CSI in Ramhipur, IGCOL-4, and DP-89 could be one of the avoidance strategies adopted by these genotypes to cope with stress.

Our results under *in vivo* PEG-induced stress indicated that P_N , E , and g_s declined in all the studied genotypes. The decreased P_N as a consequence of stomatal closure (Liu *et al.* 2005, Sourour *et al.* 2017) and leaf area reduction under water stress are the key contributors to a yield loss under drought (Legay *et al.* 2011). The reduction in P_N could occur due to the closure of stomata which resulted in a decrease in g_s . Stomatal closure is a drought-avoidance mechanism which allows the plants to minimise transpiration water loss (Chaves *et al.* 2003). Stomatal closure also restricts the diffusion of CO₂ into leaves, influences g_s and R_s (Tombesi *et al.* 2015) and thereby results in lower C_i . In our study, the genotypes DP-89, IGCOL-4, and Ramhipur exhibited lesser reductions in P_N and g_s , which could be helpful for maintaining better growth in terms of leaf area. The E highly decreased in DP-25 and DP-89 in our study which was inversely correlated with P_N . Reports have shown that g_s decreased in taro under moisture stress to minimize the rate of transpiration (Mabhaudhi *et al.* 2013). Based on our results, the genotypes DP-89, Ramhipur, and IGCOL-4 exhibited their credibility in terms of minimum fluctuation in g_s and R_s to maintain the P_N and E . A relatively stable C_i was registered by these genotypes which was influenced by uptake of atmospheric CO₂ during the course of photosynthesis. In our investigation, DP-89, Ramhipur, and IGCOL-4 managed to maintain the CE and TE under moisture stress conditions in comparison to Hunger Local.

Moisture stress influences protein synthesis in leaves and the content of total soluble leaf proteins decreases under stress (Suseela *et al.* 2015). Decreased protein contents in response to moisture stress might be due to lowering of overall protein synthesis and/or increasing protein degradation (Singh 2003). We observed a significant reduction in the total soluble protein content under PEG-mediated osmotic stress, it was lower in DP-89, IGCOL-4, and Ramhipur in comparison to Hunger Local. In support of our results, there are studies showing that the total soluble proteins significantly decreased in the presence of PEG in other crops (Gharineh and Karmollachaab, 2013). The carbohydrate content such as TSS and RS also declined under osmotic stress; they were significantly lower in DP-89, DP-25, and IGCOL-4. The decrease in the carbohydrate content in stressed plants could be attributed to the reduction in photosynthetic rate

as the later is considered to be the main source for the accumulation of soluble sugars.

Proline, an essential osmolyte (Kumar *et al.* 2011), plays a major role as an antioxidative defense molecule and a signaling molecule in plant systems under exposure to various stress conditions (Hayat *et al.* 2012). We observed an increase in the proline content under PEG stress in all the tested genotypes, remarkably higher in DP-89, DP-25, and IGCOL-4. The elevated proline content under osmotic stress is proposed to maintain plant existence (Ghorbanli *et al.* 2012) by managing osmotic balance, preventing electrolyte leakage, and lowering concentrations of reactive oxygen species (ROS) to prevent oxidative burst in plants (Hayat *et al.* 2012). Increased synthesis of total phenolics in stressed plants also exhibited a protective mechanism against the cellular structures from oxidative damage (Chakraborty and Pradhan 2012). In our study, DP-89, Ramhipur, and IGCOL-4 showed the higher total phenol content, which was evidence of cellular adaptive mechanism towards scavenging ROS during stress.

Overproduction of ROS during stress is being scavenged by enzymatic and nonenzymatic antioxidants (Sahoo *et al.* 2007), which is one of the vital mechanisms of drought tolerance (Farooq *et al.* 2009). Induction of an efficient cellular antioxidant machinery is crucial for protecting plants against the adverse effects of abiotic stresses (Pérez-Clemente *et al.* 2013). In our study, SOD and GPX activities significantly increased under osmotic stress across the taro genotypes tested, however, the induction was higher in DP-25, DP-89, IGCOL-4, and Ramhipur as compared to Hunger Local and Jhankri, which is in accordance with the report of Khanna-Chopra and Selote (2007). For SOD, two isoforms were detected in the studied genotypes which could be compared with the reports of Gomez *et al.* (2004), who suggested anodal isoforms of SOD as Cu/Zn and the cathodal isoform of SOD as Fe/Mn. The genotype DP-89 exhibited higher SOD activities with two prominent isoforms which were comparatively faint in Hunger Local. Similarly, DP-25 and Ramhipur showed higher GPX activities with two isoforms POX-1 and POX-2. Our results showed that SOD and GPX play major roles in taro leaves in scavenging of ROS during osmotic stress. Previously, we have also demonstrated a similar role of SOD (Fe/Mn and

Cu/Zn) and GPX (POX-1 and POX-2) in taro against biotic-stress tolerance (Sahoo *et al.* 2007).

The cumulative effect of morphological, physiological, and biochemical gestures under osmotic stress influences tuberization. Tolerant genotypes have the ability to maintain tuber yield under osmotic stress conditions and exhibit minimum yield reductions under scarce water conditions (Obidiegwu *et al.* 2015). Significant and strong correlation of photosynthetic parameters towards yield maintenance under osmotic stress showed its crucial role on source-sink relationship in taro. Balanced physiological and biochemical attributes with a strong enzymatic antioxidative system resulted in the low yield reduction in studied genotypes. We experienced a lower yield (< 20%) reduction in IGCOL-4, DP-89, and Ramhipur; they showed their ability to maintain the yield stability under harsh environments and proved themselves as tolerant. However, Hunger Local exhibited 41.4% of yield loss under osmotic stress which reflects its susceptibility towards the imposed stress.

Conclusion: In summary, we observed that all the studied taro genotypes exhibited various degrees of avoidance and tolerance mechanisms under *in vitro* and *in vivo* PEG-mediated osmotic stress conditions at morphological, physiological, and biochemical level. However, the genotypes Ramhipur, IGCOL-4, and DP-89 performed better growth *in vitro* and showed a moderate decrease in leaf area and RWC under stress suggesting that they were able to strike a balance between minimum water loss through transpiration while allowing biomass production to continue. This was consistent with their degree of stomatal control accompanied with the lesser decrease in CSI and RWC. *In vitro* PEG studies could be used as a reliable technique for the quick and efficient screening studies as short *in vitro* osmotic shock at cellular level triggers long-term events to combat stress. Higher photosynthesis and RWC of leaf tissues at crucial tuberization period under stress is coupled with yield maintenance in taro. Enzymatic antioxidants, SOD and GPX in particular, could be an important biochemical indicator for stress tolerance in taro. The genotypes DP-89, IGCOL-4, and Ramhipur showed the inherent tolerance traits towards osmotic stress and could be used as source materials in the future breeding program in taro.

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