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Morphological, Physiological and Biochemical evaluation of transgenic green gram (*Vigna radiata* L. Wilczek) for drought tolerance

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Abstract
Green gram (*Vigna radiata* L. Wilczek) is one of the important pulse crops in India belongs to the family Fabaceae. The production and productivity is severely affected by abiotic stresses like drought and salinity. Both control and transformed plants of green gram variety 'IPM-02-03' were evaluated for moisture stress. Soil moisture stress was imposed at the reproductive stage by withholding irrigation completely for 7 days. Morphological, physiological and biochemical parameters were evaluated for drought tolerance at 0^{th}, 3^{rd} and 7^{th} days with 100%, 50% and 25% water holding capacity, respectively during drought stress. In almost all the cases under moisture stress, transgenic plants showed higher yield and 100 seed weight. Pigments like chlorophyll and carotenoids accumulation was drastically decreases with increase in moisture stress. Higher accumulation of proline and antioxidant enzymes in transgenic plants than control showed better survival in stress condition. The present study suggests that, over expression of TIP1 gene in transformed plant can enhance better survival under drought stress condition.

Keywords: Transgenic Green gram, TIP1 gene, Drought stress, Proline, antioxidant enzymes, chlorophyll

1. Introduction
Pulses are a major source of proteins and other nutrients for most of the people in the world. Green gram is grown in many parts of India as a source of dietary protein for human consumption which contains approximately 21–25% protein. The production of green gram and other *Vigna* species overall has not been improved significantly in spite of the consistent efforts of the plant breeders to several biotic and abiotic stresses and the yield has remained almost stable during the last four decades. Despite being an economically important crop, overall production of mung bean is low due to abiotic and biotic stresses (Bangar *et al*., 2018) [12]. Drought is the most important limiting factor for Mungbean production. Drought is a multidimensional complex stress; simultaneously affect the morphological, physiological, biochemical and molecular level which affect the crop growth, yield and productivity all over the world (Basu *et al.*, 2016) [15]. As the population of the world is increasing rapidly, the available agricultural land is shrinking due to habitat use and climate change. Therefore, it is of great importance to exploit drought-affected land to meet the increasing world food demand and energy needs (Reguera *et al.*, 2012) [48]. Approximately 45% of the total agricultural lands are under perpetual or intermittent water deficits condition which is responsible for 50% of yield losses every year globally (Abdelrahman *et al.*, 2017) [1]. In the course of evolution, plants can streamline their morphological, physiological and metabolism-related responses at both organ and cellular levels to counter the drought severity (Haider *et al.*, 2017) [30].
Different morphological, physiological and biochemical parameters have been established for drought stress tolerance assessment in plants based on proline accumulation, high relative water content, leaf area index, yield components, antioxidant enzymatic activities, PEG mediation etc (Mafakheri *et al.*, 2010; Almeselmani *et al.*, 2011; Ranawake *et al.*, 2012; Alderfasi *et al.*, 2017; Swathi *et al.*, 2017) [40, 7, 46, 5, 55]. Plants response to the moisture deficit by the regulating different physiological and biochemical processes such as osmotic balance, gas exchange, photosynthesis and the metabolism of organic compounds (Osakabe *et al.*,

2014) [41] as well as adjustments of the membrane transport system (Krasensky *et al.,* 2012) [35]. However, the severity of the drought stress on physiological responses usually fluctuates in plants which constrains the photosynthesis rate, stomatal conductance and carbon dioxide (CO_2) diffusion to the chloroplast and other metabolic processes (Pinheiro *et al.*, 2011, Lovisolo *et al.,* 2010) [44, 38]. Reduced stomatal conductance decreases the intracellular CO_2 level which results in the over-production of reactive oxygen species (ROS) (Chaves *et al.,* 2009) [19]. In addition, plants accumulate compatible stress induced solutes such as proline that can stabilize proteins and sugars (raffinose and sorbitol) to prevent membrane disintegration and enzyme inactivation to reduce the turgor potential of the cell. Accumulation of proline and sugars helps in detoxification of ROS by reviving the cellular redox level (Krasensky and Jonak, 2012) [35] to facilitate water absorption by decreasing the cytoplasmic osmotic potential and remove excess of Reactive Oxygen Species (ROS) maintaining cellular redox balance (Anjum *et al.,* 2017) [8].

Plants possess an efficient antioxidant (enzymatic and non-enzymatic) defense system to cope with ROS-induced oxidative stress (Anjum *et al.,* 2011 & Ashraf *et al.,* 2015) [9, 10, 11]. Moreover, ROS enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) also play scavenging role in the degradation of free radicals (H_2O_2 and O^{-2}) and suppress the oxidative damage (Bowler *et al.,* 2003; Haider *et al.,* 2017) [17, 30]. The contribution of antioxidants to drought tolerance has been reported in several cases (Sharma *et al.,* 2012, Adebayo *et al.,* 2014 & Farooq *et al.,* 2009) [4, 27]. Plants respond to drought stress at the molecular level mainly by altered gene expression. Several different pathways are activated that interact with each other, forming a complex network which finally leads to modification of target proteins responsible for cellular responses at the physiological, biochemical, and molecular levels (Ramanjulu *et al.,* 2002 and Sreenivasulu *et al.,* 2007) [45, 53]. It is well understood that plants have its own adaptive mechanism at the physiological, molecular and cellular levels to overcome or tolerate the water balance under drought stress condition. A number of signaling network play a crucial role to counter the effect of stress responses in plants. The signal transduction activates the hydrogen pump ATPase (H+ ATPase) proteins on the plasma membrane of root hairs, which incites the biosynthesis of osmolytes to adjust water balance under decreased relative water content (RWC). Besides, various transcription factors (TFs) gene families, including WRKY TF, ethylene-responsive factor (ERF), dehydration responsive element-binding (DREB), myeloblastosis, myelocytomatosis oncogene cellular (MYC), basic helix–loop– helix TF and basic leucine zipper (bZIP) are implicated to trigger the specific genes to generate the requisite defense responses in plants (Abuzar *et al.,* 2016; Zeng *et al.,* 2017) [3, 60]. Being the largest families of TFs, WRKY proteins have been tested repeatedly against drought stress. The over expression of TFs AtWRKY57 increases the ABA level and promote the drought tolerance in *Arabidopsis* (Ren *et al.,* 2010) [49]. Similarly, the AtWRKY63 curbed the antagonistic effects of drought by using ABA-signaling pathway and also the higher activity of BdWRKY36 in transgenic tobacco assisted in drought stress tolerance (Li *et al.,* 2013; Sun *et al.,* 2015) [37, 54].

In the present investigation, we have studied the expression of stress tolerant Tonoplast Intrinsic protein I gene possibly involved in drought tolerance by regulating vacuolar membrane transport by tonoplast (Li *et al.,* 2015) [38]. The transcription factor previously reported to play a role in drought tolerance in *Vigna radiata* L. Overall this study will provide the basic information of drought tolerance mechanism under moderate and severe drought stress condition of both non transformed and transformed *Vigna radiata* L. variety 'IPM-02-03' with TIP1 gene by *Agrobacterium* mediated genetic transformation with special attention to leaf gas-exchange parameters, antioxidant capacity and membrane stability in drought conditions which can be further used for breeding of drought tolerance variety.

2. Materials and methods

2.1. Plant material

Green gram (*Vigna radiata* L. Wilczek) variety 'IPM-02-03' was selected for genetic transformation study with drought stress tolerance gene TIP1 and evaluated for drought tolerance in both control and transformed T1 plants. This variety is high yielding as well as Yellow Mosaic Virus (YMV) resistance variety suitable for different agro climatic situations in Odisha. The seeds of this cultivar were collected from Centre for Pulses Research, OUAT, Berhampur, Ganjam, Odisha and were used in the present study. The experiment was carried out at Department of Agricultural Biotechnology, College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha.

2.2. Water stress treatment

The T_1 transformed and control seeds of green gram Variety 'IPM 02-03' were placed in water for 48 hrs. The seeds were transferred to the pot prepared with Soil, Vermicompost and FYM (1:1:1) in the standard greenhouse conditions (25 ± 5°C) supplemented with 65% relative humidity and 16-h light/8-h dark photoperiod in Random Block Design (RBD) and was repeated twice. Sufficient amount of watering was done to allow normal germination and growth of plants till withdrawal of irrigation. The plants were grown in plastic pot (15 X 15 cm) filled with 1 kg soil mixture. Two seeds were sown in each pot and thinned to one after germination. Plants were supplemented with hogland solution alternatively were watered to maintained 100% field capacity. The experiment was conducted at 2 levels of treatment: no stress (control), stress at the reproductive stage (50 days after sowing) by withholding irrigation for 7 days and was released on the 8th day and normal irrigation was continued with 100%, 50% and 25% Water Holding Capacity. The control treatment received 100 ml water per plant on daily basis, while drought stress treatments (mild and severe) were levied by withholding water.

All the morphological, physiological and biochemical investigations were carried at different stages of the experiment. The third and fourth unfolded leaf from the shoot apex of control and transformed plants were collected from each replicate 1 h before the end of the photoperiod. The leaf samples were immediately put in liquid nitrogen and then stored at −80°C until further use.

Physiological observations like relative water content, cell membrane thermo stability, plant height, shape of seeds, 100 seeds weight (g), root shoot ratio, Yield per plant (g) and biochemical observations like chlorophyll, proline, catalase, peroxidise, SOD and ascorbate content were carried out on the 0th, 3rd and 7th day of drought induction in non transformed and transformed plants.

2.2.1. Morphological observations

Physical observation was taken for wilting of leaves on the 0th, 3rd and 7th day after drought induction. Leaf wilting was scored on a scale of 1–4 modified from Engelbrecht *et al*, (2007) [24] as: 1- no wilting; 2- slightly wilting; 3- wilting, where the plant showed leaf wilting only during hot hours from which the leaves recovered; and 4- severe wilting, where the wilted leaves did not recover.

2.2.2. Physiological observations

2.2.2.1. Measurement of relative water content (RWC)

The relative water content (RWC) was determined following the method described in Lv *et al.* (2009) [39]. Briefly, the fresh weights (FWs) of green gram leaves were recorded immediately after excising from plants. After soaking them in deionized water at 4 °C overnight, their turgid weights (TWs) were determined. Then their dry weights (DWs) were obtained after oven-drying the leaf samples at 70 °C for 72 hours. The relative water content (RWC) was calculated as:

$$\text{RCW (\%)} = \frac{(FW - DW)}{(TW - DW)} \times 1000$$

2.2.2.2. Cell-membrane thermo stability

Cell-membrane injury is regarded as an indicator of the ability of the plant to tolerate drought. To estimate the cell membrane injury percentage, the cell-membrane thermo stability test was carried out in T_1 generations following the method described by Farooq and Farooqe, (2006) [26]. For T_1 generation, third fully opened leaves of 50-days-old plants grown under normal irrigated conditions were used and leaves were collected on 0th, 3rd and 7th days after drought induction. Leaf discs (0.5 cm diameter) were made up to 200 mg, washed thrice with distilled water and finally 20 ml of distilled water added to each control and treatment tubes (2.5 cm x 15 cm). The tubes were covered with aluminium foil, incubated at 60 ºC in a thermostatically controlled water bath for 20 min and placed at 10 ºC for 12 h to allow the diffusion of electrolytes into the water. After recording the initial conductance at 30 ºC, the tubes were heated at 100ºC for 20 min and final conductance was recorded after cooling. Membrane Injury percentage was computed using the following formula:

$$\text{Membrane injury \%} = \left\{ \frac{\frac{(1-T_1)}{T_2}}{\frac{(1-C_1)}{C_2}} \right\}$$

where T and C refer to the values for treatment and control samples and the subscripts 1 and 2 denote the initial and final conductance, respectively.

2.2.3. Biochemical parameters estimation

2.2.3.1. Estimation of Chlorophyll and Carotenoids content

The chlorophyll A, B, total chlorophyll and Carotenoid contents were estimated by following standard procedure by Vernom, 1960 [57] and absorbance was taken at 663.2 nm (for Chl-a), 646.8 nm (for chl-b) and 470 nm (for Carotenoids) under UV – vis spectrophotometer (Elico, India**).** The amount of Chlorophyll and Carotenoids were calculated using the following formula (Microgram per ml Fresh-weight basis)

Chlorophyll A = 12.25 x A 663.2 – 2.79 x A 646.8

Chlorophyll B = 21.5 x A 646.8 – 5.1 x A 663.2

Chlorphyll A + B = 7.15 x A 663.2 – 1.71 x A 646.8

Carotenoids = 1000 x A 470 – (1.82 x A 663.2 + 85.02 x A 646.8) / 198

2.2.3.2. Estimation of proline content

The proline contents were determined using sulfosalicylic acid (3%) and final calculations were made using proline standard calibration curve (Sigma-Aldrich) at 520 nm following the method briefly explained by Abrahám *et al.*, (2010) [2].

$$\textbf{Proline (µmole/g tissue)} = \frac{\left(\frac{\mu g \text{ proline/ml x ml tolune}}{115.5} \right)}{g \text{ tissue sample}} \times 5$$

where, 115.5 is the molecular weight of proline.

2.2.4. Antioxidant enzyme assay

2.2.4.1. Determination of SOD activity

The activity of SOD enzyme was estimated by determining the ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) spectro photometrically at 560 nm described by Gupta *et al*., 1993[29]. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

2.2.4.2. Catalase and peroxidase activities

CAT (EC 1.11.1.6) activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and the enzyme. The decomposition of H_2O_2 was followed at 240 nm (Cakmak *et al.*, 1992) [18].

Calculation

$$\text{Unit/ml of enzyme} = \frac{3.45 \times \text{dilution factor}}{\text{Min} \times 0.1\text{ml}}$$

3.45- decomposition of 3.45 µ moles of H_2O_2 in a 3 ml of reaction mixture producing a decrease in the A_{240} from 0.45 to 0.4

$$\textbf{Units/mg of protein} = \frac{\text{Unit/ml of enzyme}}{\text{mg of protein/ml of enzyme}}$$

2.2.4.2. Ascorbate Peroxidase Activities

APX (EC1.11.1.11) activity was determined by monitoring the decrease in A_{290} according to the protocol described by Jimenez *et al.*, 1997 [33]. Ascorbate (AsA) was determined according to Sgherri *et al.*, 2000. The ascorbate contents were determined using phosphoric acid and 2% sodium molybdate. The final content was measured at absorbance 660 nm (Panda *et al.*, 2009) [42]

The ascorbate value was calculated using the formulae

$$\textbf{Ascorbate (µg/g FW)} = \frac{\text{Abs}_{660} \times \text{standard value 123.67 } (\mu g/ml)}{\text{Sample (g/ml)}}$$

3.13. Statistical analysis

As all the physiological and biochemical parameters were in laboratory under well-defined conditions of medium of growth, temperature and light, completely randomized design

(CRD) was employed for the different experiments. The estimated data were subjected to the one-way analysis of variance (ANOVA) using SPSS 20.0 (Chicago, USA). Linear correlation analysis was performed to study the relationship between the studied parameters. Coefficient of variance and critical differences were analysed to prove the study statistically significant. Three biological replicates were used for this investigation and means were separated using Duncan's Multiple Range test, where the value of $p < 0.05$ is considered statistically significant. The data are presented as a mean ± standard error.

3. Results

3.1. Morphological observations

Morphological observation was taken for wilting of leaves on the 0th, 3rd and 7th day after drought induction. In 0th day there was no wilting was observed in transgenic plant while slight wilting was observed in control plant with score 1. On 3rd day of drought induction, transgenic plant showed leaf wilting only during hot hours from which the leaves recovered was scored on a scale of 3. On 7th day of drought induction, severe wilting was observed where the wilted leaves did not recover in all the control plants which was scored as four where as in transgenic plants slight wilting was observed which was rated as 2 (Fig. 1).

Fig 1: Moisture stress after 7th days of drought induction (A) (transformed plant) (B) (non-transformed plant)

3.2. Physiological and Biochemical Analysis of T_1 transformed plant under moisture stress condition

Both control (Non-transformed) and transformed seeds from T_0 Plants were sown in earthen pots grown in the transgenic green house to study the rate of seed germination and growth performance. The experiment was conducted twice with three replications. There was no significant variation on rate of germination (Data not shown). After 50 days of sowing, the watering was totally stopped in both control as well as transformed plant. The observations for different physical parameters were taken after 0th, 3rd and 7th days after drought induction with 100%, 50% and 25% Water Holding Capacity (WHC). It was observed that transformed plants were growing better than control plants.

Plant height was measured by a scale from the soil surface to the highest tip of the plant. The yield of the plants was recorded from 100 seed weight and average yield from plant after harvesting. Morphological observation for seed shape and size was done after 0th, 3rd, and 7th days of drought induction (Table 1).

3.2.1. Relative water content (RWC) analysis of T_1 transformed plant

Relative water content ranged from 61.52% to 81.32%. In this study, the percentage of RWC decreased in control plant after 50 days of sowing (Table 1). The mean results showed that there was a significant decrease in physiological traits such as RWC when exposed to drought conditions at both the vegetative and the reproductive stages when compared to irrigated conditions. It was observed that plants in irrigated condition were taller than the plants in moisture stressed condition (Table 1). It was observed that plant height ranged from 23.56 to 26.32 cm in both control and transformed plant.

3.2.2. Cell-membrane thermo stability in T_1 transformed plant

The standard test for cell-membrane thermo stability was performed in the T_1 transgenic plants. Significantly lower membrane injury, which indicates higher cell-membrane stability, was observed in all the T_1 transgenic plants (46.24–83.25%) than in control plant. It was observed that there was 95.5% injury in control plant, It showed injury percentage of 50–60% with the lowest (46.24%) and the highest (95.5%) in control plant in 7th days of moisture stress (Table 1).

Table 1: Analysis of Physiological parameters after 0th, 3rd and 7th days after drought induction

S. No.	Treatments	Days to drought induction	Avg yield (gram per plant)	100 seed weight	RWC (%)	Cell membrane thermo stability (%)	Plant Height (cm)	Total fresh weight/ dry weight	Root: Shoot ratio	Shape of the seed
1	T0 (Control)	Water stress after 0th day of induction at 100% WHC	16.26	6.26	72.89	56.26	24.12	57.64/8.31	0.29	Wrinkle
2	T1 (Transformed)		17.31	7.13	81.32	46.24	28.58	58.21/8.37	0.28	Bold
3	T0 (Control)	Water stress after 3rd days of induction at 50% WHC	14.82	5.81	65.46	78.84	23.56	47.73/5.17	0.21	Wrinkle
4	T1 (Transformed)		16.6	6.02	65.28	66.42	27.64	49.42/5.29	0.24	Small
5	T0 (Control)	Water stress after 7th days of induction at 25% WHC	12.9	4.9	61.52	95.5	24.58	31.68/5.22	0.18	Rudimentary
6	T1 (Transformed)		13.2	5.4	67.84	83.25	26.32	33.24/5.45	0.21	Shrinked

Table 2: Analysis of biochemical parameter after 0th, 3rd and 7th days after drought induction drought induction

Treatments	Days to drought induction	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)	Carotenoid (mg/g)	Proline Content (µg/g)	Catalase (Unit/mg protein)	Peroxidase (Unit/mg protein)	SOD (Unit/mg protein)	Ascorbate (µg/g FW)
T_0 (Control)	Water stress after 0th day of induction at 100% WHC	134.8	181.9	252.3	168.7	2.13	3.8	3.4	3.1	1.7
T_1 (Transformed)		136.2	178.5	254.7	172.6	2.94	4.7	4.1	3.6	2.9

T_0 (Control)	Water stress after 3rd days of induction at 50% WHC	121.1	169.3	229.5	141.5	2.27	4.3	4.6	3.9	1.9
T1 (Transformed)		132.5	176.7	238.2	148.3	3.12	5.2	4.9	4.3	2.5
T_0 (Control)	Water stress after 7th days of induction at 25% WHC	104.7	141.5	197.5	126.5	2.91	4.8	5.2	4.2	2.4
T_1 (Transformed)		107.6	149.2	198.2	131.2	3.46	5.8	5.9	4.9	2.9

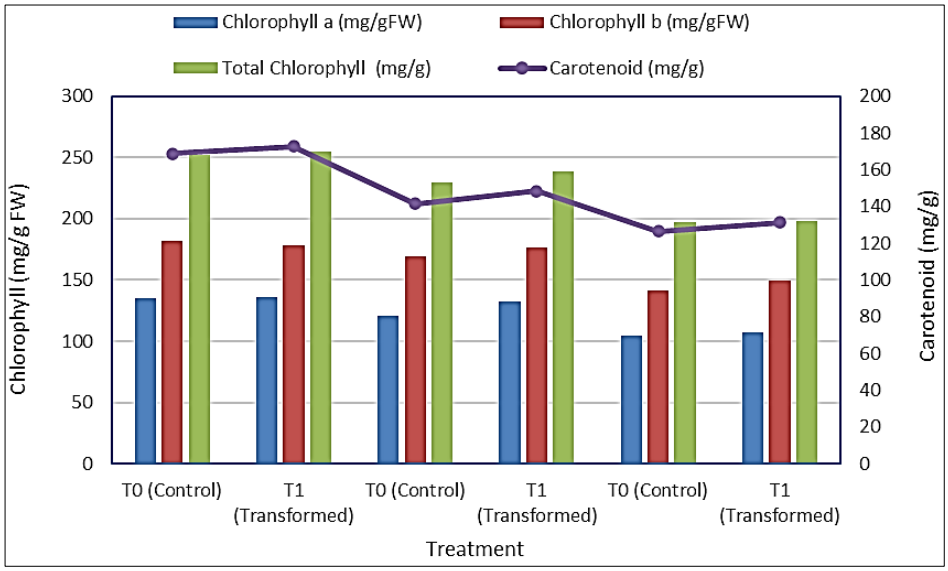

Fig 2: Accumulation of chlorophyll (a, b, total) and carotenoids after 0th, 3rd and 7th days after drought induction

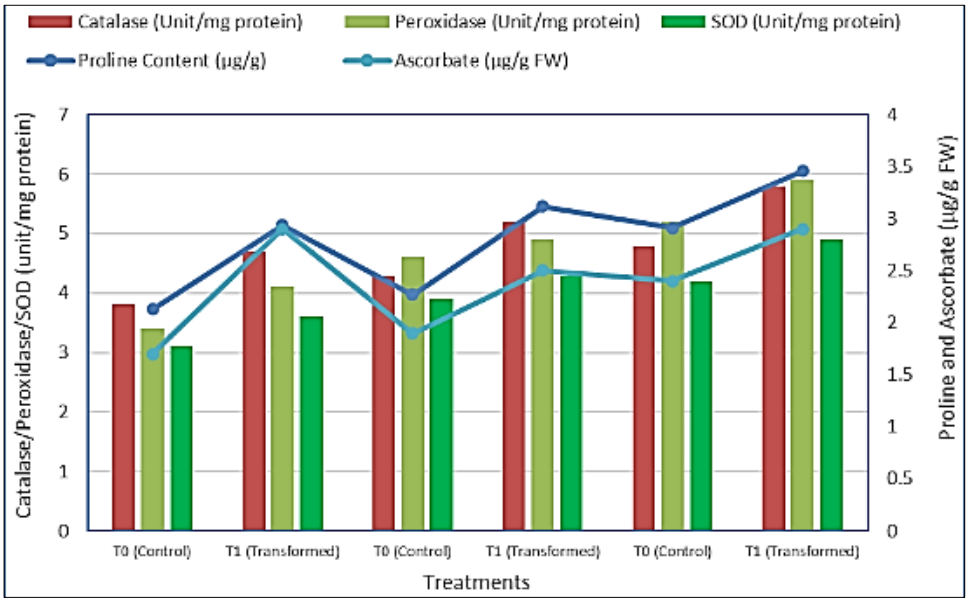

Fig 3: Accumulation of stress induced enzymes after 0th, 3rd and 7th days after drought induction drought induction

The yield performance in both transformed and non-transformed plant were analysed during moisture stress condition at different Water Holding Capacity (WHC) i.e 100%, 50%, 25%. The number of yield (gram per plant) and 100 seed weight were noted during harvest of the plant. The result showed that there was slight decrease with regards to 100 seed weight and yield per plant during control condition whereas under stress condition there was significant change of yield performance in transformed plant. The 100 seed weight was varied in both transformed and non-transformed plant (3.14g - 4.12g), Relative Water Content (RWC) (81.32% - 61.52%), Cell membrane Thermo Stability (%) 95.5%-46.24%, Plant height (28.58cm -24.12 cm), fresh weight (31.68g to 57.61 g), dry weight (5.22 g to 8.31g) and root: shoot ratio (0.18 to 0.29) were observed in 0th to 7th days with 100% to 25% WHC of drought induction and seed shape and sizes vary significantly in transgenic plant than control plant. Seed shape shrinked in higher drought induction (Table 1).

Biochemical analysis was carried out for moisture stress during growth period. Irrigation was completely stopped after 50 days of sowing in both control as well as transformed plant in transgenic greenhouse condition and biochemical analysis were taken at 0th, 3rd and 7th days after drought induction with 100%, 50% and 25% water holding capacity, respectively. It was found that the chlorophyll (a, b, total), carotenoid contents were decreases with increase in moisture stress. Highest accumulation of total chlorophyll was observed in without moisture stress condition (252.3 mg/g FW in non-transformed plant and 254.7 mg/g FW in transformed) and lowest was observed on 7th days of drought induction (126.5 mg/g FW in non-transformed plant and 131.2 mg/g FW in transformed) (Table 2 & Fig. 2). Carotenoid content was high

in without moisture stress (168.7 mg/g FW in non-transformed plant and 172.6 mg/g FW in transformed plant) and lowest accumulation (126.5 mg/g FW in non-transformed plant and 131.2 mg/g FW in transformed plant) was observed in 7th days of drought induction (Table 2). Accumulation of Proline was higher in transformed plant as compared to control plant with increase in moisture stress (Table 2 & Fig. 3). The stress induced enzymes like catalase, peroxidase, ascorbate and SOD activities were higher in transfer plant as compared to control plant. The results clearly indicate that higher accumulation of oxidative enzymes occurs during stress condition provide protection to plant cells against multiple environmental stresses.

4. Discussion

In arid and semi arid areas water scarcity is major limiting factor of crop production and productivity. Drought response is the outcome of different morphological, physiological and biochemical alteration triggered at molecular level. The comprehensive study on mechanism, responses and dynamics to limited water conditions for developing resistant lines of green gram cultivars is gaining noticeable considerations.

The drought conditions during crop growth have negative impact on water balance; hence decrease the water potential of leaves. Reduced leaf water potential and turgor inhibit the growth during water scarce condition. Therefore, Relative Water Content (RWC) indicates the degree of drought stress, as previous studies have reported that higher decreases in water potential were observed in drought-susceptible varieties than in drought-tolerant varieties (Parvin *et al.,* 2015; Chowdhury *et al.,* 2017) [43, 21]. In this study, Relative water content ranged from 61.52% to 81.32%, the percentage of RWC decreased in control plant after 50 days of sowing with Water Holding Capacity (WHC) of 100%, 50%, 25%, respectively. The mean results showed that there was a significant decrease in physiological traits such as RWC when exposed to drought conditions at both the vegetative and the reproductive stages when compared to irrigated conditions.

Significantly, lower membrane injury, which indicates higher cell-membrane stability, was observed in all the T_1 transgenic plants (46.24–83.25%) than in control plant. It was observed that there was 95.5% injury in control plant, It showed injury percentage of 50–60% with the lowest (46.24%) and the highest (95.5%) in control plant in 7th day of moisture stress. The fresh weight, dry weight and root: shoot ratio varies with WHC and days to drought induction. A root system that increases the capacity of a plant to capture water is a fundamental adaptation to drought (Jaleel *et al.,* 2008) [32]. In this study, it was seen that root length increased as drought stress increased. This increase in root length in dry soils and the establishment of a root network that goes deep into the soil would help plants to absorb moisture efficiently, and is one of the mechanisms by which green gram plants tolerate drought stress. A high root to shoot ratio observed in low soil moisture content is another strategic adaptation to develop tolerance to soil moisture deficiency. Thus plants with longer roots are able to more effectively compete for soil nutrients and water, while those with a higher proportion of shoots can collect more light energy.

Pigments like chlorophyll a, b, total and carotenoids accumulation were significantly low in drought stress. In the present investigation, the pigments accumulation decreases with increase in moisture stress. It was found that the chlorophyll (a, b, total), carotenoid content had decreased and proline content was higher. Proline content was also high in transformed plant on 7 th days of drought induction as compared to control plant. Proline accumulation is the most important physiological index for the plant's response to drought stress. Change in concentration of proline observed in green gram exposed to drought stress was higher at the reproductive stage than at the vegetative stage. This is probably because proline accumulation depends upon the leaf age, leaf position and plant age (Bharadwaj *et al.,* 2018) [16]. As with advancement in crop age, leaf water potential decreases under drought stress and free accumulation of proline occurs. It is believed that proline acts as an osmolyte and protects the plant against low water potential by maintaining osmotic regulation in plant organs (Kabbadj *et al.,* 2017; Silvestre *et al.,* 2017) [34, 52]. In addition to this, proline also plays a major role as an electron receptor and may promote damage repair ability in the plant by increasing antioxidant activity during drought stress (Yaish, 2015) [59]. Under water stress, proline accumulation was greater than that of other amino acids; therefore, proline can be used as a criterion for screening drought-tolerant varieties (Fahramand *et al.,* 2014) [25]. Overall, drought caused impairments in the processes of cell division and cell expansion and ultimate loss of cell turgor, which are responsible for reduced growth rate, plant height, leaf area, and yield traits. It was observed that irrespective of the varieties, water stress caused a greater adverse effect during the vegetative stage than during the reproductive stage; this correlates with the findings of previous studies (Allahmoradi *et al.,* 2011; Ratnasekera and Subhashi, 2015) [6, 47]. This is probably due to the water absorption capacity being low during the vegetative stage due to a shortage of soil water; consequently, grain yield and growth will be decided by the ability to grow vigorously and accumulate as much dry weight as possible before flowering (Uddin *et al.,* 2013; Baroowa and Gogoi, 2016) [56, 13]. Higher accumulation of proline betters osmotic balance in plant cells suffering from water deficit and accounted for their higher drought tolerance capacity keeping the slope of water potential at the threshold of drought stress (Baroowa *et al.,* 2015) [14]

Higher accumulation stress induced oxidative enzymes like catalases (CAT), ascorbates peroxidase (APX) and SOD were observed in transformed plant to provide protection against moisture stress. Lipid peroxidation has been associated with cellular damage caused by various environmental stress conditions (Huang *et al.,* 2009) [31]. Considering the key role of CAT in photorespiration, many authors focused on the role of CAT catalysis pathway under both drought and salt stress. So the maintenance of CAT activity in leaves of drought-stressed plants likely allowed the removal of photorespiratory H_2O_2 produced when plants are subjected to water deficit of salinity, especially under severe degrees of stress (De Pinto *et al.,* 2013) [23].

A major hydrogen peroxide detoxifying system in plant cells under abiotic stressors is the ascorbate-glutathione cycle, in which ascorbate peroxidase (APX) isoenzymes play a key role in catalyzing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor (Chen *et al.,* 2012, Correa-Aragunde *et al.,* 2013) [20, 22], particularly in the chloroplast. The genes encoding APXs are particularly important in maintaining the homeostasis of ascorbate (AsA) and glutathione (GSH), two non-enzymatic antioxidants within the context of cellular redox homeostasis and redox signaling, and directly or indirectly involved in maintaining high photosynthetic rates in plants under adverse

environmental conditions (De Pinto *et al.*, 2013, Foyer *et al.*, 2011) [23, 28].

5. Conclusion

Morphological, Physiological and Biochemical analysis of transgenic green gram variety IPM-02-03 *Vr TIP-1* gene showed enhanced tolerance to drought. Growth and survival of non-transformed plant was lower than transformed plant under drought stress condition. Similar results were also obtained in biochemical analysis of transgenic plant. Transgenic green gram variety IPM 02-3 are highly suitable to the severe drought condition under both irrigated as well as rain fed conditions.

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7. Conflict of interest

The authors declare that there is no conflict of interest in the present investigation.

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