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Physiological, Biochemical and Proteomic Responses of Rice (*Oryza sativa* L.)Varieties *Godaheenati* and *Pokkali* for Drought Stress at the Seedling Stage

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***ABSTRACT*:** *Abiotic stresses are key limiting factors that restrict rice production. Among them, drought is one of the major factors that induce several physiological, biochemical and molecular responses in plants. Screening of rice varieties against drought stress is a prerequisite to produce stress tolerant rice varieties. Two rice varieties, i.e. Godaheenati (4049) and Pokkali were screened for drought stress responses based on biochemical attributes at the seedling stage. Relative water content, the total protein content of leaves of two-week-old rice seedlings and photosynthetic pigment levels were measured over a five-day drought period. The varieties tested responded with different relative water content, protein concentrations and pigment levels, potentially due to their genetic differences. Godaheenati showed an increase of the relative water content on the fourth day under drought stress, indicative of its cell recovering capability, and thus may be drought tolerant than Pokkali. Dehydration stress resulted in the fluctuation of all three photosynthetic pigments in both varieties. On the fourth day, Godaheenati showed a significant increase of photosynthetic pigments than Pokkali, and this could be a result of recovery of plant cells due to the increase of water content. The total protein content extracted from leaves of two-week-old rice seedlings of Godaheenati exposed to drought conditions was subjected to two-dimensional gel electrophoresis. The results showed alterations in the total proteome of rice leaves, and an up regulation of genes responsible for drought stress tolerance in rice. Further screening is recommended for Godaheenati as a drought tolerant rice variety to be used in rice breeding programs.*

***Keywords*:** drought, photosynthetic pigments, rice leaf protein, relative water content

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major cereal crops that serve as staple food for more than 50% of the world population (Muthayya *et al*., 2014). Abiotic stresses such as drought, salinity and submergence are the main causes that affect the plant growth and development and hence, can be identified as the major abiotic stress factors that limit the yield potential in rice (Choudhary *et al*., 2009). Among them, drought has been identified as the single most critical threat to the world food security (Farooq *et al*., 2009), and one of the major factors limiting rice production worldwide (Rajiv *et al*., 2010), where around 130 million ha of

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paddy are annually affected by drought in Asia (Rahimi *et al*., 2013). It is estimated that drought severely impacted on the paddy production in Sri Lanka in the *Maha* season of 2013/2014, affecting 87,281 ha of paddy lands (Anonymous, 2014).

Drought stress stimulates several physiological, biochemical and molecular responses in plants and adapts the plant to survive in such stressful environmental conditions. Previous studies have reported that the initial response of plants towards acute water deficit is *via* stomatal closure, where the transpiration losses are minimized (Rajiv *et al*., 2010). However, this primarily results a reduction in the photosynthetic rate. It also affects the other physiological and biochemical activities such as respiration, ion uptake, and nutrient metabolism (Usman *et al*., 2013).

Water deficit conditions or dehydration in plant cells causes the reduction of the fresh and dry weights of the leaves, and photosynthesis pigments such as chlorophyll a, b and carotenoids (Usman *et al*., 2013; Shehab *et al*., 2010). Characters such as osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops. These are expressed in terms of relative water content (RWC), accumulation of compatible solutes like proline, and leakage of ions and electrolytes due to increased permeability of the cell membrane (Bhushan *et al*., 2007). In addition, osmotic proteins get accumulated in the cells, and they are known to be associated with the development of plant resistance against drought stress (Ghorbanli *et al*., 2012).

Apart from the biochemical and physiological conditions, plants show a variety of structural changes as responses to abiotic stresses. Water stress causes reduction of cell enlargement and thus reduces stem elongation by inhibiting inter nodal elongation. It also affects the root system and tillering capability of the plant. In rice, differences in root growth, root length and architecture could occur as a drought response. Therefore, the reduction in fresh and dry biomass is a common adverse effect of water deficit on crop plants (Sikuku *et al*., 2012).

Improving rice varieties to become less sensitive to water deficit is an essential requirement to sustain rice production (Rajiv *et al*., 2010). Identification of drought tolerance attributes such as morphological, physiological and biochemical parameters in rice would be an advantage during crop improvement (Bunnag and Pongthai, 2013; Usman *et al*., 2013; Rajiv *et al*., 2010). The RWC was identified as a key parameter to select tolerant plants under drought stress (Bunnag and Pongthai, 2013; Choudhary *et al*., 2009). Ranawake and Hewage (2014) screened 33 rice varieties against drought, salinity and submergence based on their survival rate, however, did not report a variety that can be considered as drought tolerant. The use of two-dimensional gel electrophoresis (2-DE) is also identified as an effective approach in analyzing the changes in plant proteome during stressful conditions (Choudhary *et al*., 2009; Bhushan *et al*., 2007; Abbasi and Komatsu, 2004). Both RWC and 2-DE have not been used to analyse the drought responses on Sri Lankan rice germplasm. Further, to our knowledge, no drought tolerant variety has been identified from the Sri Lankan germplasm andno research has been reported where the biochemical attributes such as chlorophyll content and protein profiles have been analysed in Sri Lankan germplasm grown under drought stress. The variety *Godaheenati* has been reported to perform well under drought conditions exposed at the reproductive stage (Wasala *et al.,* 2015). Further, *Godaheenati* has been identified as submergence-tolerant (Xiong *et al*., 2012), a trait that is in tight positive correlation with drought tolerance (Ranawake and Hewage, 2014). *Pokkali,* which is known as the standard check-variety for salinity (Moons *et al*., 1995), was used in this study as it also showed better performances for drought stress during germination (Unpublished data). Therefore, the present study was undertaken to determine responses of rice plant to drought stress using both physiochemical and proteomic approaches using the two traditional Sri Lankan rice varieties, *Pokkali* and *Godaheenati* with a view to determine whether these traits can be used for the selection of drought tolerant genotypes at the seedling stage.

METHODOLOGY

Plant materials

Two traditional rice (*Oryza sativa* L.) varieties, i.e. *Godaheenati* (4049) and *Pokkali*, were selected to screen for drought stress responses at the vegetative stage.

Plant growth, maintenance and drought induction

Seeds of *Godaheenati* (4049) and *Pokkali* were collected from the Plant Genetic Resources Center, Gannoruwa, Sri Lanka and was multiplied at the plant house of the Department of Agricultural Biology of the Faculty of Agriculture, University of Peradeniya, Sri Lanka. Rice seeds were sorted manually to eliminate broken, small and infected seeds. Healthy seeds were surface sterilized in a 5% (v/v) NaOCl solution for 20 min. The seeds were rinsed repeatedly with sterilized distilled water and air-dried at room temperature (Jamil *et al*., 2012). A single seed was planted in 8" length soil column with 1" diameter filled with a mixture of soil containing compost, top soil and clay in a ratio of 1:1:1.The plants were fertilized according to the recommendation of the Department of Agriculture, Sri Lanka, and the experiment was arranged in a complete randomized design (CRD) with three replicates. Dehydration condition was imposed to the 4-week old seedlings by withdrawing water, and tissues were harvested at every 24 h after treatment for 5 days. The collected leaf tissues were subjected to the following analysis.

Determination of relative water content (RWC)

Rice leaf tissues were collected and weighed immediately to get the fresh weight (FW). The leaf tissues were rehydrated in water for 24 h until they attained full turgidity, surface-dried and reweighed to get the turgid weight (TW). Finally the tissues were oven dried at 80°C for 48 h (until constant weight), and were reweighed to obtain the dry weight (DW). The RWC was calculated using the Equation 1 (Bhushan *et al*., 2007),

$$\text{RWC (\%)} = (\text{FW- DW/TW- DW})* 100 \text{ ------------------ Equation 1}$$

where, RWC = relative water content, FW=fresh weight, DW = dry weight, TW = turgid weight.

Estimation of total soluble protein content

About 200 mg of leaf tissue was ground in a cold mortar with 1 ml of extraction buffer containing 50 mMTris-HCL buffer (pH 8.0), 1 mM PMSF, 10% (v/v) glycerol and was homogenized with a hand held homogenizer (PRO200, PRO Scientific, USA). The homogenate was centrifuged at 12000 rpm for 20 min at 4ºC and the supernatant was taken. Protein concentration was measured using the method described by Bradford (1976) with bovine serum albumin (BSA) as a standard (Rajiv *et al*., 2010).

Pigment estimation

Tissues were harvested each day and were ground in 80% chilled acetone. The supernatant was taken for the determination of photosynthetic pigments using a spectrophotometer (OptizenPOP; Mecasys Co. Ltd., Korea). The absorbance values at 663 nm, 645 nm and 470 nm were measured and the pigment concentrations were calculated using Equations, 2, 3 and 4 (Bhushan *et al*., 2007).

Chlorophyll a (mg/g) = [(12.7*A 663 - 2.69*A 645) v/w] ------------ Equation 2

Chlorophyll b (mg/g) = [(22.9*A 645 - 4.68*A 663) v/w] ------------- Equation 3

Carotenoid (mg/g) = {[(1000*A470) - (3.27*chlorophyll a + 1.04*chlorophyll b)] /227 v/w} ----------- Equation 4

Total proteome analysis of rice leaves by two-dimensional gel electrophoresis

Tissues were harvested from two-weeks old *Godaheenati* (4049) plants, which were subjected to drought stress by withdrawing water for 10 days. A portion (400 mg) of fresh rice leaf tissues was homogenized with 1 ml of phosphate buffer (pH 7.6) containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl and 3 mM NaN_3. The homogenate was centrifuged at 15,000 rpm for 10 min at 4ºC. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and proteins were purified and precipitated with a BioRAD 2-D clean up kit.

Proteins were separated in an 18 cm immobilize pH gradient (IPG) strip gels (pH 3-10, non-linear, BioRAD), which was rehydrated with 350 µl of rehydration buffer containing the sample for 24 h and isoelectric focusing was performed at 250 V for 30 min, followed by 1000 V for 30 min (with a gradient), 2500 V for 1.30 h (with a gradient) and 2500 V for 5 h. The first-dimensional isoelectric focusing gel electrophoresis (IEF) was conducted at 20ºC and the second dimensional sodium dedocyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% acrylamide gels using an OmniPAGE WAVE (Cleaver Scientific Ltd, UK). Two gels were simultaneously assayed with 35 mA and protein spots were visualized by coommassie staining.

Data analysis

Analysis of variance (ANOVA) was performed using Microsoft Excel (Windows 7) and mean separation was done with Least Significant Difference (LSD) at p=0.05 to compare the variation in characters measured related to stress tolerating ability between the varieties and treatments.

RESULTS AND DISCUSSION

Relative water content

The RWC is considered as the best integrated measurement of plant water status, and it represents the variations in water potential, turgor potential, and the osmotic adjustment (OA) of the plant (Bhushan *et al.,* 2007) though RWC vary due to differences in OA. The Fig. 1 illustrates the variation of RWC in both *Godaheenati* and *Pokkali* over a drought period of 1-5 days. Rice variety *Pokkali* showed a gradual reduction in the RWC during the whole experimental period, whereas *Godaheenati* showed a gradual reduction of RWC up to the third day of drought induction. On the fourth day after imposing drought, *Godaheenati* showed an increase in RWC followed by a reduction on the fifth day. However, there was no significant difference ($p < 0.05$) between RWC in both *Godaheenati* and *Pokkali* during the entire experimental period in this study.

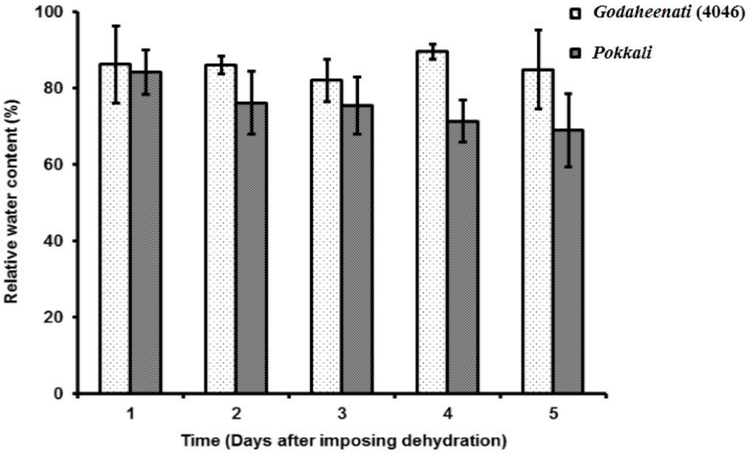

Fig.1. Effect of drought on the relative water content of rice varieties *Godaheenati* (4049) and *Pokkali*

Choudhary *et al*. (2009) screened four-weeks old rice seedlings against drought and all tested rice varieties showed a steady increase in RWC around 48-72 h (2-3 days). Thereafter, a gradual decline was reported during the initial stages. Furthermore, Chaudhary *et al*. (2009) stated that the increase of RWC during the first 2-3 days would be the cause of OA as a result of increased of proline content during the first 24-48 h. Our results showed that *Godaheenati* had an increase in RWC on the fourth day after imposing of the drought, which could be due to the same reason as suggested by Choudhary *et al*. (2009). However, *Pokkali* did not show similar results. This could be due to the varietal effects. According to Choudhary *et al*. (2009), the varietal differences of RWC during a stress period may be due to the different rates of stress development and expression of drought responses by different varieties. Kumar *et al*. (2014) stated that under water stress conditions, higher RWC values are recorded in drought stress tolerant rice genotypes compared to the susceptible genotypes. Therefore, it could be concluded that *Godaheenati* is expressing drought stress tolerance than *Pokkali.*

Total protein content

Altered gene expression during drought stress changes the regular metabolic activities and thus, creates variations in the total protein content of plants. Therefore, the change in total protein concentration is an indication of the stress response against abiotic stresses such as drought (Qureshi *et al.,* 2007). Further, certain proteins can get expressed only when exposed to specific stress conditions. Therefore, both qualitative and quantitative changes in proteins are expected when the plants are exposed to abiotic stresses. The changes in the total protein content during drought stress are an indicator of their drought tolerance ability defined by their genetic composition (Choudhary *et al*., 2009).

Fig. 2 illustrates the variation of total protein content in the leaves of rice varieties *Godaheenati* and *Pokkali* over the drought imposed period. *Godaheenati* showed a negligible change in the protein concentration on the first and second day after imposing drought stress. However, a severe reduction of total protein content was shown on the third day after imposing drought stress followed by a drastic increase on the fourth day and a further increase on the fifth day. As reported by Choudhary *et al.* (2009) and Qureshi *et al.* (2007), the changes in the total protein content of both *Godaheenati* and *Pokkali* during drought stress may be a result of altered gene expression in response to water deficit. The highest total protein content for *Godaheenati* was observed on the fifth day after imposing drought stress. *Pokkali* showed a gradual decrease of the total protein content in the first three days and a rapid increase on the fourth day after imposing drought stress. Rice variety *Pokkali* may have expressed the ability to withstand drought stress, however, the tolerance responses got initiated few days after the induction of drought stress. In this study, a comparatively higher protein concentration was observed in *Godaheenati*, which could be due to drought tolerance.

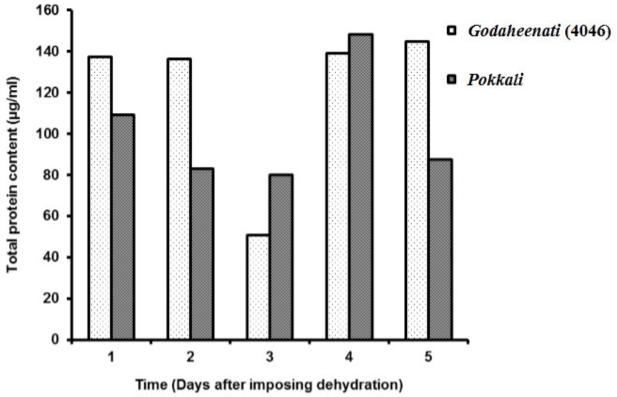

Fig.2. Effect of drought on total protein content in *Godaheenati* (4049) and *Pokkali*

Photosynthetic pigments

Both the varieties showed similar variation pattern in chlorophyll-a (Chl-a), chlorophyll-b (Chl-b) and carotenoid contents (Fig. 3A, B and C).The decrease in chlorophyll content under drought stress has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation. The previous findings showed that the chlorophyll content would either increase or decrease or even remain unchanged during drought stress depending on the duration and severity of the drought condition (Anjum *et al*., 2011).

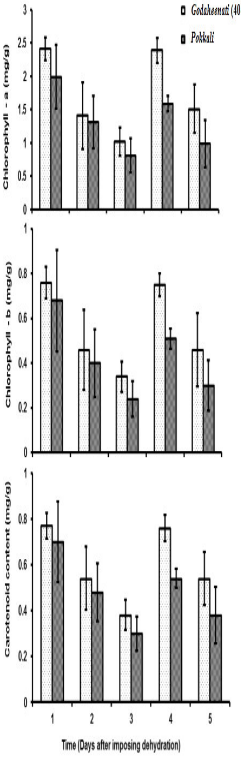

Fig.3. **Effect of drought on chlorophyll-a (A), chlorophyll-b (B) and carotenoid (C) in *Godaheenati* (4049) and *Pokkali***

Both *Godaheenati* and *Pokkali* showed a significant ($p<0.05$) gradual reduction of all three pigments during the first three days, and a rapid increase on the fourth day followed by a reduction on the fifth day (Fig. 3). Our results conclude that the dehydration stress causes the fluctuation of photosynthetic pigments in rice leaves over time. Furthermore, there is no significant difference ($p<0.05$) among the two tested traditional rice varieties in all three pigment concentrations during the drought period except on the fourth day. On the fourth day, *Godaheenati* showed a rapid increase of the photosynthetic pigments, which was significantly ($p<0.05$) higher than that of *Pokkali.* The results are also in agreement with Pandey and Shukla (2015), who revealed that the loss or reduced synthesis of photosynthetic pigments during drought stress is a common phenomenon, which is closely associated to reduction of plant biomass and yield.

Our results indicated that RWC in *Godaheenati* was reduced over the first three days and increased on the fourth day, following a similar variation pattern to that of Chl-a and Chl-b in the variety. Since plant cells reached their usual status due to the increase of water content, rapid increase of the chlorophyll content would be an evidence for the incidence of photosynthesis inside the plant. Choudhary *et al*. (2009) revealed that the increase of RWC during the first 48-72 h is due to proline accumulation in rice plants exposed to drought stress is to facilitate maintenance of regular metabolic activities and physiological processes *via* osmotic adjustment. Therefore, the increase of photosynthetic pigments on the fourth day in *Godaheenati* would be due to the increase of water status of the cell creating normal conditions inside the plant.

Furthermore, Choudhary *et al.* (2009) stated that higher carotenoid levels may result in an enhanced protection from damage caused by dehydration, preventing the production of reactive oxygen species. Therefore, increased carotenoid content on the fourth day would facilitate the recovery of damaged cells due to drought stress, that enabling those cells to proceed with photosynthesis.

Total proteome analysis of rice leaves by two-dimensional gel electrophoresis

The number of protein spots reported in the present study (Fig. 4A and 4B) is lower compared to that reported by Ali and Komatsu (2006), where a total of 698 proteins were identified from two-week old rice leaves exposed to 2 - 6 days of drought stress. Abbasi and Komatsu (2004) reported the spotting of 500 rice leaf sheath proteins in a total protein extracts of two-weeks old seedlings exposed to a 24 h salt stress. However, in the present study only 50 protein spots were detected which could be due to either the degradation of proteins during extraction, gel assaying conditions and/or less sensitivity of the used staining process.

According to Ali and Komatsu (2006) and Zhao *et al.* (2005), protein spots obtained from drought stress exposed two-week old rice leaf sheaths were separated in a basic pH gradient of 5.0 - 9.0 and 5.0 -7.0, respectively. The present study reported the separation in a pH range of 5.0 – 8.0. Zhao *et al.* (2005) showed that most of the rice leaf proteins in field grown rice plants had a size range of 14 to 66 kDa. The few protein spots obtained in this study also had a size range of 15-75 kDa.

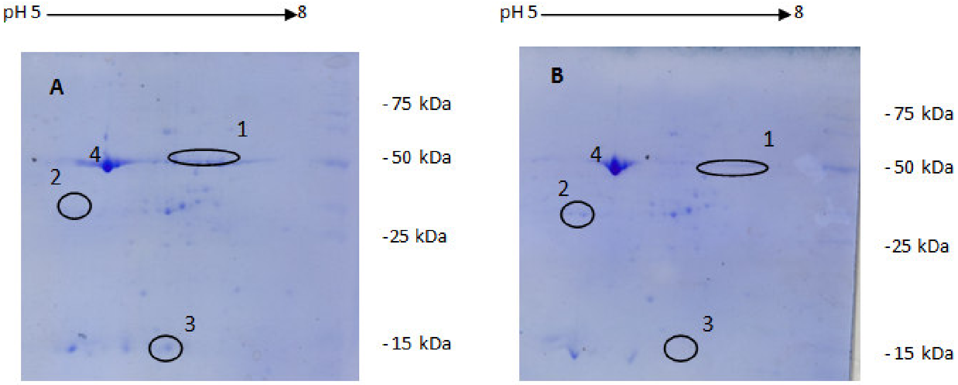

Fig. 4. **Differentially expressed proteins of two-week old rice leaves of *Godaheenati* (4049): control (A) and drought affected (B) (A representative fraction of the 12% acrylamide gel)**

Proteins 1 and 3 (Fig. 4) were up-regulated in control plants (Fig. 4A) but not in drought affected plants (Fig. 4B). Protein 2 (Fig. 4) was up-regulated in drought affected plants (Fig. 4B). Ali and Komatsu (2006) identified 12 proteins that clearly responded to drought as early as in 2-5 days after imposing stress. Among them, 10 proteins were up-regulated during drought stress, while two were down-regulated. One such down-regulated protein was identified as RuBisCO small chain (100) (N-XQVWPIEGIK), which was 17 kDa in size and was spotted in a pH of 6.4. In the present study, protein 3 was in the pH range of 6.0 – 7.0 and the size was around 15 kDa. Even though this protein was not further analyzed due to unavailability of protein identification techniques, it is possible to anticipate that protein 3 is small subunit of RuBisCO.

During this study, a large protein spot (4 on Fig. 4) was identified around 50 kDa size in pH 5.5 region. Ali and Komatsu (2006) identified RuBisCO large chain (100) protein (N-blocked I-MTLGFVDLLR) at pH 6.0 with a 50 kDa size. Zhao *et al*. (2005) also identified 52 kDa size protein spot around pH 6.5 as RuBisCO large subunit in field grown rice plants. Therefore, protein 4 could be a RuBisCO protein based on similarities of the size and the pH range. However, further studies such as protein fingerprinting by mass spectrometry are needed to confirm our findings.

Ali and Komatsu (2006) further identified a protein of 40 kDa size in of pH 5.6 called photosystem II oxygen evolving complex protein (N-EGVPPXLTFD) and stated of its role in light harvesting, which could potentially yield crop plants that are more resistant to environmental stress and prevent inhibitory effects on photosynthesis. In the present study, protein 2 (Fig. 4) was up-regulated during drought stress and appeared around pH 5.0 witha size of 37-40 kDa thus suggesting that protein 2 could possibly be the same as described by Ali and Komatsu (2006).

Wasala *et al*. (2015) screened forty six rice accessions, including traditional Sri Lankan rice germplasm, for drought stress by inducing drought conditions at the reproductive stage. Interestingly, *Godaheenati* (4049) performed well under induced drought conditions. Our findings also have revealed that *Godaheenati* (4049) performed well under drought even at germination (unpublished data by the author) and vegetative stages. Hence, further screening of *Godaheenati* (4049) for drought tolerance would be an advantage to produce drought tolerant varieties through rice breeding programmes.

CONCLUSION

The RWC, photosynthetic pigment concentration and total protein content of rice leaves fluctuate in the vegetative stage in both *Godaheenati* (4049) and *Pokkali* due to drought stress. The two rice varieties studied responded differently for RWC, protein concentrations and pigment levels indicating the varietal difference in stress development. Results on RWC revealed that *Godaheenati* (4049) may have a higher drought tolerance than *Pokkali*. Increase of photosynthetic pigments on the fourth day in *Godaheenati* (4049) could be due to the recovery of photosynthesis in the affected plants owing to plant cells reaching a normal water status.

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