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Research article

Starch accumulation in rice grains subjected to drought during grain filling stage

Prathap V[a], Kishwar Ali[a], Archana Singh[a], Chandrapal Vishwakarma[b], Veda Krishnan[a], Viswanathan Chinnusamy[b], Aruna Tyagi[a,*]

[a] Division of Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi, India
[b] Division of Plant Physiology, ICAR-Indian Agricultural Research Institute, New Delhi, India

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ABSTRACT

Drought stress during the grain filling stage severely affects the quality and quantity of starch in rice grains. The enzymes such as ADP-glucose pyrophosphorylase (AGPase, *EC* 2.7.7.27) and starch synthase (SS, *EC* 2.4.1.21) are the key regulatory enzymes involved in the starch biosynthesis. In this study, the activity of the AGPase and starch synthase (SS) was correlated with the qualitative and quantitative parameters such as sucrose, starch, amylose, amylopectin, and resistant starch in leaves, roots, and grains of drought tolerant (N22) and drought susceptible (IR64) cultivars under applied water deficit stress (WDS). Drought stress enhanced the remobilization of stored starch from leaves to developing rice grains which was positively correlated with a decrease in the starch and starch synthase activity in leaves. Starch accumulation in developing grains was positively correlated with an increase in the AGPase and SS activity under drought. It was found that starch, amylopectin, and sucrose content in developing grains increased under water deficit stress (WDS), while amylose content decreased in both the varieties. However, in leaves, the SS activity decreased while AGPase activity was found to be increased under WDS in both varieties. Decreased starch content in matured grains was due to shortening of grain filling stage as drought stress caused early plant senescence. Yield reduction under drought was more in susceptible variety IR64 as compared to tolerant genotype N22.

1. Introduction

Plant are sessile as they can't move from one place to another. Under field conditions, plants are continuously subjected to various biotic and abiotic stresses. Abiotic stresses are estimated to cause more than 50% of crop yield losses (Mawlong et al., 2014; Ramalingam et al., 2015). To improve crop productivity, it is necessary to understand the mechanism of plant responses to drought with the goal of improving crop performance in the vast areas of the world where rainfall is limiting or unreliable. It is estimated that 50% of the world rice production is affected by drought (Soren et al., 2010). About 90% of the dry matter of rice grain is starch (Yoshida, 1972; Cao et al., 1992). Starch, a polymer of D-glucose linked at (α 1–4) glycosidic bond, usually consists of a linear fraction, amylose, and a branched fraction, amylopectin. The X-ray diffraction experiments suggest that about 70% of the starch granule mass is regarded as amorphous (mainly amylose) and remaining 30% as crystalline or semi-crystalline (amylopectin) (Sajilata et al., 2006). Starch content in rice grain ranges from 81.23 to 92.73% (Omar et al., 2016). The amylose to amylopectin ratio varies from plant to plant, amylose generally ranges from 20 to 25% and amylopectin is about 75–80% by weight, whereas rice typically has 22% of amylose and 78% amylopectin (Tester et al., 2004; Jane, 2009). The grain filling in cereals depends on two carbon sources; photosynthetic carbon assimilation and remobilization of stored reserves (Yoshida, 1972; Kobata et al., 1992; Tyagi, A., and Chandra, A. 2006). The reserve starch contributes about 1/3rd of the final grain weight of the rice (CocK and Yoshida, 1972). The water deficit stress during the grain filling stage enhances the plant senescence and remobilization of carbon store from vegetative tissue to grains that could increase the grain starch content (Yang et al., 2002). The water deficit stress, promotes the reallocation of stem reserve of prefixed C14 to the grain, thereby reducing the grain filling period, and increasing the rate of grain filling (Yang et al., 2001). The sucrose is the major transportable form of disaccharide in the plants involved in photosynthate trafficking.

Among 33 enzymes involved in starch biosynthesis pathway; starch synthase (EC 2.4.1.21) and adenine diphosphoglucose pyrophosphorylase (EC 2.7.7.27), are key regulatory enzymes (Nakamura et al., 2002). The whole starch biosynthetic pathway separated from the

* Corresponding author.
E-mail address: arunatyagi19@yahoo.com (A. Tyagi).

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cytosolic sucrose biosynthetic pathway exclusively resides in the chloroplast. The immediate precursor for the starch synthesis in the grain is ADP-glucose which is synthesized from glucose-1-phosphate and ATP, and the reaction is catalyzed by ADP-glucose pyrophosphorylase (ADPG-PPase or AGPase) (Neuhaus et al., 2005; Streb et al., 2009; Stitt and Zeeman, 2012). There are two types of starch synthase enzymes which are classified based on their localization; soluble starch synthase (SS) and granule-bound starch synthase (GBSS) which are involved in amylopectin and amylose synthesis, respectively (Delrue et al., 1992). However both SS enzymes utilize ADPG as the substrate, but a non-physiologically high concentration of the granule-bound enzyme may use UDPG as well. (Neuhaus et al., 2005; Streb et al., 2009; Stitt and Zeeman, 2012). Soluble starch synthase (SS) is highly sensitive to water stress and it responded earlier, and to a greater extent than the other enzymes (Ahmadi and Baker, 2000). The activity of soluble starch synthase (SS) and starch branching enzyme (SBE) in the grains were substantially enhanced, while ADP- glucose pyrophosphorylase activity was enhanced with starch assimilation rate (SAR) with a smaller coefficient under water deficit in rice and wheat (Yang et al., 2004).

The starch content is a key factor affecting grain quality and quantity but not much information is available on starch accumulation and related enzyme activities under WDS during grain filling stage in rice. So far there are no reports on the effect of WDS on quantitative and qualitative changes in starch accumulation during grain filling stage. The present study was aimed to unveil link between the key regulatory enzymes involved in starch biosynthesis and starch quality and quantity in two contrasting i.e. drought tolerant (N22) and susceptible (IR 64) rice genotypes during grain filling stage.

2. Material and methods

2.1. Plant materials and cultivation

The present study was conducted in the Division of Biochemistry, Indian Agricultural Research Institute, New Delhi. Two contrasting rice genotypes N22 (drought tolerant) and IR64 (drought susceptible), were grown under field conditions. The experiments were carried out in replicates. The transplanting was done 30 days after sowing. The well-watered (WW) pots were irrigated regularly, whereas water deficit stress (WDS) was induced by withholding irrigation during the booting initiation (45 days after transplanting) for 21 days. After drought treatment, re-watering was done when the plants showed the symptoms like chlorosis, drying and rolling of leaves, etc.

2.2. Sampling

The samples for the present study i.e. leaves, roots and developing grains were collected before re-watering. The samples were dipped in liquid nitrogen and stored at −80 ^{O}C. Matured grains were collected from the field after drying of the harvested crop.

2.3. Relative water content

The relative water content of leaf was measured on clear days at mid-day (11:30 am) after stress treatment. Leaf samples from WW and WDS plants were weighed to record fresh weight and then saturated in water for 4 h. The turgid weight of the leaf samples was recorded. The leaf samples were packed in butter paper bag and oven dried at 65 °C for 48 h. The dry weight of samples was taken after ensuring that the samples were completely dried up (Barrs and Weatherley, 1962). Relative water content was calculated as follows:

Relative water content = (Fresh weight (FW) – Dry weight (DW) / Turgid weight (TW) – Dry weight (DW)) X 100

2.4. Soil moisture content

The soil moisture content was measured in both WW and WDS condition, before re-watering the WDS plants. The soil samples were collected in the pre-weighed boxes using the sampling auger tool. From each plot, two soil samples (top 15 cm and below 15 cm) were collected in triplicate. Soil samples from WW and WDS plots were weighed to record fresh weight and kept in oven to dry at 105 °C–110 °C for overnight. The dry weight of the soil samples was recorded after ensuring that the samples were completely dried up. Soil moisture content was calculated as follows (Schmugge et al., 1980):

Soil moisture content = (Weight of wet soil (Ww) – Weight of dry soil (Wd) / Weight of wet soil (Ww)) X 100

2.5. Photosynthetic rate (Pn)

Photosynthetic rate was measured on flag leaves of both the varieties under WW and WDS on day before re-watering, while for recovered plants Pn was measured on four days after re-watering using a portable photosynthesis system (Li-COR LI-6200), attached to an infrared gas analyzer (Li-COR LI-6250) (Al-Khatib, K., and Paulsen, G. M. 1990).

2.6. Estimation of starch, amylose and amylopectin content

Starch and amylose content was determined according to Clegg (1956) and Williams et al. (1970), respectively. For the starch estimation, 100 mg flour from samples was extracted with hot 70% ethanol. After centrifugation, the residue was washed with 70% hot ethanol and dried to remove the excess of ethanol. To the residue, 5 ml of water and 6.5 ml of 52% perchloric acid was added and shaken for 5min followed by centrifugation at 10000 rpm for 10 min. This step was repeated for two to three times using 5 ml fresh perchloric acid and supernatant was pooled, made up the volume to 100 ml with DDW. The 5 ml of anthrone reagent was added to 0.1 ml aliquot of extract for glucose estimation. The intensity of the color formed was measured at 620 nm after heating on boiling water bath for 10 min and rapidly cooled. The glucose concentration was estimated using a standard curve prepared from different glucose concentration.

For amylose content estimation, 100 mg of finely powdered rice sample was stirred with 10 ml of 0.5 N KOH thoroughly with a magnetic stirrer for 10 min and volume was made up to 100 ml. To the 10 ml of the aliquot, 5 ml of 0.1 N HCl and 0.5 ml of Iodine reagent was added. The intensity of the blue color formed was measured at 625 nm after making up the volume to 50 ml using DDW. The amount of amylose was calculated from a standard curve prepared by using a standard solution containing different amount of amylose (0.2–2 mg) and amylopectin (0.8–8 mg) keeping the ratio of amylose and amylopectin constant (1: 4 w/w). The amylopectin content was determined by subtracting the amylose content from the total starch content.

2.7. Estimation of resistant starch

The resistant starch (RS) was estimated using the Megazyme kit. Finely ground samples were incubated at 37 °C for 16 h in a shaking water bath along with the pancreatic α-amylase and amyloglucosidase (AMG). The hydrolysis reaction was terminated by the addition of an equal volume of ethanol and the RS which was later recovered as a pellet by centrifugation. The pellet was washed with ethanol (50% v/v), followed by centrifugation. The pellet was dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution was neutralized by acetate buffer. The AMG hydrolyzed starch to D-glucose which was quantitatively measured with glucose oxidase or peroxidase reagent (GO/POD). Absorbance was read at 510 nm

against the reagent blank. The total RS was expressed in percentage.

2.8. Estimation of sucrose

Sucrose content was determined according to Finley and Fellers (1973) modified the anthrone method. For the sucrose estimation, 1 g of powdered sample was boiled with 50 ml of 80% ethanol in 100 ml volumetric flask for 15 min. After cooling extract was diluted to 100 ml with 80% ethanol. To the 1 ml of aliquot added 9 ml of Fehling solution and heated in a boiling water bath for 15 min. Cooled it to room temperature and from which 1 ml of the aliquot was taken and 10 ml of anthrone reagent was rapidly added to it. It was held for 30 min at 40 °C. Simultaneously reagent blank and a standard of 1 ml of 15% w/v of sucrose were also run side by side. The absorbance was read at 610 nm against a reagent blank. Sucrose content was calculated as follows:

Percent of sucrose = $(A_{sample} / A_{standard})$ X 15

2.9. Enzyme extraction and assay

The chemicals and enzymes for activity assay were obtained from Sigma Company. Both the enzyme assays were optimized for substrate concentration and pH with respect to protein concentration and incubation time. Protein estimation was done as per the Bradford (1976) method using BSA as standard. Enzyme activity was expressed in nm/min/mg. For SS extraction,1g of the sample was homogenized in 10 ml of ice-cold extraction medium containing HEPES buffer (0.05 M, pH 7.0), EDTA (10 mM), DTT (5 mM) and 1% insoluble polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000 g for 20 min at 4 °C, after being filtered through four layers of cheesecloth. The supernatant was used for the estimation of starch synthase activity. For the assay of SS activity, 0.3 ml of the reaction mixture (RM) consisting of glycine Buffer (pH 8.3, 0.08 M), EDTA (4 mM), amylopectin (50 mg/ml) and glutathione (40 mg/ml) was taken and reaction was initiated by the addition of enzyme extract as the last component. The reaction mixture was then incubated in a shaking water bath at 37 °C for 4 h. After 4 h, 20 µl each of phosphoenol pyruvate (10 mM) and pyruvate kinase (10 units, freshly diluted with 0.1 M $MgSO_4$) were added. The contents were incubated again at 37 °C for another 15 min. After the end of incubation period, 0.2 ml of dinitriphenyl hydrazine reagent was added. After 5 min 0.2 ml of 10 N NaOH was added followed by the addition of 2 ml of 95% ethanol. The contents were mixed, centrifuged and absorbance of brown color formed was measured at 520 nm (Leloir et al., 1961). The enzyme activity was expressed as the change in OD at 520 nm/min/mg protein.

For AGPase extraction, samples were taken in a pre-chilled mortar and homogenized thoroughly with 10 ml of ice-cold extraction buffer containing Tris-HCI buffer (0.1 M, pH 7.9), 5 mM glutathione and 1 mM EDTA. The homogenate was centrifuged at 15,000 g for 20 min at 4 °C, after being filtered through four layers of cheesecloth. The supernatant was used for the estimation of AGPase activity. For the AGPase activity assay, 3 ml of the reaction mixture (RM) consisting of Tris-HCI buffer (0.4 M; pH 7.9), $MgS0_4$ (0.06 M), Cysteine (48 mM), BSA (2.4 mg/ml), ADPG (4 mM), Sodium pyro- phosphate (20 mM), 3-Phosphoglycerate (PGA, 30 mM), Glucose-6-Phosphate Dehydrogenase (4 units), phosphoglucomutase (4 units) and enzyme extract (0.1 ml) was taken in a test tube and the reaction was initiated by the addition of NADP as the last component. The absorbance was read at 340 nm in a spectrophotometer at 30 s interval at 30 °C (Turner, 1969). The enzyme activity was expressed as the change in absorbance (OD) at 340 nm/min/mg protein.

3. Results

3.1. Relative water content (RWC)

To assess the extent of stress level, RWC was measured under well-watered (WW) and WDS. In N22, RWC values were 93.71% and 78.31% in N22 whereas in IR64 values were 87.29% and 67.3% under WW and WDS respectively (Fig. 1). The water deficit treatment substantially reduced RWC in both the varieties. However, IR64 showed a more significant reduction in RWC (20%) than the N22 (15.4%). A significant reduction in RWC in both the varieties under the stress indicated that plant experienced drought during the grain filling stage (Fig. 1).

3.2. Soil moisture content

The soil moisture content was found to be more or less similar in the N22 and IR64 under controlled as well as in drought condition. The soil moisture content for N22 and IR64 under the controlled condition was 34% and 34.9%, respectively, in top 15 cm, while it was 31.8% and 33.3% in below 15 cm soil (Fig. 2). Drought stress reduced the soil water potential to 5.21% and 5.24% in the top 15 cm soil of N22 and IR64, respectively, while it reduced to 7.48% and 7.06% in below 15 cm soil. Both the varieties were exposed to the same level of drought stress during the grain filling stage (Fig. 2).

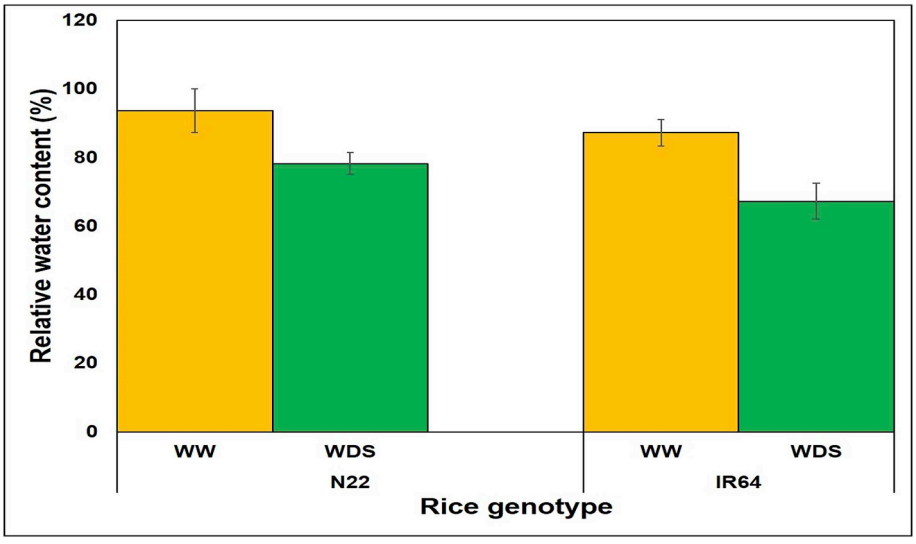

Fig. 1. Relative water content (RWC) of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions.

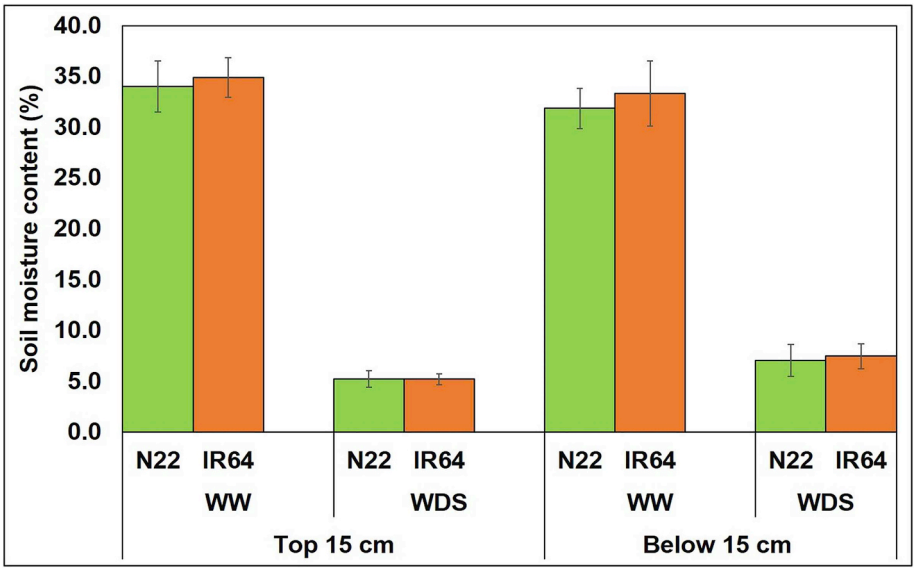

Fig. 2. Soil moisture content (RWC) of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions from top 15 cm and below 15 cm.

3.3. Photosynthetic rate (Pn)

The photosynthetic rate was significantly reduced in both the varieties under the drought stress. The photosynthetic rate reduced from 24.4 to 10.36 (μm/m^2/s) and 24.26 to 11.86 (μm/m^2/s) in N22 and IR64, respectively under the drought stress. However, after re-watering Pn increased to 15.13 and 12.63(μm/m^2/s) in N22 and IR64, respectively. The drought stress during the grain filling stage significantly reduced the photosynthetic rate in both cultivars (Fig. 3). However, N22 recovered well and showed better Pn after rewatering compared to IR64.

3.4. Starch

WDS affects starch content which is a key factor affecting grain quality and quantity. The starch content was estimated in leaves, roots, developing grains and matured grains. Maximum starch content was observed in matured grains followed by developing grains, leaves, and roots (Fig. 4 a and b). Almost negligible starch content was observed in roots, however, it decreased from 0.28% to 0.16% and from 0.29% to 0.19% under WDS in N22 and IR 64 respectively. A decrease in starch content was observed in leaves, roots and matured grains under WDS whereas an increase was observed in developing grains. Starch content in leaves decreased from 2.56% to 1.37% in IR64 and from 2.12% to 2.08% in N22 under WDS. The starch content in matured rice grains decreased from 88.12% to 76.35% in N22, whereas in IR64, it decreased from 90.1% to 72.5% under WDS. A significant reduction in starch content was observed in both the varieties when the plant was exposed to stress during grain filling stage, however, percent reduction was more in IR64 (17.6%) as compared to N22 (11.77%). At the same time, the starch content of developing grains increased under drought in both the varieties. The starch content increased from 53.6% to 62.5% in N22 and 48.6% to 55.6% in IR 64 under WDS as compared to WW (Fig. 4b). However, the increase in starch content in developing grains

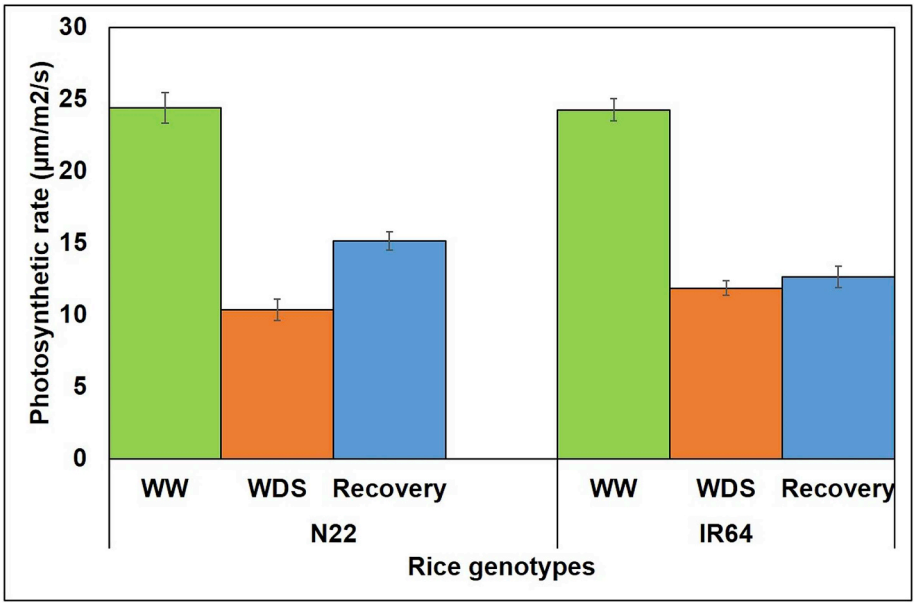

Fig. 3. Photosynthetic rate of rice genotypes- N22 and IR64 under well-watered (WW), water deficit stress (WDS) and after recovery.

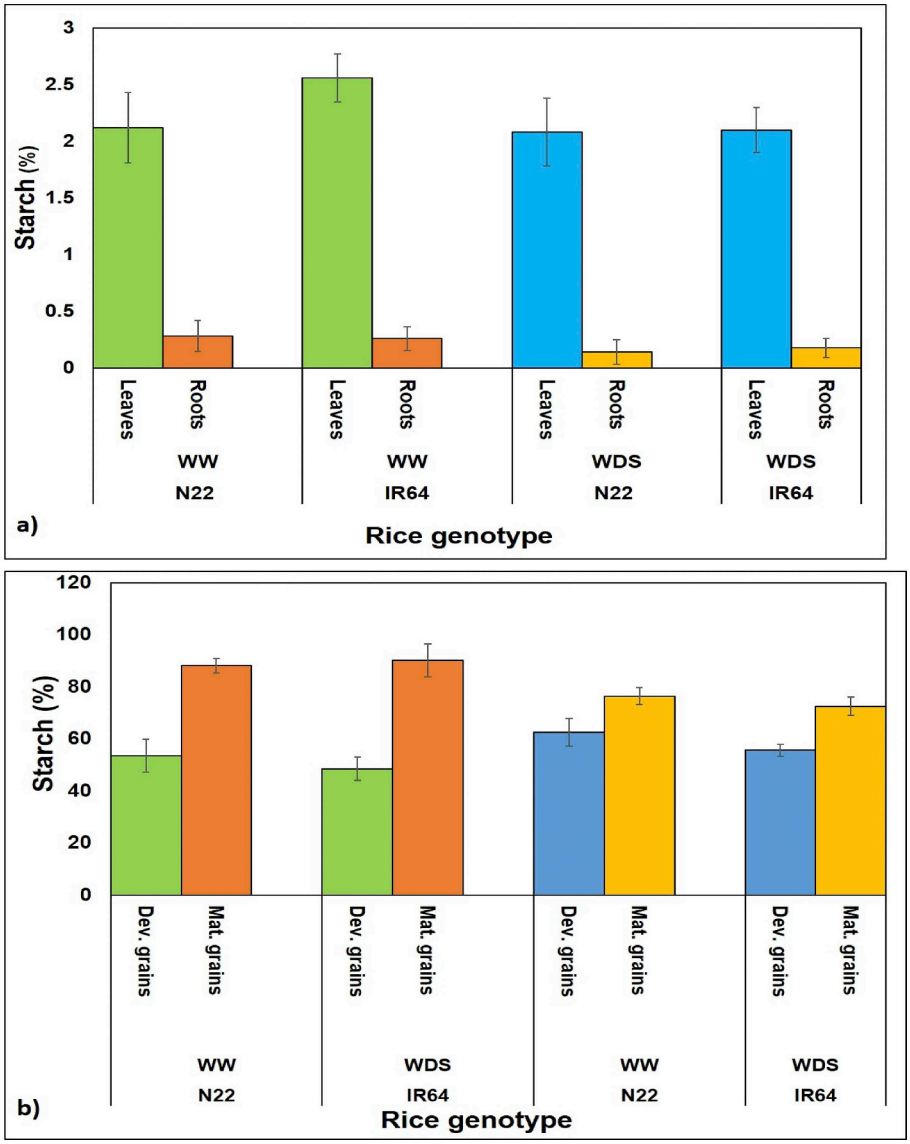

Fig. 4. Starch content of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions. a) Leaves and roots b) Developing grains and matured grains.

was higher in N22 (8.9%) as compared to IR 64 (7%).

3.5. Amylose and amylopectin

The amylose content was more in matured grains than the developing grains in both cultivars under the WW and WDS (Fig. 5). In the developing grains, amylose content decreased from 9.95% to 6.81% and 9.91% to 7.25% under WDS in N22 and IR64 respectively. In matured grains, amylose content was found to decrease from 17.92% to 13.41% in N22, whereas in case of IR64, it decreased from 17.01% to 13.92%, under WW and WDS, respectively. The amylose content significantly reduced under the WDS in both N22 (4.51%) and IR64 (3.1%).

The amylopectin was estimated in developing and matured rice grains (Fig. 6). The amylopectin content in developing grains was found to be increased from 43.64% to 55.71%, and from 38.65% to 48.37% under WDS in N22 and IR64 respectively. The increased amylopectin content under WDS was more in N22 (12.07%) than IR64 (9.72%). The matured grains showed a reduction in amylopectin content from 70.2% to 62.94% in N22 whereas, in IR64, the decrease was from 73.09% to 58.58% under WDS. The decrease in amylopectin content under WDS in matured grains was 7.26% and 14.51% in N22 and IR64, respectively. However, IR64 showed more reduction in amylopectin than N22 (Fig. 6).

As very low and almost negligible starch content was observed in leaves and roots respectively, no detectable amylose and amylopectin fractions were observed in leaves and roots as the content of amylose and amylopectin was lower than the sensitivity of the method employed to estimate it.

3.6. Sucrose

The sucrose accumulation trend in leaves, roots and developing grains under WW and WDS was estimated. The sucrose content was high in developing grains followed by leaves and roots (Fig. 7). The sucrose content decreased in leaves and roots while it increased in developing grains under WDS. The sucrose content decreased from 2.21% to 1.13% in N22 and from 2.1% to 0.62% in IR64 leaves when WW plants were subjected to WDS during the grain filling stage. However, there was no significant difference in the reduction of sucrose under WDS between two varieties. At the same time, it was also found that in developing grains the sucrose content increased from 3.05% to

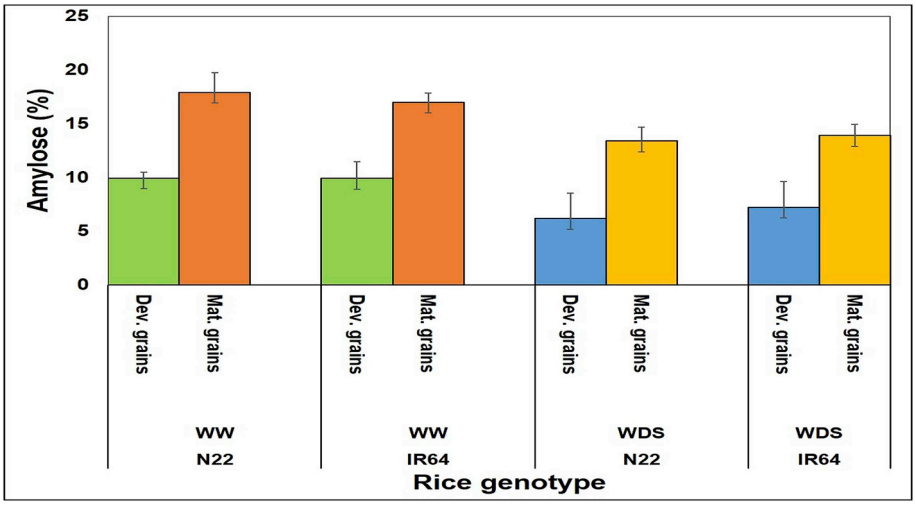

Fig. 5. Amylose content in developing grains and matured grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions. Amylose content was not detectable in leaves and roots.

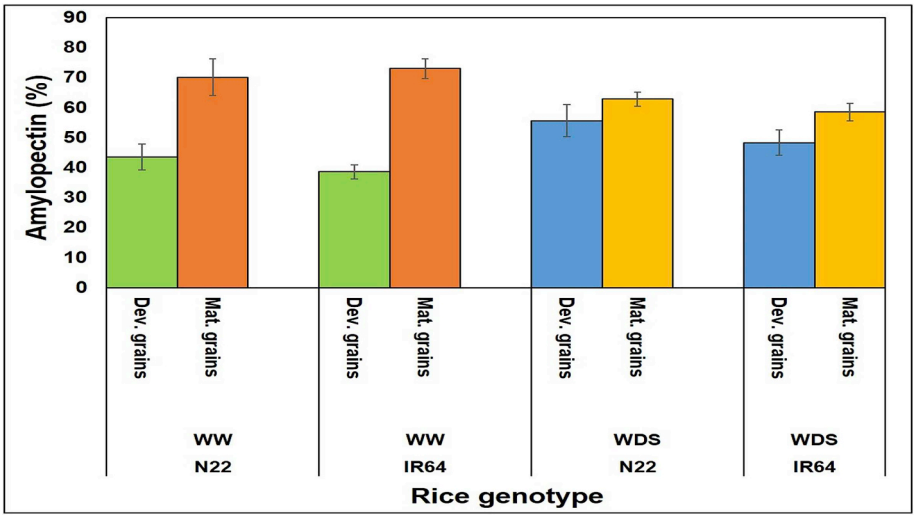

Fig. 6. Amylopectin content in developing and matured grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions. Amylopectin content was not detectable in leaves and roots.

5.33% in N22 and 3.24% to 4.78% in IR64 under WDS (Fig. 7). Increase in sucrose accumulation was more in N22 (2.28%) than IR64 (1.54%).

3.7. Resistant starch (RS)

The accumulation of resistant starch in developing and matured grains was affected under WDS during grain filling (Fig. 8). However, results showed that RS accumulation in developing grains was much higher as compared to matured grains. The resistant starch decreased from 5.41% to 4.99% and from 3.73% to 3.07% in IR64 and N22 developing grains under WDS (Fig. 8). The RS content significantly differed between N22 and IR64. However, there was no significant difference in the reduction of RS accumulation under WDS in both varieties. The RS content of matured grains too decreased under WDS and was more in N22 than IR64 (Fig. 8). The RS content of matured grains decreased from 2.88% to 2.33% in N22 and 1.69% to 0.91% in IR64, under WDS.

3.8. Change in enzyme activity

The activity of two key regulatory enzymes of starch biosynthesis pathway was examined in leaves and grains of rice under WW and WDS conditions. Both SS and AGPase activities were determined by the indirect method as mentioned earlier.

3.8.1. SS activity

The SS activity was found to be higher in developing rice grains of IR64 than N22, which is 1.73 (nm/min/mg) in N22 and 2.16 (nm/min/mg) in IR64 (Fig. 9). However, the SS activity in developing rice grains increased under WDS from 1.73 to 2.56 (nm/min/mg) in N22 and 2.16 to 2.37(nm/min/mg) in IR64. The SS activity in leaves decreased from 1.32 to 0.93 (nm/min/mg) in N22 and 0.95 to 0.85 (nm/min/mg) in IR64, under WDS (Fig. 9). There was no significant reduction in SS activity under WW and WDS in the leaves of both the varieties, while there was a significant difference in activity of enzyme between the varieties. The SS activity was not detected in roots as starch content in roots was very low.

3.8.2. ADP glucose pyrophosphorylase (AGPase) activity

The ADP glucose pyrophosphorylase (AGPase) activity was determined in leaves and developing grains. The AGPase activity was more in developing grains (1.2 ± 0.5) than leaves (0.25 ± 0.01) (Fig. 10). The AGPase activity in developing rice grains increased from 1.02 (μm/min/mg) to1.27 (μm/min/mg) in N22, and from 1.27 to 1.41

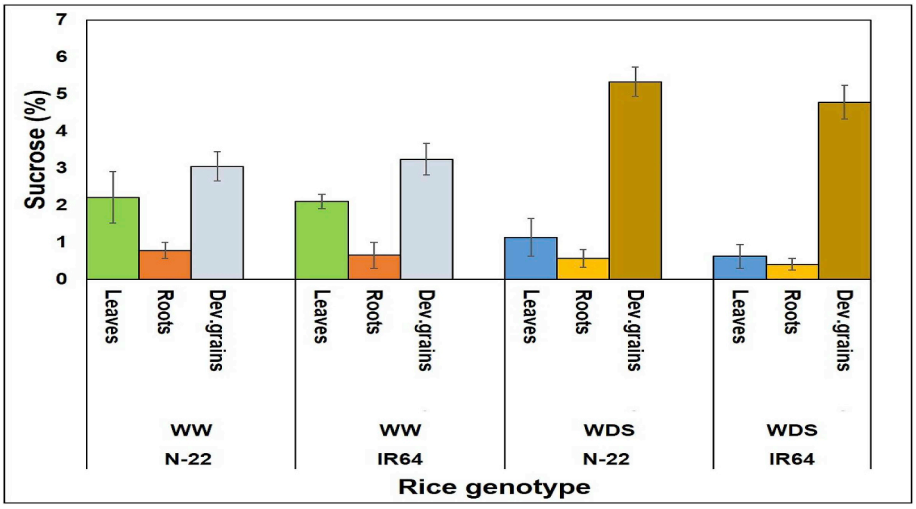

Fig. 7. Sucrose content in leaves, roots and developing grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions.

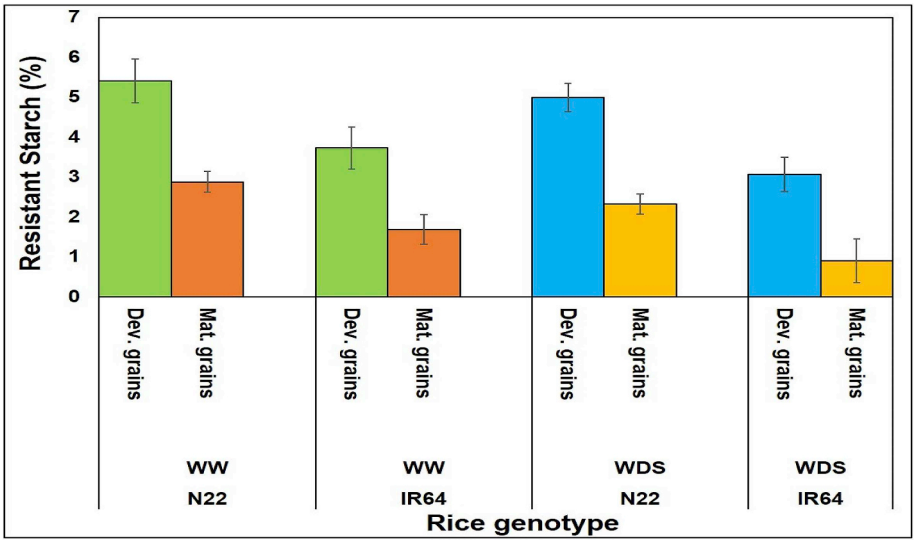

Fig. 8. Resistant starch content in developing grains and matured grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions.

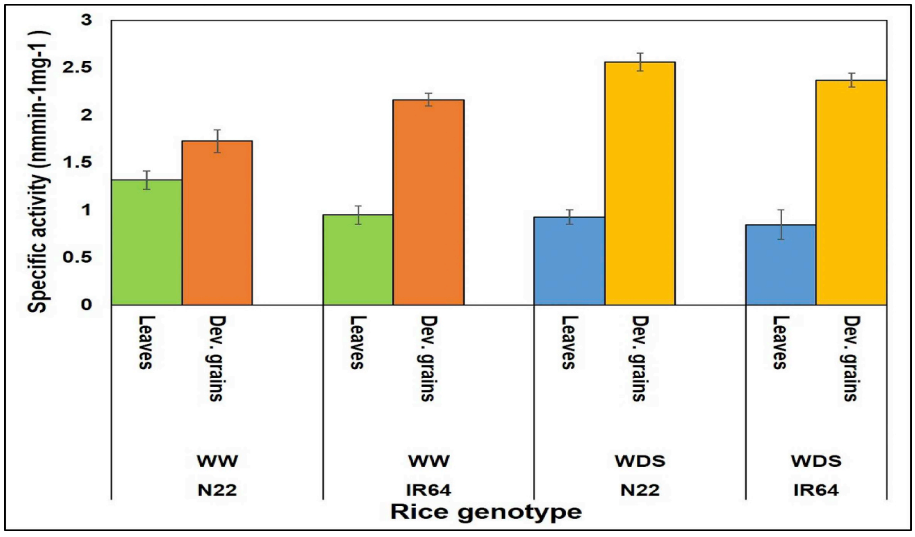

Fig. 9. Starch synthase activity in leaves and developing grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions. Starch synthase activity was not detectable in roots.

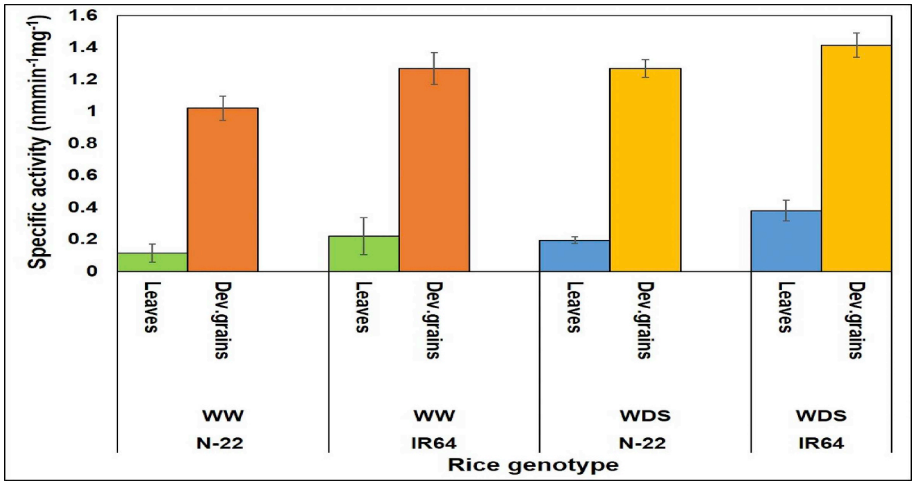

Fig. 10. AGPase activity in leaves and developing grains of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions. The AGPase activity was not detectable in roots.

(μm/min/mg) in IR64 under WDS. The results showed no significant difference in the increased AGPase activity between N22 and IR64 (Fig. 10).

Similarly, in leaves also, AGPase activity increased from 0.115 to 0.195 (μm/min/mg) in N22 and 0.22 to 0.39 (μm/min/mg) in IR64 under WDS (Fig. 10). However, there was no significant difference in change in the activity under WDS in both the varieties. These results indicate that the AGPase was differentially regulated under WDS in the leaves and developing grains of rice during the grain filling stage.

3.9. Drought-induced morphology and size variations of starch granules

The grain yield was measured after drying of grains from 20 harvested plants. The weight was 234.9 g and 147.25 g in N22 while in IR64, the weight was 317.3 g and 136.45 g under WW and drought stress respectively. The decrease in grain yield was by 37.31% (87.65 g) and 56.99% (180.85 g) under WDS in N22 and IR64 respectively. In IR64 grain yield reduction was 19.68% (93.2 g) more than the N22 (Fig. 11).

3.10. Grain yield under WDS

To evaluate the morphological features of starch granules of two contrasting rice varieties, starch granules were viewed and revealed by the scanning electron microscope (Fig. 12). The changes in the starch granules of both the varieties under well-watered and drought conditions were observed using a scanning electron microscope (SEM). The results showed that drought treatment clearly induced ultrastructural changes in the arrangement of starch granules in both rice varieties as compared to the WW. The drought-induced effect on morphological changes and arrangement of starch granules were clearly visible. The morphological characteristics such as the arrangement of starch granules, shape and size of the starch granules exhibited differences. Drought stress affects the packaging of starch granules thereby affecting the starch composition. The starch granules were bold, round to oval shaped, structured, compact and intact under WW (Fig. 12A1, and 12B1) as compared to defragmented, irregular in shape, loosely packed, pleated and less numbered under drought stress (Fig. 12A2, and 12B2). However, compared to starch granules of N22 under drought, starch granules of IR64 under drought were less disintegrated and loosely arranged.

3.11. Correlation analysis

Correlation analysis of all the parameters revealed a positive correlation of WDS with sucrose content and negative correlation with amylose, resistant starch, and yield (Table.1). A positive correlation was observed between starch and sucrose; amylopectin and SS activity.

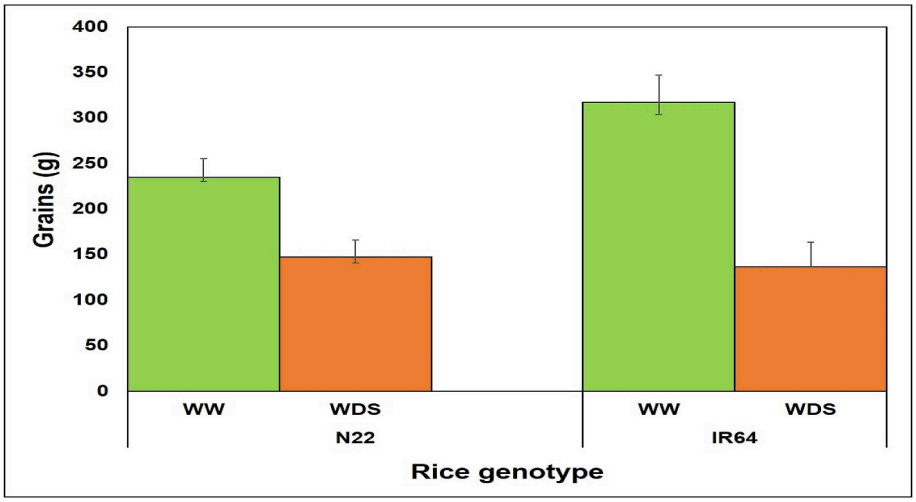

Fig. 11. Grain weight of twenty plants of rice genotypes- N22 and IR64 under well-watered (WW) and water deficit stress (WDS) conditions.

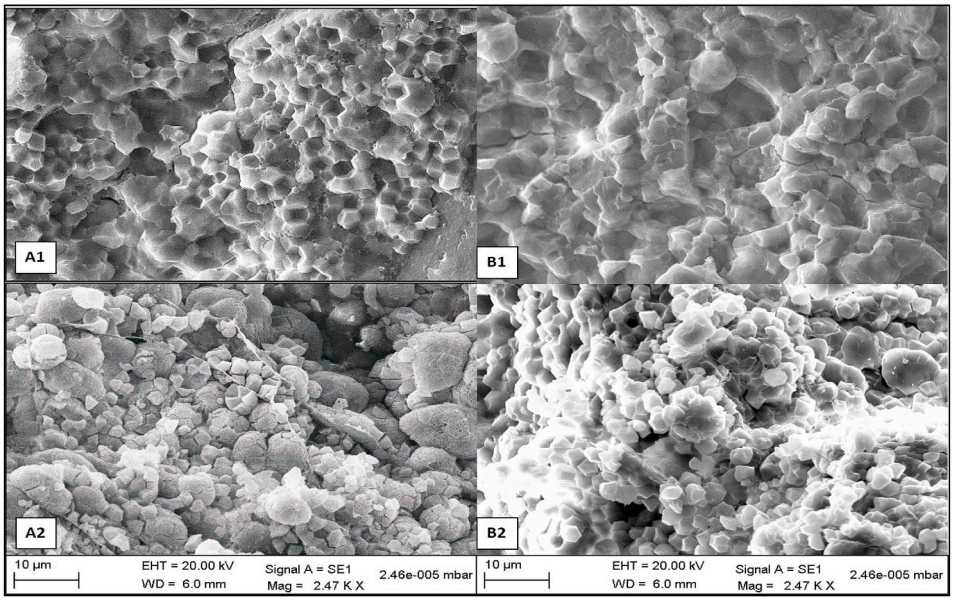

Fig. 12. Scanning electron micrographs of endosperm starch granules of N22 and IR64 under WW (A1 & B1) and WDS (A2 & B2) conditions.

Table 1
Correlation table for physio-biochemical parameters of matured grains under WDS.

	RWC	Starch	Sucrose	Amylose	Amylopectin	Resistant starch	SS activity	AGPas activity	Total soluble protein	20 plant grain weight
RWC	1									
Starch	−0.325	1								
Sucrose	−0.747[b]	0.788[b]	1							
Amylose	0.858[b]	−0.726[b]	−0.885[b]	1						
Amylopectin	−0.482	0.985[b]	0.868[b]	−0.830[b]	1					
Resistant starch	0.686[a]	0.427	−0.093	0.311	0.269	1				
SS activity	−0.321	0.632[a]	0.725[b]	−0.390	0.640[a]	0.332	1			
AGPas activity	−0.281	0.070	0.473	−0.072	0.117	−0.041	0.766[b]	1		
Total soluble protein	−0.414	0.015	0.454	−0.126	0.086	−0.183	0.748[b]	0.960[b]	1	
20 plant grain weight	0.746[b]	−0.787[b]	−0.818[b]	0.884[b]	−0.858[b]	0.073	−0.605[a]	−0.139	−0.253	1

[a] Significant at 5% level.
[b] Significant at 1% level.

Similarly, sucrose content was positively correlated with amylopectin and SS activity and a negative correlation was observed with amylose and yield. Amylose content was negatively correlated with amylopectin and positive correlation was observed with yield. Amylopectin showed a positive correlation with SS activity and negative correlation with yield. SS showed a positive correlation with AGPase and both SS and AGPase showed a positive correlation with total soluble protein.

4. Discussion

4.1. Effect of drought stress on water status

RWC provides the information of the plant's response to different environmental conditions; and is a more stable parameter (Sade et al., 2009, 2012). RWC determination is an important indicator of soil water content, which defines the water stress in plants (Hayatu et al., 2014; Todaka et al., 2017). It is an important physiological parameter which directly reflects the soil water content (Sarker et al., 1999) and the water stress-induced reduction in leaf RWC has been observed in various crops (Yao et al., 2012; Meena et al., 2014; Kumar et al., 2015, 2018). Sinclair and Ludlow (1986) had proposed that RWC was a better indicator of drought stress. In this study, we found, that RWC was found to decrease in the leaves of both varieties when plants were subjected to WDS during the grain filling stage in the rice, especially at the booting stage. There was no difference in percentage reduction in RWC in both the varieties under the stress, indicating that plants experienced comparable WDS during the grain filling stage (Fig. 1). The soil moisture content under the WDS was greatly reduced in both the verities. The results showed that both the verities were exposed to the same level of WDS during the grain filling stage.

4.2. Effect of drought stress on photosynthetic rate

The photosynthetic rate was almost comparable in both the varieties under WW conditions and decreased during the grain filling stage under WDS. Though reduction was relatively high, drought tolerant N22 showed better Pn and recovery after re-watering compared to the IR64. The reduced photosynthetic rate under the WDS was mainly attributed to WDS induced early senescence. The WDS during grain filling stage could damage the photosynthetic machinery thereby affecting the photosynthetic rate. The mild WDS during grain filling would not much affect the photosynthetic rate (Yang et al., 2000; 2001; Tyagi and Chandra, 2006). In rice, reduction of photosynthetic under the WDS was attributed to the loss of chlorophyll and early senescence (Yang et al., 2002; Biswas and Choudhuri, 1980; Kaiser, 1987; Siddique et al., 1999).

4.3. Effect of drought stress on starch

Cereals need the initiation of whole plant senescence to remobilize the pre-stored carbon from stems to grains (Gan and Amasino, 1997). Grain filling in cereals depends on carbon from two sources: current assimilation

and remobilization of stored reserves in the other parts (Kobata et al., 1992). The main storage form of carbohydrate of rice is starch. Drought stress during grain filling enhances whole plant senescence thus shortening the grain filling duration and accelerating the remobilization of prestored carbon in the stem of rice through regulating the key enzymes involved leading to increased starch. The biosynthesis and accumulation of starch were greatly influenced by drought (https://www.sciencedirect.com/science/article/pii/S0733521015300709 He et al., 2012), heat (https://www.sciencedirect.com/science/article/pii/S0733521015300709 Hurkman and Wood, 2011), salinity (https://www.sciencedirect.com/science/article/pii/S0733521015300709 Chen et al., 2008) and soil acidity (https://www.sciencedirect.com/science/article/pii/S0733521015300709 Mishra and Dubey, 2008).

The WDS accelerates grain filling rate and reduces total starch accumulation; these changes are directly related to productivity (https://www.sciencedirect.com/science/article/pii/S0733521015300709 He et al., 2012). Grain weight was greatly reduced for plants under the WDS treatment during the day (Yang et al., 2004). Moreover, starch content was increased under the drought in developing grains. These contradictory results were attributed to several reasons.

Our studies showed that increase in the starch content in developing grains of both the varieties was positively correlated with the enhanced mobilization of carbon source towards the sink which in turn positively correlated with the decreased starch content in leaves. This indicates that WDS during grain filling enhanced the translocation of carbon source from the sink to the source. The N22 showed better performance with respect to translocation of transitory starch than IR64 under WDS. A decrease in starch content of the leaves was observed under the drought condition in both the cultivars, while tolerant genotypes maintained their yield under WD conditions (Mahla et al., 2017**).** The PEG-induced WDS reduced the leaf starch content in wheat (Wei et al., 2014). The starch content in the leaves decreased that were treated solely with ABA (Akihiro et al., 2005; Ali et al., 2011). The decrease in the starch content in leaves was positively correlated with the decreased activity of SS enzyme. This could be due to the remobilization of transitory starch from leaves to grains. The transitory starch acts as an immediate source of carbon under the adverse climate condition (Weise et al., 2011). The increased total starch content of the developing grains under WDS was mainly attributed to senescence-enhanced grain filling rate during the developmental stage. The reduced starch content in matured grains was due to WDS induced shortening of grain filling stage (Krasensky and Jonak, 2012). However, WDS induced senescence during grain filling stage in wheat and rice increased the grain filling rate and shortened the grain filling period (Aggarwal and Sinha, 1984; Nicolas et al., 1985; .

4.4. Effect of drought stress on sucrose

Sucrose is a major transportable form of sugar in the plants. Sucrose is mainly synthesized in the cytoplasm of the photosynthetic tissue and gets transported to non- photosynthetic tissue where it is utilized as a carbon source. The sucrose content in the leaves and roots was found to be decreased under the drought, while in developing grains, it increased in both the cultivars. The decrease in the sucrose in leaves and roots under the drought might be due to increased remobilization of sucrose to developing grains. It has been reported that SuSase activity decreased 18 days after WDS in rice (Yang et al., 2003, 2004). Sucrose synthase is a major enzyme involved in the hydrolysis of sucrose in the developing grains which were not studied in the present study. The increase in sucrose content in the developing grains could be due to the reduction in the activity of the SuSy which is the main enzyme involved in the breakdown of sucrose. The ABA treatment during the grain filling stage increased the sucrose content, SuSy activity, thereby enhancing grain weight in rice (Tang et al., 2009). The sucrose content increased under WDS and had a positive correlation with ABA content in rice (Mukherjee et al., 2015).

4.5. Effect of drought stress on amylose and amylopectin

The amylose content was significantly reduced under the WDS in developing and matured grains of both the varieties, while there was no significant difference in reduction between the two varieties. However, amylopectin content decreased in matured grains but increased in developing grains under WDS. Thus, WDS during the grain filling stage in rice affects the amylose and amylopectin content. The reduction in the amylose and amylopectin content under WDS was positively correlated with the decreased starch content of the matured grains. It has been reported that reduced amylose content was due to repression of SS (*Wx*) expression or decreased GBSS activity (Wang et al., 2006). Further, amylose content of starch was found to be lowered in drought-treated wheat (Fabian et al., 2011; Singh et al., 2008) and rice (Gunaratne et al., 2011; Liu et al., 2010). Reports are there which suggested an increase of amylopectin content under stress in rice (Beckles and Thitisaksakul, 2014). Overexpression of SBE and SS under water-deficit stress during grain filling also might be responsible for enhanced amylopectin (Yang et al., 2003).

4.6. Effect of drought stress on RS

The resistant starch content was higher in developing grains compared to matured grains. The RS content decreased in both matured and developing grains under the WDS. The decrease in RS content under drought was positively correlated with the decreased amylose content in both the varieties It has been reported that lipid content of the cereal endosperm is found to be complexed with the amylose (Tester and Morrison, 1990). The decrease in the RS content could be due to reduced amylose content under WDS. It has been also reported that WDS reduces the starch-lipid composition in wheat (Fabian et al., 2011). The SSIIIa deficient plants showed an increase in amounts of both amylose and extra-long chains in amylopectin (Fujita et al., 2007; Hanashiro et al., 2008). The high amylose complexes with lipids and contributes to RS formation (Raigond et al., 2014)

4.7. Effect of drought stress on AGPase and SS activity

The grain filling in cereals is the process of accumulation of starch from sugars and depends upon the sink strength (Liang et al., 2001). The sink strength is a product of the size and activity of the sink. The activity of the sink depends on the key enzymes of carbon metabolism and other factors (Wang et al., 1993). The enhanced remobilization of carbon resources from source to sink was mainly attributed to the change in the activity of the key enzymes of carbon metabolism.

The increase in SS activity in N22 was more compared to IR 64 which was positively correlated with the increase in amylopectin content and total starch content. Different isoforms of SS, BE, and DBE has a distinct role in amylopectin biosynthesis (Nakamura et al., 2002). It has been reported that ABA treatment has no effect on wheat TaSSIIIa which is similar to SSIIC of rice (Mukherjee et al., 2015). WDS during grain filling stage enhanced the grain starch in rice and wheat and correlated to increased activity of SuSy, SBE and SS (Yang et al., 2003). However, heat stress reduced starch content in wheat grains (Chinnusamy and Khanna-Chopra 2003).

4.8. Effect of drought stress on morphology and arrangement of starch granules

In cereals reduced water supply alters endosperm starch granule, size distribution and amylose content (Brooks et al., 1982; Fabian et al., 2011; Singh et al., 2008). The amylose, mainly present in the amorphous region of the starch influences the arrangement of amylopectin in crystalline lamellae (Copeland et al., 2009). The decrease in amylose content might be responsible for the loosening of the starch granules under the drought stress. The amylose, mainly present in the

amorphous region of the starch influences the arrangement of amylopectin in crystalline lamellae (Copeland et al., 2009). The ratio of amylose and amylopectin might be responsible for loosening of starch granules under the drought which could be clearly seen in images. The disintegration of starch granules observed under the drought might be due to activation of the catabolic enzymes.

The observed microstructural changes including the small cavities and pits resulted from the action of hydrolytic enzyme produced in the aleurone layer during the seed germination. The microstructural changes on the surface of granules caused by drought were related to the action of enzymes. However there are no prior reports on rice starch granules microstructure in response to drought, our results imply that the change in the microstructure of the starch granules under the drought may be due to an imbalance in the activities of the hydrolytic enzymes. In rice shape of the starch granules were polyhedral (Tester et al., 2004). The WDS caused the breakdown of starch granules (Gunaratne et al., 2011). It has been reported that high temperature during the endosperm cell division phase produced thermal disruption and thus reduced the total number of starch granules. The pits appeared in starch granules from the kernels exposed to a high temperature by an increase in susceptibility to enzymatic hydrolysis (Li et al., 2017).

4.9. Effect of drought on grain weight of 20 rice plants

To evaluate the yield potential of the experiment, 20 plants were taken. The grain weight was higher in the WW condition in both cultivars than the WDS. The decrease in yield under the drought was more in drought susceptible IR 64 than the drought tolerant N22 variety. The reduction of yield could be due to a decrease in the grain filling period under the drought. The yield reduction was not only dependent on the starch content but also depends on other factors such as the number of tillers, panicle numbers, number of grains per spike, etc.

Many reports are there which suggest that plant growth and productivity of rice is adversely affected by various biotic and abiotic stress factors. Tolerant genotypes maintained their yield under drought stress conditions (Mahla et al., 2017). In the present study, the correlation between quantitative and qualitative changes in starch accumulation are summarized. There is a decrease in starch and sucrose deposition in leaves under WDS in both N22 and IR 64. However, the decrease was more in IR64 than N22. Further, under drought stress there is increased remobilization of reserves from leaves to developing grains and duration of grain filling is shortened. As a result, starch deposition in developing grains increases under stress and increase was more in N22 as compared to IR 64 because transitory starch and sucrose were more in N22 than IR64. However, increased starch in developing grains doesn't get translated into increased starch in matured grains because of shortened grain filling period under drought condition and activation of starch catabolic enzymes. Further, under the stress condition rate of demand for the carbon source exceeds the rate of CO_2 assimilation. Thus, though starch deposition was reduced in matured grain the yield was higher in N22 than IR64 because the accumulation of transitory starch and sucrose was higher in developing grains than IR 64.

5. Conclusion

The WDS induced senescence during the grain filling stage enhanced the starch accumulation in developing grains by increasing remobilization of transitory starch and sucrose from leaves and stem. This was positively correlated with an increase in the starch content, AGPase and SS activity under the drought. Drought tolerant genotype N22 showed better performance under the drought as compared to susceptible variety IR64. WDS enhanced the remobilization of transitory starch from leaves to grains and a positive correlation was observed with decreased starch and starch synthase activity in leaves. A decrease in amylose and RS content in developing and matured grains was observed under WDS in both the varieties. The SS and AGPase are differentially regulated in leaves, roots and developing grains under the WDS. WDS also affects the packaging of starch granules thereby affecting the starch composition. However, still further studies are required on expression and activity profiling of various metabolic enzymes to understand the regulation of starch biosynthesis in developing grains to improve crop yield under drought stress.

Compliance with ethical standards

Conflicts of interest

The authors declare no competing financial interests.

Author contributions

PV carried out all the research work and framed the manuscript, Kiswar Ali provided the study material, supervised and assisted in research, AS and VK assisted in research. CV maintained rice fields and collected Pn and soil moisture data under supervision of VC. Aruna Tyagi conceived of the research area, supervised statistical analysis, contributed scientific advice, correction and final revision of the manuscript.

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