Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 1211-1218
Received: 12-05-2020
Accepted: 14-06-2020

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Starch content and activities of starch biosynthetic enzymes in wheat, rice and millets

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DOI: https://doi.org/10.22271/phyto.2020.v9.i4q.11883

Abstract
The aim of the study was to correlate the accumulation of amylose and total starch with activities of starch biosynthetic enzymes (AGPase, SSS and GBSS) in cereals like rice (PB-2, PD-19), wheat (UP-262, PBW-343) and millets (Barnyard millet, Finger millet and Foxtail millet) at different stages of endosperm development. ADP-glucose pyrophosphorylase (AGPase) activity was found higher in all varieties during developmental stage followed by granule bound starch synthase (GBSS) and soluble starch synthase (SSS). Accumulation of amylose was positively influenced by activity of AGPase and GBSS. Total starch accumulation was positively correlated with activity of AGPase and SSS. In all these varieties rate of enzymes activity was found higher at S3 stage. Higher ratio of AGPase and GBSS contributes lesser resistant starch, amylose content and am/ap ratio. Ratio of AGPase and GBSS can be used as an index for amylose content when it was studied in large sample size of similar varieties.

Keywords: starch, amylose, total starch, AGPase, SSS, GBSS

Introduction
With the growing in health-conscious consumers, there has been an increase in demand for high-quality wheat four. The endosperm starch content and composition are two critical parameters for evaluating the quality of wheat flour. The physiochemical properties of starch can be influenced by enzyme activities and has been used in different industrial application (Steve 2004 and Smith, 2008) [14, 13]. The endosperm starch content (approximate 70% of the dry weight) not only influences the grain weight and quality but also reflects the capacity of the sink tissues. Regarding the source-sink relationship, the photoassimilates produced in source tissues as leaf, stem and root were transported into the amyloplast in the form of sucrose, which is the main carbohydrate transported in higher plants. Sucrose is degraded to fructose and uridine diphosphate glucose (UDPG) which are the main precursors of starch synthesis by sucrose synthase. The ability of the sink tissues to accept and convert photoassimilates can be affected by the sink strength, which is an important limiting factor to wheat grain yield, and the activity of SUS can be considered as an indicator of sink strength. A coordinated series of enzyme-catalysed reactions in wheat endosperm result in starch synthesis including ADPG pyrophosphorylase (AGPase: EC 2.7.7.27), starch branching enzyme (SBE: EC 2.4.1.18), starch debranching enzyme (DBE), granule bound starch synthase (GBSS: EC 2.4.1.21) and soluble starch synthase (SSS: EC 2.4.1.21). GBSS and SSS are the two forms of starch synthase. For catalysing the first unique step in starch synthesis, AGPase is the rate-limiting enzyme and is the most important determinant of seed sink strength. Starch synthases catalyse the elongation of the linear glucan chains moreover the different genetic characterization assigns preferential functions for individual isoforms to synthesize amylose or amylopectin. It is generally known that in the endosperm, amylose is synthesized by AGPase and GBSS (Zi *et al*., 2018) [21]. The objective of this study was to investigate the activities of AGPase, SSS and GBSS in relation to starch accumulation in different cereals like rice, wheat and millets.

Material and Methods
Field experiments were conducted at N.E. Borlaug Crop Research Centre of GBPUA&T, Pantnagar, Uttarakhand. The experimental material of study consisted of two cultivars of rice (PB-2 and PD-19), wheat (UP-262 and PBW-343) and millets namely finger millet (VL Mandua-352), barnyard millet (VL Madira-207) and foxtail millet (DHFT-109-3). During endosperm development cereals grains were taken for enzymatic study as well as analysis of starch and amylose accumulation. Plant spacing, weeds, insects and diseases were managed as recommended agronomic practises to avoid yielding loss.

Extraction of AGPase, SSS and GBSS

Extraction of enzymes was done by Nakmura *et al*., (1989) [11] with some modifications. Immature grain seeds were taken after 7,14,21,28 days after flowering (S_1, S_2, S_3 and S_4) from watery stage to grain filling stage for the assay of endosperm enzyme. 100 mg immature seeds was weighed and homogenized in pre-chilled mortar & pestle with 2 ml of extraction buffer containing 100 mM HEPES-NaOH (pH 7.5), 8 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT/ 50 mM 2-mercaptoethanol, 12.5% glycerol (v/v) along with addition of insoluble PVP. The homogenate was centrifuged at 10000g for 20 minute and the supernatant was used for ADP- glucose pyrophosphorylase **(**AGPase) and soluble starch synthase (SSS) enzyme assay. The pellet of starch granule was washed with 2-4 ml of extraction buffer twice by centrifugation to remove all traces of soluble starch synthases (SSS). The pellet of starch granule was resuspended by 2.0 ml of extraction buffer and stored at 4ºC for granule bound starch synthase (GBSS) activity.

Measurement of the activities of AGPase, GBSS and SSS:

Twenty kernels weighed at different filling stages were tested. AGPase, GBSS and SSS activity were assayed according to Nakamura *et al*., (1989) [11].

Assay of ADP- glucose pyrophosphorylase (AGPase)

The reaction mixture contained 100 mM HEPES-NaOH buffer (pH 7.5), 3 mM PP_i, 5 mM $MgCl_2$, 4 mM DTT, 1.2 mM ADP-glucose, 0.1 ml enzyme extract and distilled water in a total volume of 1.0 ml. After 20 minutes, the reaction was terminated by incubation at 100ºC for 1 minute and it was centrifuged at 6000g for 10 minute. The supernatant was transferred in new tube and PGM (0.4U), 10 mM NADP and glucose-6-phosphate dehydrogenase (0.35U) were added to it. AGPase activity can be quantified by measuring NADPH concentration at 340 nm. One unit of AGPase activity is defined as the amount of enzyme required to convert 1 nmole of substrate into product per minute.

Assay of Soluble starch synthase (SSS)

The reaction mixture contained 50 mM HEPES-NaOH buffer (pH 7.5), 15 mM DTT, Amylopectin (100 mg/ml), 1.6 mM ADP-glucose, 0.2 ml enzyme extract and distilled water in a total volume of 0.45 ml. After 20 minute, the reaction was terminated by incubation at100ºC for 1 minute and it was centrifuged at 6000g for 10 minute. Supernatant was transferred in new tube and 0.2 ml freshly prepared pyruvate kinase reaction mixture and pyruvate kinase (1.2U) was added. After 30 minute, it was incubated at 100ºC for 1 minute. Then it was centrifuged at 6000g for 10 minutes and supernatant was transferred in a new test tube. After that 0.4 ml freshly prepared glucose-6-phosphate dehydrogenase reaction mixture, hexokinase (1.4U) and glucose-6-phosphate dehydrogenase (0.35U) were added. Soluble starch synthase activity can be quantified by measuring NADPH concentration at 340 nm. One unit of SSS activity is defined as the amount of enzyme required to convert 1 nmole of substrate into product per minute.

Assay of Granule bound starch synthase (GSSS) assay

The assay procedure for granule bound starch synthase (GBSS) was similar to soluble starch synthase (SSS). In GBSS; amylopectin and enzyme extract were not added while 0.2 ml aliquot of the starch granules pellet suspension was added as a source of both enzyme and reaction substrate.

Amylose content and Total starch determination

Amylose content was determined by method described by McCready *et al*. (1950) [9] with some modifications. Total starch was determined by method adopted from Dubois *et al*. (1956) [3] with some modification.

Statistical Analysis

The experimental data were done in triplicate and analysed as mean ± standard error (SE) using Completely Randomized Design. Values with different letters in the same column are significantly different with $p < 0.05$. One-way analysis of variance (ANOVA) and correlation analysis was carried out using WASP1.0 (Web Agri Stat Package 1.0 ICAR-CCARI, Goa) and OP STAT. Correlation analysis was shown in Table 1.

Results and Discussion

Accumulation of amylose and total starch in cereals at different stages of development

The accumulation of amylose and total starch was observed at different stages (S1, S2, S3 and S4) of endosperm development (Fig.1).

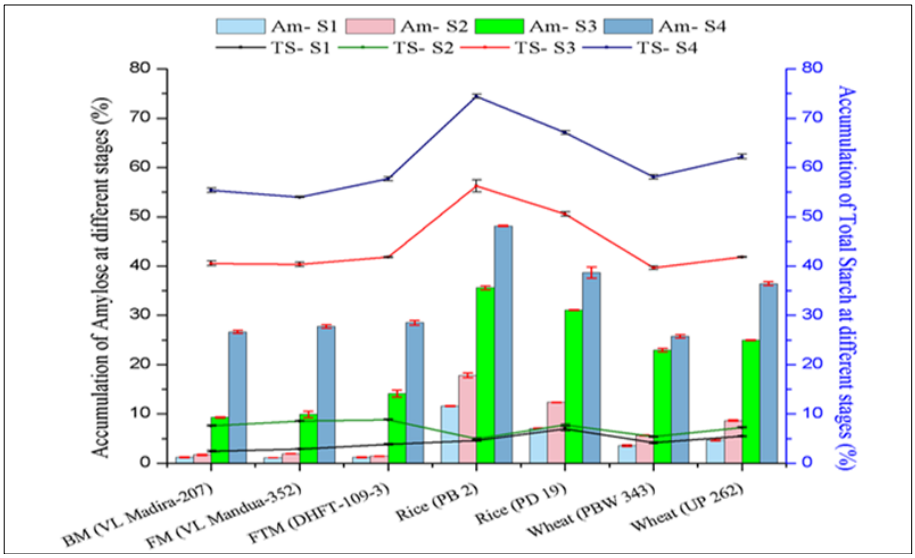

Fig 1: Accumulation of amylose and total starch in cereals viz. millets, rice and wheat during different stages of development (S1 to S4 stages). Data represents Mean±SD of triplicates. Treatments found significant at 5% level of significance at different stage of development.

In S1 to S4 stages of development, accumulation of amylose was found maximum in PB-2 followed by PD-19. In case of wheat, the accumulation of amylose was high in UP-262 followed by PBW-343 at different stages of endosperm development. In millet at S4 stage the amylose accumulation was found significantly different while in S1 to S3 stage accumulation of amylose was found to be similar. In all the varieties it was observed that accumulation rate of amylose was higher in S3 stage. Wambugu *et al.* (2018) [17] analyzed 100 progenies of interspecific cross between African rice and *O. sativa* to determine amylose content and it was found that amylose content varies from 19.2 to 26.2%. Asaoka *et al.* (1985) [2] investigated the developmental changes in the structure of rice endosperm starches using *waxy* starch and it was found that amylose content in *nonwaxy* starch was increased during the endosperm developmental stage. They noticed that accumulation of amylose was stopped earlier than that of amylopectin biosynthesis during different stages of endosperm development. At the early stage of development the endosperm starch was greatly influenced by temperature. The proximate amylose content in rice endosperm has been reported to increase. They also observed that amylose content was increased from 17-25% during the endosperm development of Japanese cultivars while 23 to 27% in Indian cultivars. Yu *et al.* (2015) [19] reported that amylose content in wheat pericarp varies from 28-25% during caryopsis development. They noticed that amylose content at earlier stage was slightly higher than later stage. In the present study it was observed that accumulation of amylose positively correlated with the activity of granule bound starch synthase enzyme which is responsible for amylose biosynthesis during endosperm development. The GBSS activity was not significantly different at S1-S3 stage corresponding to amylose accumulation in all millets. But at S4 stage the GBSS activity was higher in foxtail millet followed by finger millet than barnyard millet while accumulation of amylose was significantly same in all these verities of millets. These results suggested that at S4 stage amylose formed might be involved in starch packaging. In wheat and rice amylose accumulation corresponds the activity of GBSS. During the endosperm development total starch was determined at S1, S2, S3 and S4 stages. At S1 stage accumulation of total starch was found maximum in PD-19 followed by UP-262. Total starch accumulation in PB-2 was minimum comparatively to other rice and wheat cultivars but significantly same to PBW-343. In millet total starch accumulation at S1 stage was lower than rice and wheat. Total starch was observed maximum in foxtail millet followed by finger millet and barnyard millet. In S2 stage of development total starch accumulation was found lower in PB-2 comparatively to all cultivars. Foxtail millet and finger millet has higher total starch accumulation during S2 stage than other cultivars. In both S3 and S4 stage total starch accumulated was higher in PB-2 followed by other cultivars following the order rice (PB-2>PD-19) > wheat (UP-262 > PBW-343) > millet (Foxtail millet > Finger millet > Barnyard millet). The rate of accumulation of total starch was found higher at S3 stage. Yu *et al.* (2015) [19] reported total starch accumulation in pericarp of wheat and found that initially total starch content increased from 2 to 6 days after anthesis (DAA) and then slightly decreased from 6 DAA to 20 DAA. Li *et al.* (2012) [8] observed that pattern of starch accumulation during developmental stage of wheat grain. Starch granules were detected in the pericarp from 3 DAA and had almost disappeared from 15DAA. But few starch granules were observed at 3 DAA in the endosperm. Endosperm starch granules accumulation increased rapidly from 6 DAA to 21 DAA. The accumulation rate increased at about 15-18 DAA. Total starch accumulation in endosperm was found until maturity. Prioul *et al.* (2008) [12] investigated transcriptome analysis of maize endosperm development and starch filling through cDNA libraries at kernel feeling stage ranging from 12 to 40 days after anthesis (DAP) and found that sucrose and amino acid converted into starch and storage proteins and deposited in the endosperm. Yu and Wang (2016) [18] reported that starch granule packaging was completed in the whole endosperm at 20 days after flowering (DAF). They also emphasized that starch granule packaging and biosynthesis in cereals endosperm committed an integrated mechanism of starch biosynthesis enzymes and other metabolism. The effect of AGPase activity at S1 and S2 stages were found non-significant correlation during the initial stage of starch accumulation. However at later stage of development the AGPase activity was highly correlated with total starch accumulation. These results suggested that total starch accumulation was higher at maturity stage.

Table 1: Correlation analysis of starch biosynthesis enzymes with amylose and total starch during endosperm development

	AGPaseS1	AGPaseS2	AGPaseS3	AGPaseS4	SSS1	SSS2	SSS3	SSS4	GBSS1	GBSS2	GBSS3	GBSS4	Amy1	Amy2	Amy3	Amy4	TS1	TS2	TS3	TS4
AGPaseS1	1	0.992**	0.978**	0.964**	0.667*	0.522**	0.511**	0.641NS	0.941**	0.896**	0.915**	0.548NS	0.849*	0.793*	0.636**	0.837*	0.171NS	-0.574NS	0.909**	0.845*
AGPaseS2		1	0.990**	0.958**	0.597*	0.447**	0.444*	0.601NS	0.931**	0.895**	0.932**	0.563NS	0.858*	0.798*	0.660**	0.825*	0.159NS	-0.648NS	0.884**	0.847*
AGPaseS3			1	0.947**	0.589*	0.440*	0.448*	0.600NS	0.898**	0.868*	0.916**	0.495NS	0.899**	0.850*	0.715**	0.866*	0.216NS	-0.693NS	0.885**	0.876**
AGPaseS4				1	0.695**	0.578**	0.578**	0.728NS	0.881**	0.876**	0.890**	0.597NS	0.874*	0.826*	0.700**	0.869*	0.298NS	-0.507NS	0.949**	0.878**
SSS1					1	0.982**	0.973**	0.948**	0.756**	0.669*	0.544*	0.029NS	0.535*	0.749NS	0.673NS	0.727NS	0.631NS	-0.217NS	0.861*	0.763*
SSS2						1	0.995**	0.941**	0.794**	0.613**	0.583**	0.068NS	0.544*	0.674NS	0.630NS	0.642NS	0.701NS	-0.098NS	0.778*	0.681NS
SSS3							1	0.951**	0.762**	0.687*	0.574**	0.094NS	0.584*	0.719NS	0.693NS	0.674NS	0.766*	-0.141NS	0.786*	0.717NS
SSS4								1	0.691**	0.644*	0.640**	0.040NS	0.7591*	0.803*	0.784*	0.719NS	0.713NS	-0.343NS	0.867*	0.800*
GBSS1									1	0.978**	0.964**	0.711NS	0.640**	0.664**	0.660*	0.681*	-0.133NS	-0.417NS	0.749NS	0.642NS
GBSS2										1	0.974**	0.770*	0.603**	0.526**	0.625*	0.652*	-0.166NS	-0.381NS	0.707NS	0.594NS
GBSS3											1	0.775*	0.685**	0.610*	0.648**	0.722*	-0.068NS	-0.491NS	0.736NS	0.686NS
GBSS4												1	0.528**	0.645*	0.640NS	0.653*	-0.242NS	0.026NS	0.403NS	0.303NS
Amy1													1	0.993**	0.941**	0.946**	0.610NS	-0.692NS	0.930**	0.984**
Amy2														1	0.958**	0.946**	0.681NS	-0.660NS	0.911**	0.977**
Amy3															1	0.855*	0.801*	-0.667NS	0.814*	0.934**
Amy4																1	0.599NS	-0.467NS	0.933**	0.965**
TS1																	1	-0.173NS	0.540NS	0.653NS
TS2																		1	-0.455NS	-0.593NS
TS3																			1	0.951**
TS4																				1

* Significant at $p < 0.05$
** Significant at $p < 0.01$
NS Non-significant

Assay of starch biosynthetic enzyme in cereals at different growing stage

Activity of starch biosynthetic enzymes like AGPase, SSS and GBSS were observed during endosperm development at S1, S2, S3 and S4 stages in cultivars (Fig 2, 3, 4).

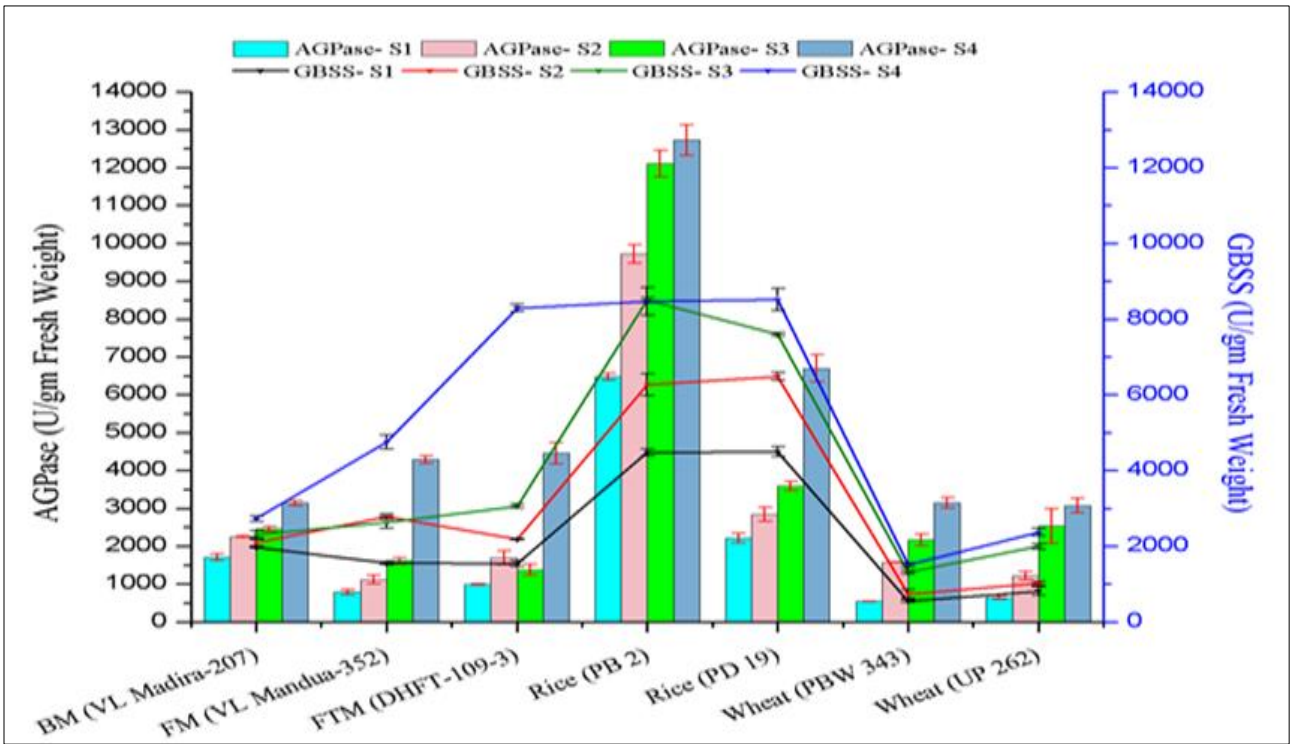

Fig 2: Starch biosynthesis enzymes like AGPase and GBSS activity in endosperm of cereals viz. millets, rice and wheat during different stages of development (S1 to S4 stages). Data represents Mean±SD of triplicates. Treatments found significant at 5% level of significance at different stage of development.

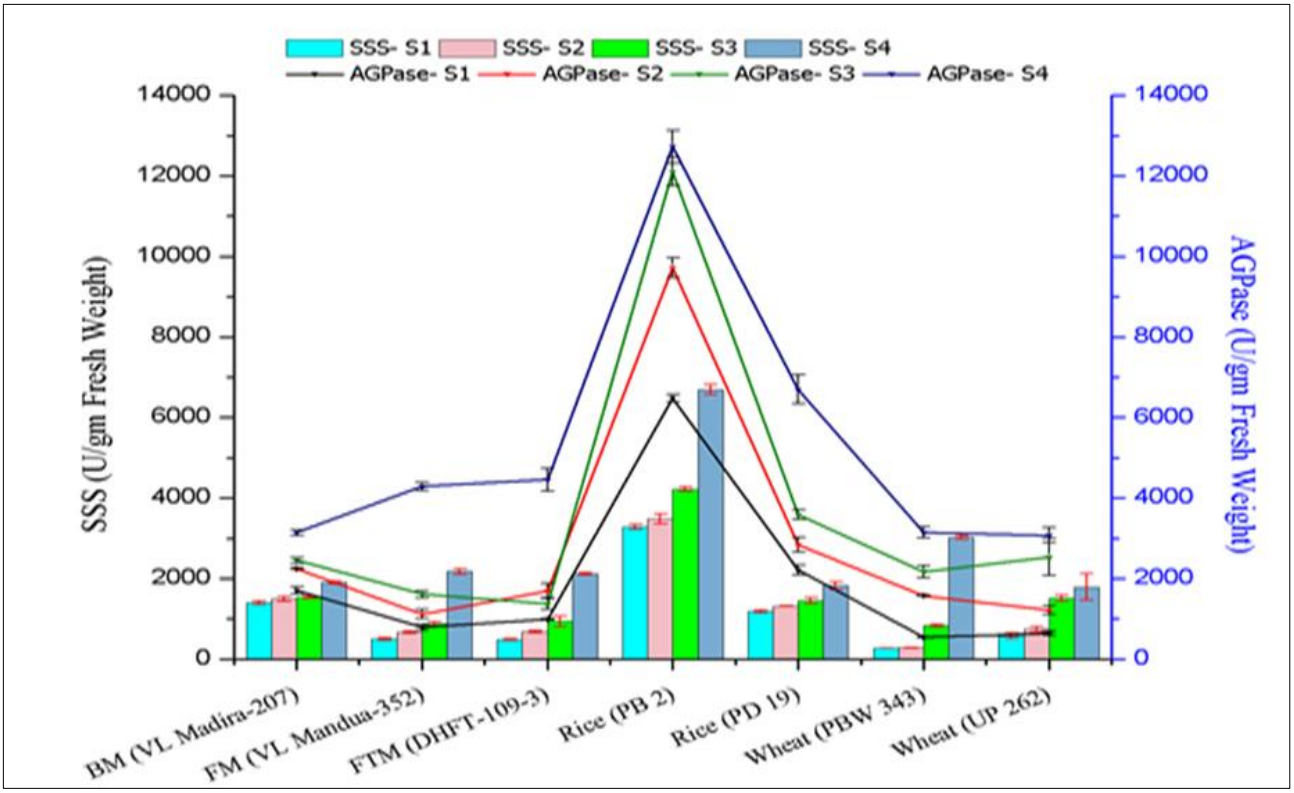

Fig 3: Starch biosynthesis enzymes like AGPase and SSS activity in endosperm of cereals viz. millets, rice and wheat during different stages of development (S1 to S4 stages). Data represents Mean±SD of triplicates. Treatments found significant at 5% level of significance at different stage of development.

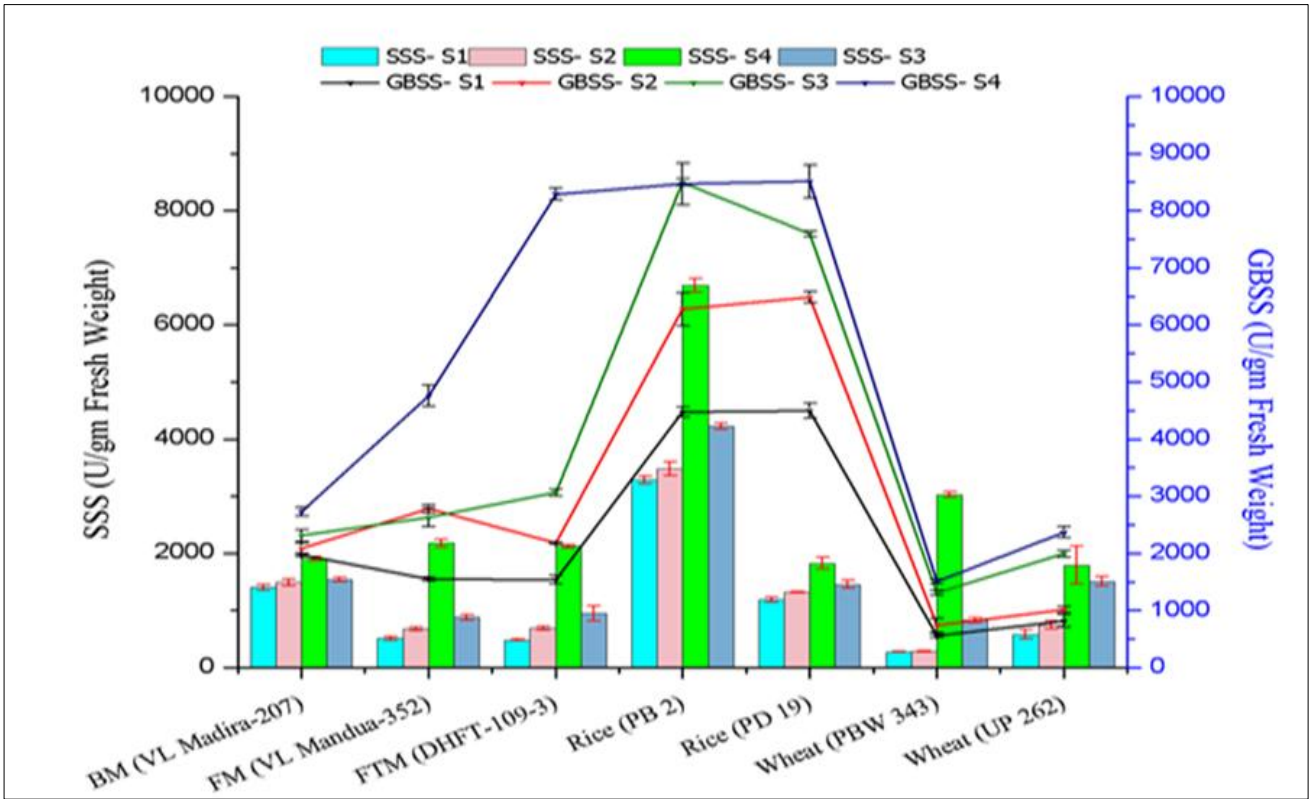

Fig 4: Starch biosynthesis enzymes like GBSS and SSS activity in endosperm of cereals viz. millets, rice and wheat during different stages of development (S1 to S4 stages). Data represents Mean±SD of triplicates. Treatments found significant at 5% level of significance at different stage of development.

In all these cultivars AGPase activity was found maximum followed by GBSS and SSS during endosperm development. In barnyard millet AGPase, SSS and GBSS activity was slightly increased during endosperm development. At different stages of the endosperm development the activity of these enzymes are found significantly different. In finger millet the AGPase activity was slightly increased till S3 stage but at S4 stage the AGPase activity showed elevation of greater than 2 fold compared to S3 stage. Similar pattern was observed in activity of SSS while in GBSS the activity at S4 stage was 1.5 fold higher than S3 stage. In foxtail millet the activity of these enzymes are increased during endosperm development while at S4 stage the activity of these enzymes was almost 3 times of S3 stage. In all the cultivars of millet the activity of starch biosynthesis enzymes are progressively increased from S1 to S4 stage. The activity of enzyme was observed in two cultivars of rice viz. PB-2 and PD-19 at different stages (S1, S2, S3 and S4) of endosperm development. In both the cultivars AGPase, SSS and GBSS activity were increased during endosperm development. However, at S1 to S3 stage AGPase activity in PB-2 was found higher than PD-19 by 3 folds. At S4 stage it was found higher by 2 folds. Higher activity of AGPase at those stages in PB-2 might be responsible for higher accumulation of amylose and total starch. Activity of SSS in both the cultivars of rice increases during endosperm development, but PD-19 had higher activity than PB-2. It was observed that at S2 stage activity of SSS in PD-19 was 2 folds higher than PB-2. Activity of GBSS in both the cultivars increases but PB-2 had higher activity than PD-19. During the endosperm development the activity of GBSS was found maximum in PB-2 than PD-19 by 2-3 folds. The activity of starch biosynthetic enzymes was observed in two cultivars of wheat viz. PBW-343 and UP-262 at different stage of development. It was examined that the activity of AGPase, SSS and GBSS increases throughout the developmental stages. AGPase activity was maximum in both cultivars comparatively to SSS and GBSS. In PBW-343 the GBSS activity was higher till S3 stage as compared to SSS activity. At S4 stage the SSS activity was 2 folds higher compared to GBSS activity in PBW 343. In UP-262 activity of GBSS was found higher compared to SSS activity throughout the development process. Similar results were obtained in millets. Among millets the activity of starch biosynthetic enzymes corresponds to accumulation of amylose and total starch (Fig. 1). The function of starch biosynthetic enzymes was previously discussed. In rice (PB-2 & PD-19) the higher ratio of AGPase and GBSS corresponds higher resistant starch, amylose content and am/ap ratio. As discussed in previous section, in rice amylose and am/ap ratio might be the factor associated with resistant to digestion of starch. Among millets the factors associated with resistant to digestion of starch may be influenced by dietary fiber, starch-protein interaction and flavonoids present. In millets it was observed that ratio greater than 0.5 contributes good amount of resistant starch. In wheat (PBW-343 & UP-262) higher ratio of AGPase and GBSS contributes lesser resistant starch, amylose content and am/ap ratio. It might be possible because higher ratio indicates lower activity of GBSS and higher activity of SSS in both the wheat verities. The ratio of AGPase and GBSS was different in varieties under observation might be due to botanical origin, starch packaging and environmental effects. Ratio of AGPase and GBSS may be the index for resistant starch and amylose content this may be confirmed by studying large sample size in these crops. Leroux *et al.* (2014) [7] reported that key enzymes for starch biosynthetic enzymes during endosperm development have been extensively studied in the major cereals like rice wheat and maize. Zeeman *et al.* (2010) [20] reported that AGPase is the committed enzymes in starch biosynthesis catalyzed the conversion of Glucose-1-P and

ATP to ADP-Glucose and it acts as glucose donor to elongate the α-1,4 linked glucan chain to synthesize amylose and amylopectin to which starch granule formed catalyzed by SSS and GBSS respectively. Jiang *et al.* (2003) [6] reported that effect of nitrogen treatment on starch biosynthetic enzymes in two varieties of wheat viz. superior (Lumai 22) and inferior (Lumai 14) during endosperm development. They found that AGPase activity in superior grains was higher than the inferior grains during the whole grain filling stage. Nitrogen increased AGPase activity in inferior grain during initial stage of development but the later stage the activity was decreased. Therefore the average activity of AGPase was same in both grains during grain filling stage. During the grain filling the SSS activity was also found higher in superior grain than inferior one but it was noticed that the activity of SSS in superior grain 30.1% higher than inferior grain. Changes in the activity of GBSS were found similar *wrt* to SSS but GBSS activity in superior grain was found 73% higher than inferior grain. In the endosperm, the activity of key enzymes of starch biosynthesis (AGPase, SSS and GBSS) could play important roles in starch biosynthesis during grain filling at elevated temperature (Jenner, 1991 a, b) [4, 5]. Zi *et al.* (2018) [21] studied the key enzymes of starch biosynthetic enzymes in waxy and non-waxy wheat and they reported that the activity of AGPase and SSS was found maximum in 25th days after anthesis (DAA) whereas the activity of SSS was found maximum between 25th to 30th DAA. During the grain filling stage GBSS activity was found lower in *waxy* wheat compared to those of *non-waxy* wheat. In contrast the SSS activity was found maximum in *waxy* wheat compared to non-waxy wheat between 20th to 30th DAA. The lower activity of GBSS in waxy wheat could be the result of absence of *waxy* protein. Tetlow *et al.* (2003) [15] investigated the AGPase activity in wheat cultivars from 8 days after pollination (DAP) to 30 DAP and found that AGPase activity was gradually increased during the endosperm development. The maximum AGPase activity was found at 25 DAP. Nakamura and Yuki (1992) [10] investigated the change in the activities of starch biosynthetic enzymes in rice endosperm at different days after anthesis. They studied various enzymes activities involved in carbohydrate metabolism for starch biosynthesis in relation to starch accumulation. They found that the enzyme activities for most enzymes increased up to stage III. However, the accumulation pattern of AGPase and Q-enzymes are differed among other enzymes. These enzymes continued to increase up to stage IV. The GBSS activity was found higher than SSS when starch biosynthesis was active. The higher activities of AGPase and Q-enzyme were almost parallel leads to higher accumulation of starch in rice endosperm. From the above discussion it may be concluded that starch biosynthetic enzymes are independently controlled by genetically in rice endosperm and AGPase play an important role in starch production in rice endosperm Tsai *et al.* (1970) [16] analysed starch biosynthetic enzymes in C_4 plant like maize endosperm. However similar pattern were observed in starch biosynthetic enzymes including AGPase, SSS and GBSS during endosperm development. It may reflect that differences in botanical origin of species have similar pattern for starch biosynthetic enzymes but it might be affected by temperature (Ahmed *et al.*, 2008) [1].

Conclusion

From the above study it was observed that activity of starch biosynthetic enzymes like AGPase, SSS and GBSS was increased throughout the developmental stages. Among all the varieties AGPase and GBSS were found higher in rice (PB-2) which corresponds to higher accumulation of amylose and total starch. However, activity of SSS was initially minimum in all these varieties while at maturity activity was increased significantly. At later stage higher activity of SSS leads to the synthesis of branch chain resulting in the higher accumulation of starch comparatively to initial stage of endosperm development. The activity of starch biosynthetic enzyme corresponds to positive correlation of amylose and total starch accumulation. So, it is important to manipulate the starch biosynthetic enzyme to fulfil the demand of starch for growing population, growing food industries and protect our environment from non-degradable biopolymer.

Acknowledgement

We are grateful to Department of Genetics and Plant Breeding and Department of Agronomy, GBPUAT, Pantnagar for providing experimental fields. Our sincere thanks to DST-FIST for providing equipment facility and Directorate of Experiment Station (DES) Pantnagar for utilization of infrastructure. I greatly acknowledged ICAR (Indian Council of Agricultural Research) for providing financial support.

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