



## Original article

## Determinants of resistant starch accumulation in wheat endosperm

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## ABSTRACT

A part of the big three cereal crops in the world, wheat has become a major constituent of the everyday food chain and is grown at a massive scale to meet global demands. This makes it an important crop from an economic as well as food security perspective. Selection of high-quality cultivars and consistent trait enhancement for such cultivars is crucial, and in light of new challenges from climate change, this has become an absolute necessity of time. In this regard, we conducted a detailed qualitative and quantitative trait analysis for multiple commercially viable varieties of wheat, and corresponding results were subjected to a series of critical statistical analyses. Final results have shown that five cultivars including Uqaab-2000, Faisalabad- 85, Anmol-19, NARC-2009, and Pirsabak-2004 depicts higher levels of various essential qualitative and quantitative traits (including Starch content, grain weight, RS content, Protein content, etc.) and are most viable varieties for further growth and trait enhancements to meet regional and global food challenges.

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## 1. Introduction

Wheat (*Triticum aestivum* L.) is among the major cereal crops grown worldwide and is an important constituent of the human food and animal feed chain. The crop has been a primary focus of world food systems ever since the beginning of the “Neolithic or new stone age revolution”, as humans begin to transit from hunting to cultivation-based food growing methods, the wheat crop was the primary cereal crop choice (Nesbitt, 1998, Dubcovsky and Dvorak, 2007). Early evidence shows first cultivation maneuvers for the wheat crop were carried out in the Levant region some 10,000 years ago and from there crop was gradually dispersed all across the globe by the early 18th century (Feldman et al., 2001, Shewry, 2009). Seed of wheat is the primary source of many nutri-

tional biomolecules, and the main storage compound in wheat seeds is starch, which accounts for about 65–75% of the final grain's dry weight making out an overall large proportion of the seed and is a major source of carbohydrates in the food system. The starch product is mainly formed inside the amyloplast present in the endodermal cells after anthesis for about 4 days, during this period the overall structure of the endoderm remains the same. This starch is the most important component of granules and a key energy reservoir within the plants. This starch is further composed of a mixture of two groups of molecules; amylose and amylopectin. Amylose molecules are of relatively lower molecular weight and are composed of small but significant proportions of long branched chains having 1–4- $\alpha$  linear linkages, whereas the amylopectin molecules have relatively larger molecular weights and with a large proportion of branched short chains inside the molecule having 1–6- $\alpha$  linear linkage points in it (Takeda et al., 1993).

Starch synthesis is a complex biological process involving a series of enzyme types playing a particular role in this process, the most important of these are; Starch synthases (SS), starch branching enzyme (SBE), and debranching enzyme (DBE)(Gous et al., 2013). Combine action of all these enzyme groups which ultimately determines the number of biochemical process responses from distribution of chain lengths to the degree of polymerization of amylose and amylopectin chains is a key component in determining the final proportion of two chains which imparts

**Abbreviations:** AC, Amylose content; Apc, Amylopectin Content; PC, Protein content; RS, Resistant Starch content; SC, Starch content; TGW, Thousand-grain weight.

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key functional and nutritional values to final crop seed that is detrimental in product finishing as food, feed or other industrial-based products as it determines factor which influences the retrogradation property and gelatinization properties which affects its final products (Salman et al., 2009, Li et al., 2019). Furthermore, the quality and yield of seeds are determined both environmentally and genetically. The status of soil water, specifically during the development of grain, ranks as the most significant environmental factor which affects the grain quality and seed in cereals (Lu et al., 2015). Quality of the grain is a somehow complex trait having several determinants, for instance, physical appearance, cooking and eating quality, and nutritional value. Such factors are also beneficial for the consumers and are related to the physicochemical properties and determinants of crop starch, which include hydration, expansion, gelatinization, and digestion properties (Zhang et al., 2017).

Owing to its rapid expansion worldwide wheat has become an important cereal crop and now accounts for most of the starch consumption around the globe. It has become the primary source of glucose consumption and is important for maintaining blood sugar levels. Often during starch consumption, a fair proportion of total starch content is not digested properly as escapes into the colon region in this undigested form and is termed to be the resistant starch (RS). A key benefit of this RS is that it acts as a source of fermentable substrate for microbiota in the large intestine and produces a large number of healthy small chained fatty acids. It has been observed that amylose content percentage can greatly influence the levels of RS in the colon region thus influencing the human gut health. In this regard, to impart a certain level of nutritional value to the overall diet the appropriate configuration of amylose and amylopectin content in wheat starch is of great significance and is crucial for the production of a wide range of health benefiting products from wheat sources (Bird & Regina, 2018; Newberry et al., 2018).

In this regard, the current study is mainly focused on assessing multiple traits of wheat grains for 50 cultivars and how various factors influence these changes, for the selection of the best possible cultivars for product development.

## 2. Materials and methods

### 2.1. Plant material

For the current study, germplasm of a total 50 varieties were collected from Wheat Research Institute (WRI) of the Ayub Agriculture Research Institute (AARI), Faisalabad. Each of the germplasm was cultivated, grown under field conditions, harvested and subjected to detailed quantitative and qualitative analysis.

### 2.2. Starch content assay (SC)

The wheat grains were initially ground to whole-meal in a cyclone mill machine. It was achieved by using a sieve with a size of 0.5 mm (Cyclote 1093, Tecator, Sweden). The Starch element of the ground wheat was tested using the AACC method 76.13 it was achieved by using 20 mg of whole-meal for each of three identical samples (Konik-Rose et al., 2007).

### 2.3. Assay of amylose content (AC)

The contents of starch were determined by a slight modification of the method of Morrison and Laignelet (1983). The first 2 mg of starch was added to 1 ml of 85% methanol solution and was heated at 65 °C for 1 h with random vortexing of solution, followed by centrifuging at 13000 rpm for 5 min, after this the supernatant was

discarded. The starch pellet was subjected to one hour of drying at 65° followed by dilution into 1 ml Urea DiMethyl Sulphoxide (UDMSO) solution (0.6 M urea in 90% dimethyl sulphoxide). The mixture was vortexed and followed by immersion in a hot water bath at 95 °C for 1 h with periodic vortexing for the complete dissolution of the starch content. An aliquot of the starch-UDMSO solution (50 µl) was nursed with 20 µl of I2-KI solution (1:10 w/w in water) and made up to 1 ml with water. The sample absorbance of 200 µl solution was measured with an "Emax Precision Microplate Reader" at 620 nm along with standards containing amylose ranging from 0 to 100%, made from combined potato amylose and corn amylopectin. Absorbance values were converted to percentage amylose using a regression equation derived from the standard samples.

### 2.4. Resistant Starch content (RS)

Resistant starch content was estimated following the protocol explained by Englyst et al., (1992).

### 2.5. Thousand-grain weight (TGW)

The grain weight from all genotypes was measured using an electric weighing balance. The average grain weight measured was the mean of the total triplicates of 1000 grains for each genotype.

### 2.6. Analysis of starch A and B granule size

The Granule's distribution of size by volume of the starch slurry was determined using a laser diffraction particle size analyzer. The total % age of the small B-type starch granules was deduced using a defined cut-off diameter. The diameter of A-type starch granules was calculated as the diameter of starch granules at the peak of A-type starch granules. The particle size characteristics of the starch were determined using an MS-2000 laser particle size analyzer (Malvern, England). The instrument uses laser light scattering to measure sizes between 0.2 and 2000 µm. The dispersed phase was absolute ethyl alcohol. Starch granule size distributions were measured using the native instrument software and expressed as percentages.

### 2.7. Assay of protein content (PC)

Protein content for the wheat germplasm was estimated using a near-infrared analysis approach applied using a NIR 5000 (FOS) based on protocol explained by Cavanagh et al., (2010).

### 2.8. Granule bound starch synthase activity

Protein molecules adhered to starch granules were initially isolated and later separated via an SDS-PAGE gel (Rahman et al., 2000). Silver staining was used to stain these proteins followed by scanning of gels for image analysis (Li et al., 2000).

Initially, the grain samples (180–200 mg) were taken and homogenized in a pre-cooled pestle and mortar with extraction buffers containing HEPES-NaOH (pH 7.6), MgCl<sub>2</sub>, dithiothreitol (DTT), EDTA, insoluble polyvinylpyrrolidone, and glycerol added to it. The homogenized mixture was then spun at 12000 rpm for 10 mins, the corresponding supernatant for the solution was collected and examined for SSS (soluble starch synthase) assay. After collecting the supernatant, an extraction buffer was used for re-suspension to analyze the GBSS activity. The GBSS and SSS activities in these grains were measured by the process which was developed by Wang et al. (2020). The units used to express enzyme quantity were nmol mg<sup>-1</sup> protein min<sup>-1</sup>.

### 2.9. Statistical analysis

Data were collected for each trait/parameter and subjected to statistical analysis. Analysis of variance (ANOVA) for each of the traits was carried out, keeping in view the cultivar and time, complete randomized design (CRD), followed by LSD test with a 5% probability ( $p < 0.05$ ) and correlation estimation for factors under study. In addition to this, a cluster analysis was also carried out to align various cultivars into multiple groups for better estimation of high-value traits among various commercially viable cultivars. IBM SPSS and R-studio were used for statistical analysis of collected data and its plotting.

## 3. Results

### 3.1. Starch content (SC)

Starch content levels were assessed for all cultivars under investigation, each of them showed a stable response as the overall deviation among the cultivars and within the cultivars (for 3 replication) was minimum. Overall, the cultivars NARC-2009, Faisalabad-85, Sehar-2006 showed the highest level of starch content whereas cultivars Amin-2000 and BARS-2009 showed the lowest levels of starch contents. The difference between the two

extremes of starch response was less than 10%, all other remaining cultivars exhibited a moderate response with recorded values lying in between the two extremes. Fig. 1 gives graphical representation of all cultivar values.

### 3.2. Amylose content (AC)

Amylose is one of the two major structured molecular chains within the seed storage material, the cultivars under study were evaluated for their particular levels of amylose, all the cultivars exhibited significantly identical responses with each giving a fair amount of amylose content. Overall, the cultivars Zarghoon and Meraj-08 showed the highest levels of amylose content. Fig. 2 gives graphical representation of all cultivar Amylose content values.

### 3.3. Amylopectin content (Apc)

Amylopectin is the second major component of starch reserves present inside seed grain, these are chained molecules and are usually present in a higher concentration in comparison to amylose. In this Uqaab-2000, Faisalabad-85 and Abadgar-93 showed the best response to the assessment whereas Zarghoon and Meraj-08 showed the lowest levels of amylopectin contents, remaining of the cultivars showed moderate responses with minimum deviation

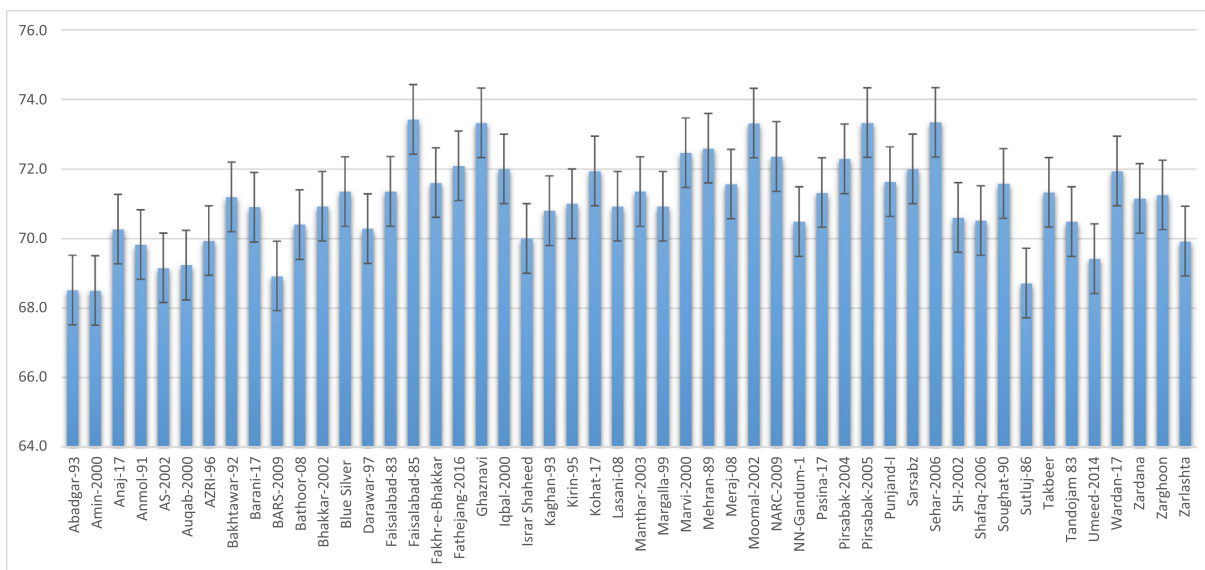


Fig. 1. Starch content of studied wheat genotypes.

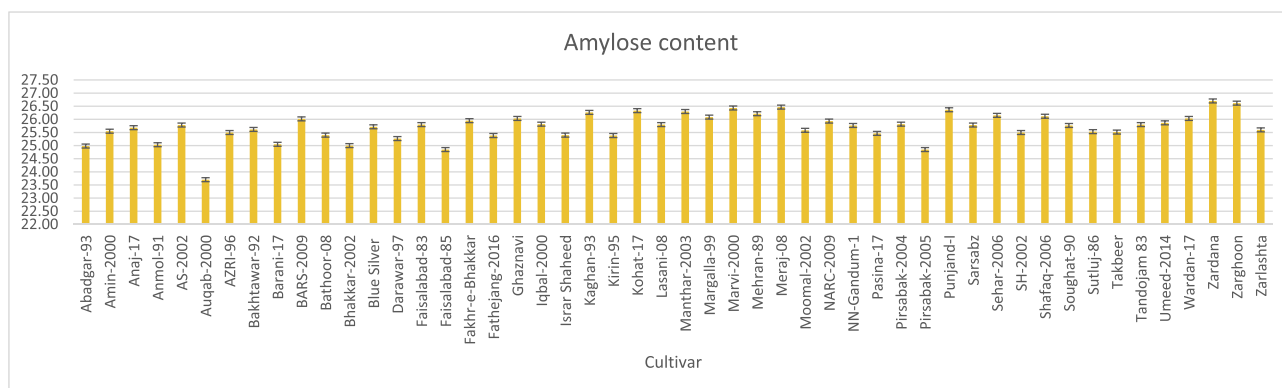


Fig. 2. Amylose Content of studied wheat genotypes.

among recorded values. Apc content for all cultivars are shown graphically in Fig. 3.

### 3.4. 1000-Grain Weight (TGW)

Grain weight is an important standard for overall yield assessments and performance evaluation of all cultivars with better seed size indicating higher yield responses. For this trait, a higher degree of variation was observed among all varieties under observation, overall AS-2002, Uqaab-2000, Tandojam 83 showed the highest 1000 grain weight values. Followed by this a 15–20% difference was observed between these and the next best-performing cultivars including Anaj-17, Barani-17, Fakhr-e-Bhakkar, and BARS-2009. Two of the varieties exhibited the worst responses overall; Zardana and Darawar-97 which showed minimum results overall and a difference of 40–45% grain weight response was recorded between the highest and lowest recorded values for these cultivars. Fig. 4 gives TGW values of all cultivars.

### 3.5. Protein content (PC)

Protein content assessment for food products is often marked as an important part of food evaluation systems, being a key element to all biological processes protein consumption is crucial to the stable biological performance of living entities. In this regard, the protein content for these given cultivars was assessed and corresponding outcomes were recorded. NARC-2009, Sarsabz, Takbeer, AS-2002 showed equally effective responses and were overall the highest recorded values for PC. Fig. 5 gives graphical representation of protein content values.

### 3.6. Analysis of starch A and B granule size

Starch granule A and B are the two major types of starch granules present in mature wheat grain. A major difference between these two given types of granule is in the existence of a type of amylopectin chains. Among all the varieties assessed the A-type granule was the most frequent type of starch granule, although the B-type granule was not that dominant still it was observed in sufficient quantities among all the given cultivars. The A-type granule concentrations were maximum in Anmol-91 and Wardan-17 whereas all other cultivars showed prominent results for A-type starch granule as only about 5% differences were observed between the highest and lowest recorded values. For B-type granules, the concentrations were relatively lower as most of the cultivars showed a response 50% less than the A types. Overall values for B granule were consistent among all the cultivars with minimum variations. Fig. 6 gives graphical representation of Starch granules A and B.

### 3.7. Resistance starch (RS)

Resistance starch concentrations are assessed for estimating health benefits of wheat varieties with the increased importance of RS content reported consistently the content evaluation now has a key detrimental effect for commercial selections of varieties. A certain level of variation was observed among recorded RS content for all varieties with some exhibiting higher levels, whereas others showed slightly moderate to low results. Fig. 7 gives graphical representation of all cultivar values.

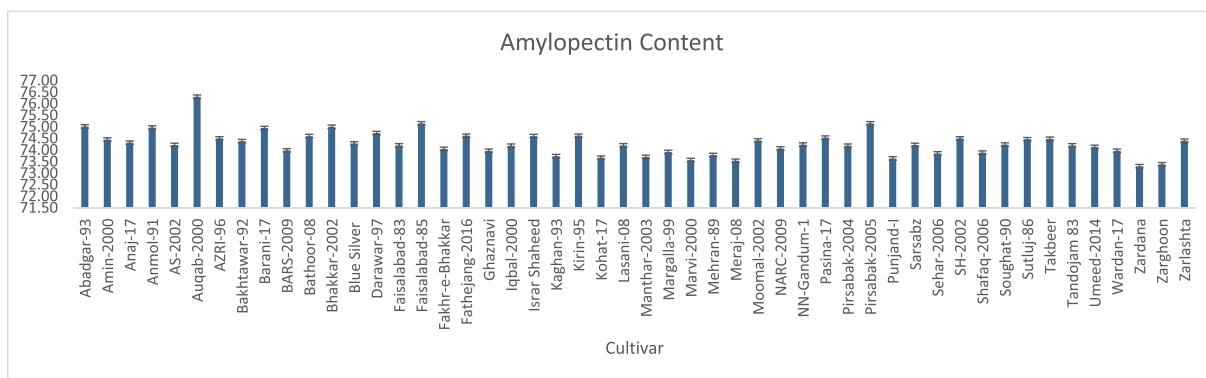


Fig. 3. Amylopectin content of studied wheat genotypes.

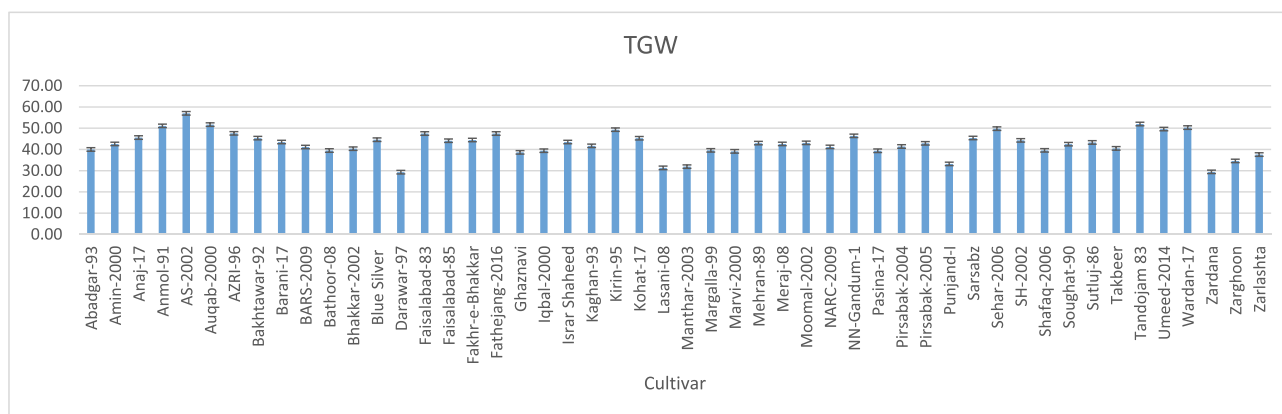


Fig. 4. Thousand Grain Weight of studied wheat genotypes.

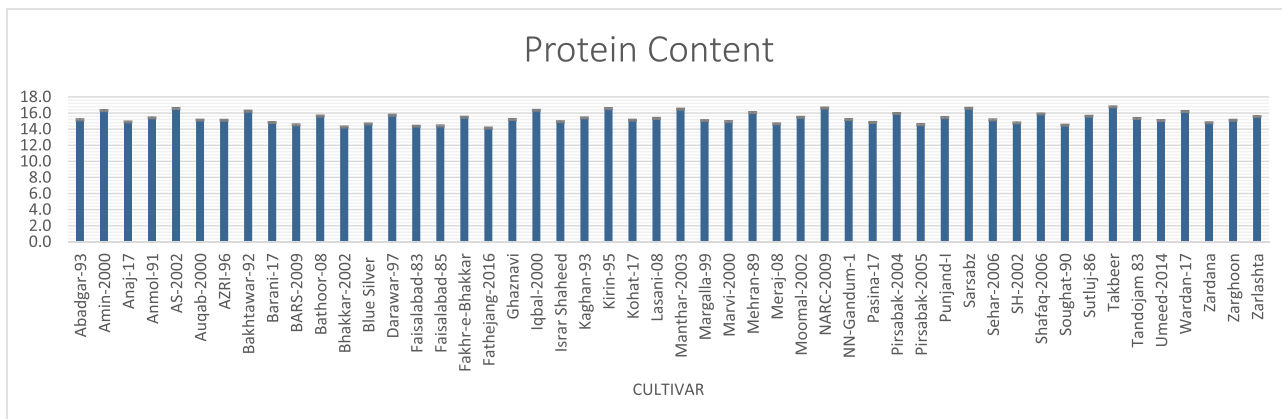


Fig. 5. Protein Content of studied wheat genotypes.

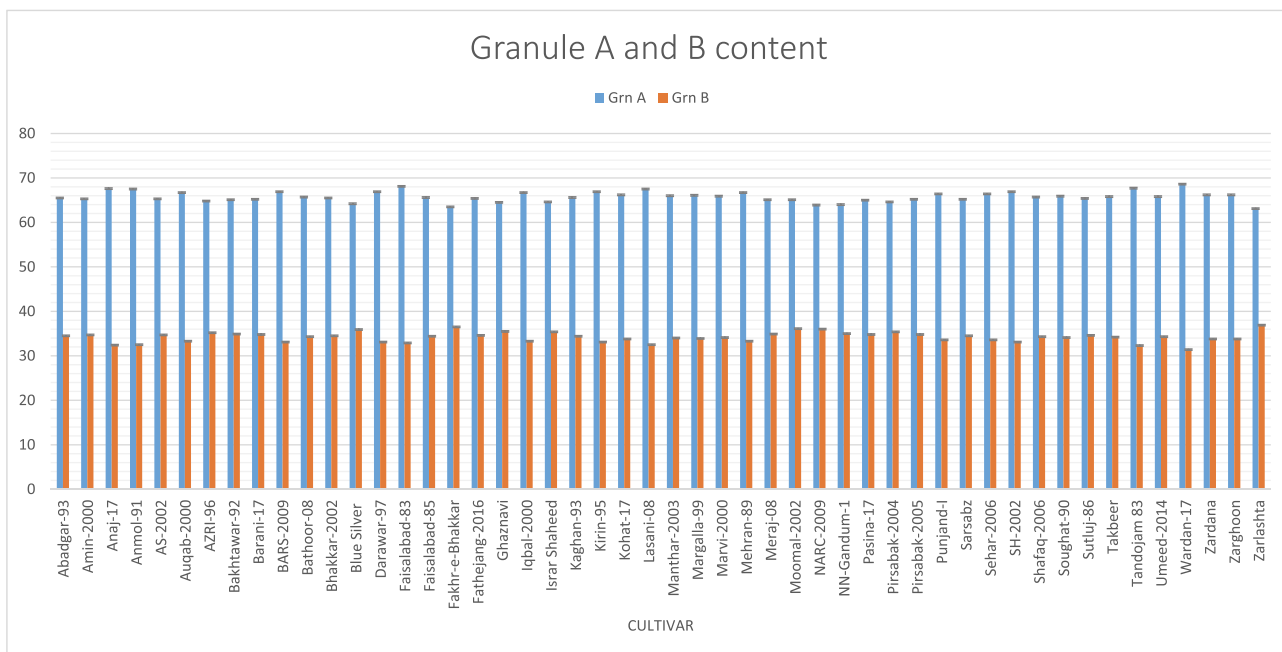


Fig. 6. Granule A and B content of studied wheat genotypes.

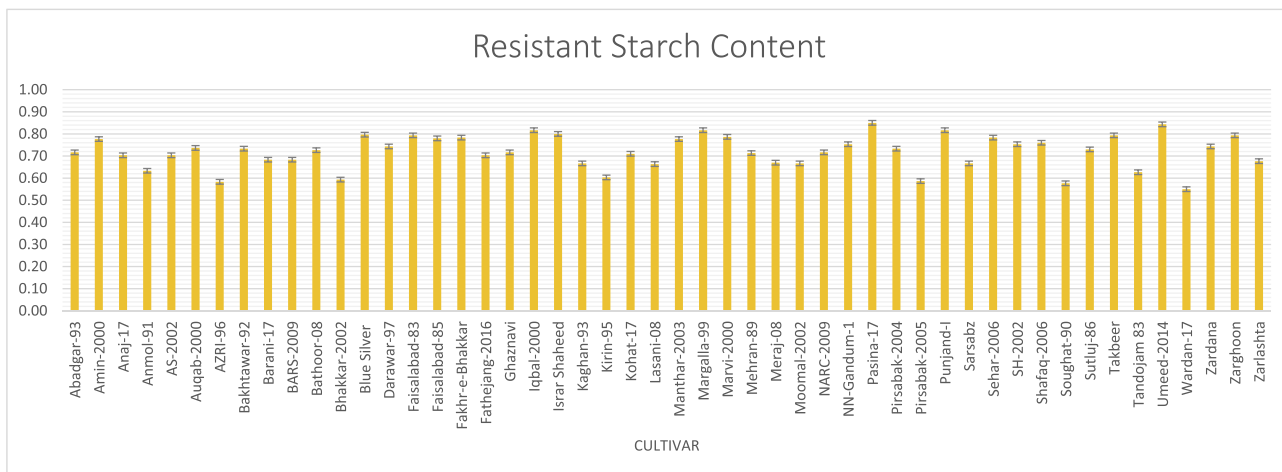


Fig. 7. Resistant Starch Content of studied wheat genotypes.



### 3.8. Statistical analysis

By analyzing the mean values of different traits and variation coefficients, the level determination and tested varieties stability was found. The different variables which were analyzed were amylose content, amylopectin content, resistance starch content, average yields, 1000 grain weight (TGW), granule bound starch synthase activity, soluble starch synthase activity as well as chain length distribution of tested wheat varieties. The comparison of values together and average showed that NARC-2009, and Zarghoon, were the best performing varieties amongst all.

### 3.9. Cluster analysis

Cluster analysis was carried out for multiple varieties grown under controlled greenhouse conditions. For each of the trial cluster analyses focuses on the grouping of cultivars in such clusters where each of the given cluster varieties gives highly similar responses to external stimuli and the corresponding traits showed high resemblance in their outputs.

For controlled experimentation, the relationships within fifty varieties were estimated by cluster analysis of trait similarity matrices. According to the dendrogram which was obtained for different wheat varieties, the 50 varieties were grouped into various clusters. This dendrogram is separated into different clusters. The first cluster is composed of four observations which are 'Abadgar-93', 'BARS-2009', 'Amin-2000', and 'Sutluj-86' varieties, the second cluster contained 'Bakhtawar-92', 'Sarsabz', 'Kohat-17', 'Faisalabad-85', 'Pirsabak-2005', and 'Mehran-89', the next cluster contained 'Barani-17', 'Israr Shaheed', 'Kaghan-93', 'Meraj-08', 'Sougah-90', 'Moumal-2002', 'NARC-2009', 'Pirsabak-2004', 'Blue Silver' and 'Fakhr-e-Bhakkar.' 'Bathoor-08', 'Shafaq-2006', 'Margalla-99', 'Iqbal-2000', 'Takbeer', 'Bhakkar-2002', 'Pasina-17', 'Ghaznavi', 'Marvi-2000' and 'Zarlashta', whereas 'Zarlasta' is the counterpart of other nine varieties of this cluster. 'Darawar-97', 'Lasani-08', 'Zardana' are closely related and placed in the same cluster whereas 'Manthar-2003', 'Punjnand-1', and 'Zarghoon' are the counterpart of the previous varieties placed in the same cluster. The next cluster consists of five varieties, which included

'Anaj-17', 'SH-2002', 'AZRI-96', 'NN-Gandum-1', and 'Fathejang-2016.' The last cluster included 'Anmol-91', 'Tandojam 83', 'Wardan-17', 'Faisalabad-83', 'Sehar-2006', 'Kirin-95', 'Umeed-2014', and 'Uqaab-2000' wheat varieties. 'AS-2002' is the unrelated one therefore it is the out-group. The last cluster only had one variety—AS-2002, which proved to have the lowest yield because of its low TGW. The above analysis shows that 'Abadgar-93', 'BARS-2009', 'Amin-2000', and 'Sutluj-86' are among the best wheat varieties for various agricultural traits as shown in Fig. 8.

### 3.10. Heat-map

The heat map is a graphical data representation where individual values present in the matrix are represented in the form of colors (Toddenth, 2014). A heat-map is a way to visualize hierarchical clustering. Heat maps permit to visualize features and sample clusters. The categorical phenotypes for instance wheat varieties can be annotated in the rows whereas several continuous parameters such as assay of amylose content (AC), starch content (SC), and others can be annotated in the columns heat map figure as shown. These varieties were further divided into several groups which were then labeled by particular color at the cut-off level. To demonstrate heat-map, various wheat varieties were used to check the response given under various parameters. First, differential analysis was performed to compare expressions given by different varieties. Various varieties showing greater standard deviations were selected and then randomly selected 25 varieties (as shown in Fig. 9) to generate a heat map. Several important parameters for the demonstration were included.

The selected variables were grain yield, amylopectin content, thousand-grain weight, resistant starch content, an assay of protein, starch, and amylose content. Using this data, a heat map was generated having column side and row side annotations and a legend color bar (Fig. 2). The dendrogram shows that amylopectin content and thousand-grain weight (TGW) showed the most similarity. The same was observed in the case of amylose and starch content as they are the most similar subgroups. This heat-map also shows that PC and ApC showed the best result. Functional properties of wheat and its associated products could

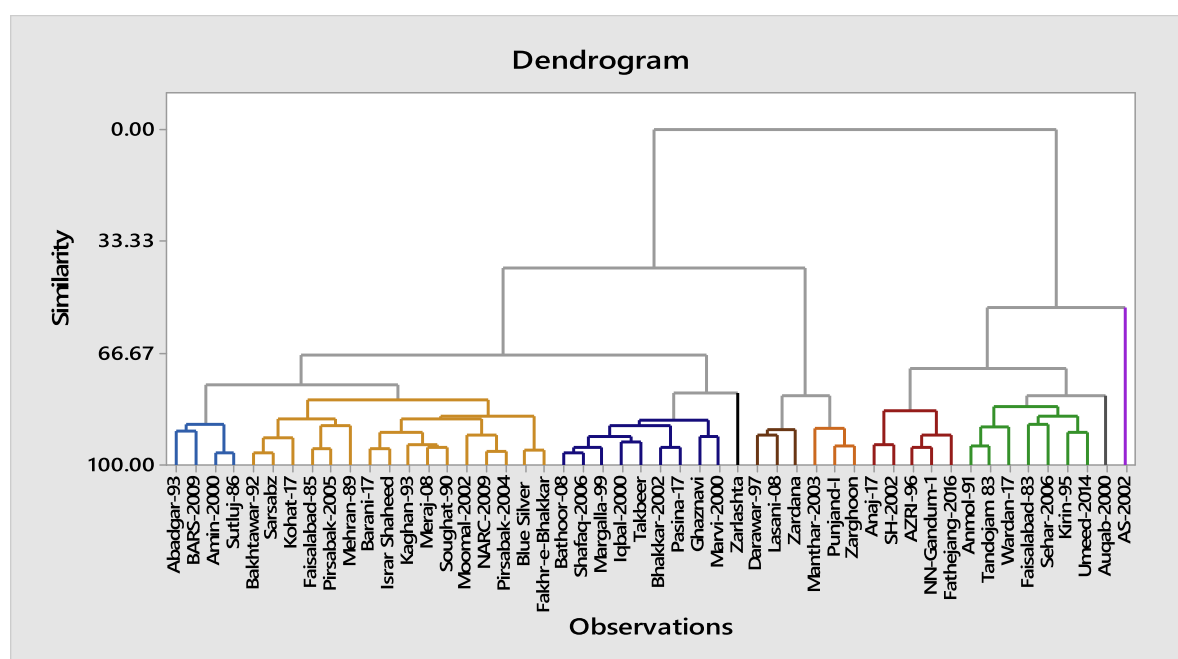
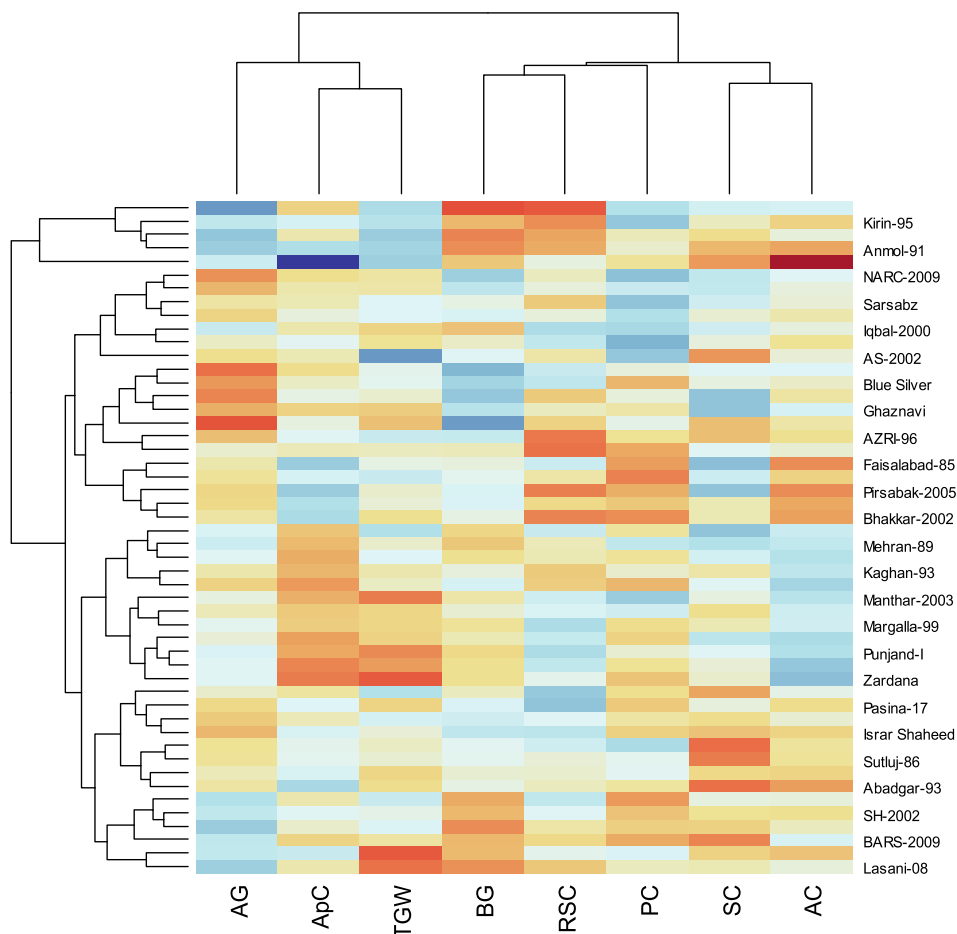


Fig. 8. A dendrogram showing the result of the hierarchical clustering.



**Fig. 9.** An example of the heat-map. This heat map was generated by using 25 genotypes of wheat. Based on the correlation between varieties, the dendrogram was further divided into two parts. The clusters and dendrograms were generated and all varieties were selected by the standard deviation.

be affected by the total proportion of D-glucose polymers that are present in granules of starch, which are amylopectin and amylose content. Computational multivariate analysis of data were also performed as shown by the dendrogram. The lowest amylose content (AC) was found in Anmol-91 whereas the same variety showed the highest amylopectin content (ApC). The response of these varieties to Resistance starch content (RSC) was also checked which was found to be of moderate value in Kirin-95. TGW (1000 grain weight) was calculated and it was found that each variety showed mixed results. The significantly largest (TGW) 1000 grain weight was found for the varieties and was further assessed and utilized in the clustered analysis.

Based on the results obtained from the heat-map, it was inferred which specific variety showed the best activity under defined conditions and designated standards, that further defines their important role. Results showed that the heat-map can be useful in the selection of traits that best indicate phenotypes of the samples (Fig. 10).

### 3.11. Correlation analysis

Correlation analysis, correlation is used to measure the strength of the linear relationship between various quantitative variables. Every cell in the table represents the correlation between any two variables. The correlation matrix is used for summarizing the data, putting it as an input, and changing it into an advanced analysis, and for advanced diagnostic analyses. There are two correlations. A negative correlation is on the right-hand side, on which the scale shows red color while a positive correlation is towards

blue. This color scale follows a gradient that is based on the strength of correlation. The coefficient of correlation 'r' measures the direction and strength of the linear relationship, '1' represents exact positive correlation, '-1' represents exact negative correlation, 0 shows that no relationship is present between different variables. The values between 1 and -1 show the strength of correlation.

### 3.12. Principal component analysis

We have 8 variables on both the vertical and horizontal axis. We also have a spectrum in which dark red indicated a strong negative correlation whereas dark blue indicated a strong positive correlation. Moving along the diagonal, variables' autocorrelation is shown. Correlation coefficients were assessed to find out interdependence between various variables. For example, SC shows perfect correlation with SC, the same is the case for other variables. Other correlations are shown in two ways, one is by the color and the other by the shape, which is indicative of the strength of correlation. In the figure, it can be seen that ApC and AC showed a very weak negative correlation. Similarly, AG and BG also showed a weak negative correlation. A very strong positive correlation can be seen between RSC and SC, AG and AC, PC and RSC. Similarly, TGW and PC showed the strongest positive correlation. The best properties were given under RSC in which most of the results found that a strong correlation was found. Whereas in the case of PC, the same pattern was observed. These wheat varieties had several favorable properties, for instance, TGW.

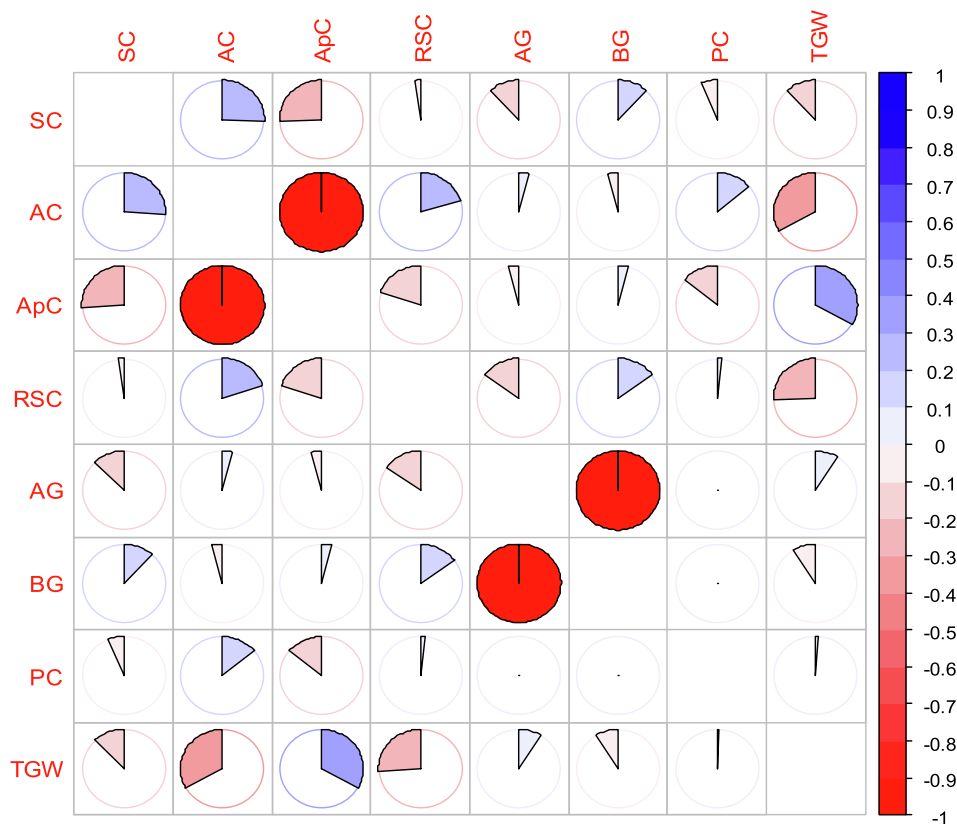


Fig. 10. Phenotypic correlations for the studied wheat genotypes following different parameters.

### 3.13. Variable plot

The principal component analysis represents expression levels of different variables at baseline. It also shows relationships between all the variables. Variables-PCA graph represents variable correlation plots of principal component analysis. It takes place with the contribution of variables expression level shown with a color gradient. This is known as control. Scree plot, the center, shows the percentage of variance which is explained by every dimension. Dimensions 1 and 2 explain 30.2% and 25.8% of the variance respectively. Positively correlated variables ApC and BG are grouped. TGW positioned towards Dim2. Whereas negatively correlated variables PC, SC, RSC, AC, and AG are in the opposite quadrant (Fig. 11).

## 4. Discussion

Wheat is among the big three cereal crops and is cultivated on a massive scale across the globe, ultimately serving as a major source of daily nutrition for a massive proportion of the world population (Kumar et al., 2013). Phenotypic and biochemical trait analysis is considered a major option for better estimation of cultivar's intrinsic values and the corresponding response can further be utilized for better cultivar selection.

In the current study, multiple analyses have been performed for the estimation of key traits and ultimately the selection of these cultivars for further cultivation and breeding purposes to develop more nutritious and climate-resilient varieties. Assessment for traits such as resistance starch (RS) was carried out owing to its immense health benefits; the RS plays a major role in healthy bacterial growth in the intestinal tract as it acts as a nutrient-rich food source for healthy bacteria and thus improving digestive function-

ality of the tract (Silvi et al., 1999). Also, the RS content assists in processes such as mineral absorptions (including Calcium and iron) and helps in maintaining blood cholesterol levels in humans (Coudray et al., 1997; Nugent, 2005). Similarly, other traits have a significant beneficial impact and were assessed for the determination of best cultivars.

Recorded traits were statistically analyzed and subjected to cluster analysis. According to the dendrogram which was obtained for different wheat varieties, six main clusters were formed with each of them consisting of varieties that have exhibited closely related responses for the evaluated traits. In addition to the cluster analysis, all traits were also subjected to heat-map based assessment, and a close trend was observed as high performing varieties (Uqaab-2000, Faisalabad- 85, Anmol-19, NARC-2009, and Pirsabak-2004) exhibited closely related responses for numerous important traits including, grain size, protein content, amylose content, and resistant starch content.

The principal component analysis is used for the extraction of the important information, which is taken from a multivariate table containing data and to show that this information is a set of some variables known as **principal components**. These variables relate to the linear combination of originals. The principal components number is equal to or less than the original variables. Information in the given data set agrees to the total variation it holds. The goal of PCA is the identification of directions (or **the principal components**) in which variation in data is maximum. PCA reduces the dimensionality of multivariate data to either two or three main components, which can be graphically visualized, with minimal information loss. This multivariate analysis approach has helped in assessment of qualitative traits of samples. Principal component analysis represent expression levels of different variables at baseline. It also shows relationships between all the variables. Variables-PCA graph represents vari-



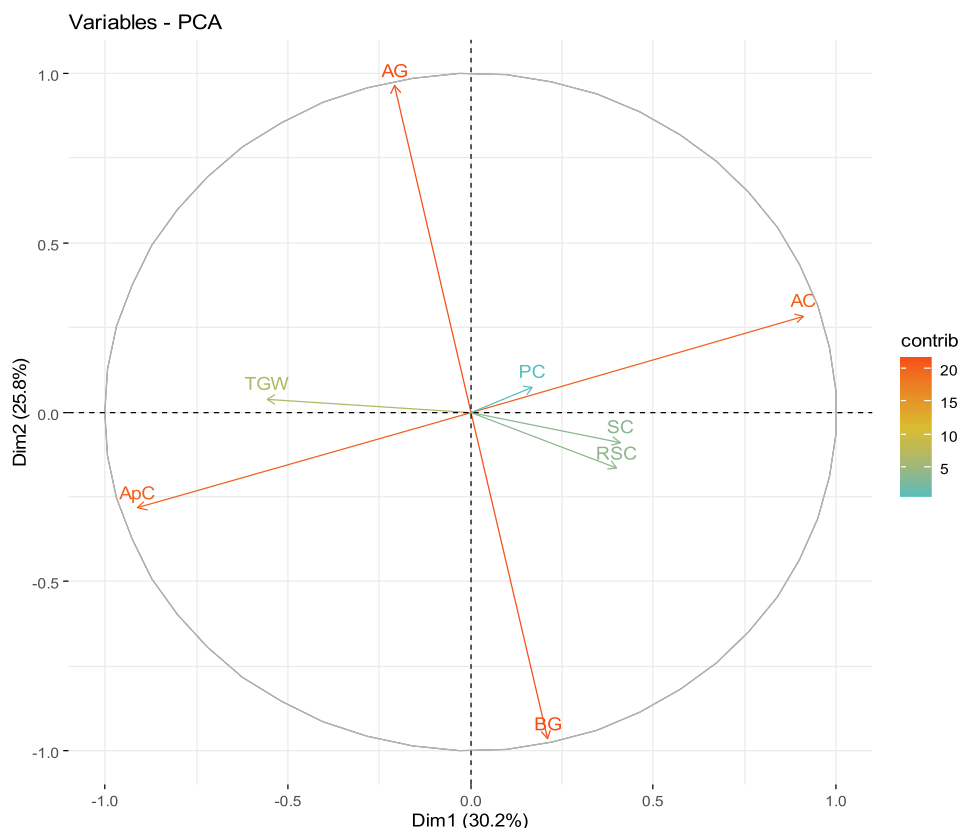


Fig. 11. Variable plot of the principal component analysis (PCA) for different variables in the studied wheat genotypes.

able correlation plots of principal component analysis. It takes place with contribution of variables expression level shown with color gradient. This is known as contribution. Scree plot, the center, show percentage of variance, which is explained by every dimension. Dimensions 1 and 2 explain 30.2% and 25.8% of variance respectively. Positively correlated variables ApC and BG are grouped together. TGW positioned towards Dim2. Whereas negatively correlated variables PC, SC, RSC, AC and AG are in the opposite quadrant.

Pre-determination of several cultivar traits can be of great help for estimating the potential yield and nutritional impact of grown cereals. In this regard, the current study focused on a better understanding of these complex qualitative and quantitative yield traits and have established an approach where statistical evaluation for such complex traits can ultimately be used for better seed storage, crop planning, and increased harvest.

## 5. Conclusion

The results of the present study provided information about traits of wheat varieties, which are crucial in the assessment and selection of suitable varieties as part of effective agricultural production strategies. The resultant crops are superior in traits and purposefully important for better growth, better yield outputs, and nutritious crop which can be used in daily food chains. The tested wheat varieties were divided into groups having low and high appropriateness for this particular system as per agricultural features. The varieties which were most beneficial for cultivation were found to be Uqaab-2000, Faisalabad-85, Anmol-19, NARC-2009, and Pirsabak-2004. These varieties are recommended for growing purposes and recom-

mended for further exploration in yield and qualitative trait enhancements.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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