Advances in Bioresearch
Adv. Biores., Vol 7 (3) May 2016: 109-118
©2016 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV Value 8.21 [2014]

Advances in Bioresearch

ORIGINAL ARTICLE

Real Time PCR Analysis of α-amylse Gene Expression in Spring Wheat during Pre-Harvest Sprouting

A.Ahmadpour Malakshah
Department of Environmental Sciences, Savitribai Phule Pune University, Pune, India.

ABSTRACT
The changes in gene expression of α-amylase (RT-PCR) and biochemical attributes during pre-harvest spouting (PHS)in different wheat genotypes of northern Iran were analyzed using sprouted grains of different stages .The results revealed that amongst the wheat genotypes investigated, N-87-8 had shown very high level of gene expression, high CT and subsequently stimulated α-amylase activity, confirming its high sensitiveness to PHS. It was also supported by very high reduction in starch accumulation, kernel weight and yield. While the variety Nai60 had shown comparatively low expression of gene, low CT and low level of α-amylase activity, with less reduction in kernel weight and yield, revealing its high tolerance to pre-harvest sprouting(PHST). The remaining cultivars (N-80-19 and N-87-12) were marked as medium sensitive, on the basis of above studies. On the basis of biochemical analysis N-86-12 had higher content of starch, illustrating its tolerance to PHS. From this study it can be concluded that molecular assay coupled with biochemical analysis will serve as the better tool for screening of PHST in wheat, where its cultivation is prone to this phenomena. The results of molecular and biochemical analyses were tested in field experiments using mist irrigation (MI). The regression analysis ofα-amylase activity in sprouted grains of spike (after MI) showed lower linear model in both N-86-12 and Nai60, fully confirmed their PHST. The increasing level of α-amylase with increasing days of MI also supported the PHS susceptible nature of N-87-8 in field trial.
Keywords: *wheat, gene expression, α-amylase, starch, yield, PHS*

Received 18/01/2016 Accepted 04/04/2016

How to cite this article:
A.Ahmadpour Malakshah. Real Time PCR Analysis of α-amylse Gene Expression in Spring Wheat during Pre-Harvest Sprouting. Adv. Biores.Vol 7 [3] May 2016: 109-118. DOI: 10.15515/abr.0976-4585.7.3.109118

INTRODUCTION

PHS is one of the main disadvantages of wheat crop particularly when harvest time coincides with rainfall and high humidity, leading to heavy losses in grain yield and quality [1]. Many options have been proposed for the control of sprouting damage, including molecular analysis [2]. Several genes have been characterized that play an important role in embryo development, maturation and induction of dormancy during germination [3, 4]. Gerjets *et al.* [5] noted that biochemical analysis and monitoring of α-amylase at transcriptional and post-transcriptional levels during kernel development is pivotal in PHS.

Similarly Derycke *et al.*[6] and DeLaethauwer *et al.*[2] confirmed that α-Amy2 peaked in the beginning of kernel development, while α- Amy1 increased towards harvest maturity and the relative α-Amy1 expression was higher in the PHS-susceptible varieties using RT-PCR. Damage caused by PHS has often been associated with increased levels of α-amylase activity in the kernel because it converts starch into soluble sugars and induce germination [7, 8].The high α-amylase activity negatively affect the nutritional and end-use quality of grain [8, 9].

Although different levels of α-amylase activity had been detected in cereals like wheat, rye and triticale, they all show a typical pattern during kernel development [10]. The expression of α-Amy genes is positively controlled by a transcription factor GAMYB which binds to the gibberellins (GA)-responsive element (GARE), present in the promoter region of α-Amy genes [11]. Seed dormancy is generally induced during kernel development and depends on environmental conditions as well as biochemical process and genetic makeup of cereals. The full range of crosstalk between them is not yet fully established. Although some of these genes are possibly involved in both PHS and late maturity α- Amylase (LMA), little information is available on the behavior and expression of these genes during kernel

development. Hence the study was attempted to focus on the α- Amylase genes expression using RT-PCR and biochemical attributes during PHS in different spring wheat genotypes of north Iran.

MATERIAL AND METHODS

Molecular Analysis of α-amylase:

Changes in expression levels of candidate genes during pre-harvest sprouting of four wheat lines such as N-87-12, N-87-8, N-87-19 and Nai60 were determined by Real time PCR. The primers were: ATTGGGAATCGTGGAAGACA (forward) and CTGGATCAGCGACTTGAGGT (reverse) for amylase and TCAATGTTCCCGCCATGTAT(forward) and AGACCACTGGCA
TAGAGGGAAG(reverse) for beta-actine. α- Amylase Quantification of gene expression of amylase was done using the Qiagen SYBR-green. Reproduction reaction and information analysis was performed using a commercial kit based on the Kurbot methodology.

RNA Extraction:

The QIA GENE Kit was used to extract RNA and its quantity was determined by spectrophotometer (Nanodrop) and its integrity was confirmed using agarose gel electrophoresis.

Primer Design for RT-PCR:

Primer 3 was used to design primers for amplification of beta-actin and alpha-amylase genes. The designed primers were edited using BioEdit and verified by blasting with the sequences registered in NCBI website.ForcDNA synthesis, 1 µl oligo-dT was added to 11 µl extracted RNA before, the sample was heated at 70 ˚C for 5 min and returned to ice afterwards. Then 1 µl Riboblock (RNAse inhibitor), 2 µl dNTP and 4 µl Reverse transcriptse (RT) buffer and 1 µl of the RT enzyme were added to the reaction. The reaction mixture was incubated at 42˚C for 1 hr, then transferred to 70 °C for 10 min (for inactivation of the enzyme) and finally stored at 20°C. Also a small quantity of the reaction mixture was applied to gel electrophoresis to ensure the quality and integrity of the synthesized cDNA.

Quantitative RT- PCR

The Kit (USA) was used to quantify α-amylase gene at mRNA levels. Gene amplification and information analyses were carried out using real time Corbett PCR system (QIAGENE; USA).The process of melting curve internally set by the Corbett Thermo cycler was used, followed by detection using gel electrophoresis. SYBER green was used to follow the rate of amplification in each cycle. These data were used to draw a graph specific for each reaction and subsequently determine temperature cycle. The PCR products were electrophoretically resolved in 1% agarose gel.

Experimental Layout for Field Evaluation of Wheat Genotypes under Mist Irrigation (MI)

The split plot design was followed to evaluate PHS tolerance / sensitiveness in four genotypes such as, N-80-19, N-87-12, N-86-12 and N-87-8 with one local cultivar Nai60 for field evaluation under different conditions of MI at Baye kola Agriculture Research Station, Iran. About 20g of seeds of each genotype was sown in six rows per plot. MI was given for 7, 14 and 21 days. Observations were recorded on respective days and the results were analyzed by SPSS software (version 18).

Biochemical Analysis

Starch content was estimated from one gram of grain flour sample using Anthrone reagent (Thayumanavan and Sadasivam 1984) The α- amylase activity in composite grain flour samples was determined by following the method of Sadasivam and Manikum [12].

Statistical Analysis of Data

For analyzing the genetic adjectives, the cross- test exam was followed and for analysis of gene expression from standardize delta CT from T-test and ANOVA was used. For the statistical analysis SPSS software (Version 16) was used.

Model Fitting

The relationships between studied traits and MI were evaluated by fitting linear and non-linear regression models by SAS software [Version18]. In this study a segmented model was applied as non-linear model as follows: $Y= a + bx$if $x<x0$ [1]$Y = a + bx0$ if $x\geq x0$ [2], where Y is the studied physiological parameters, a is intercept, b is the rate of increase or decrease in studied traits, $x0$ is turning point between two phases and x is mist irrigation duration. The internal validity of the models was tested by coefficient of determination (R^2).

RESULTS AND DISCUSSION

The results on gene expression of α- amylase during sprouting of grains in wheat genotypes indicated that there was significant difference between the selected genotypes such as Nai60, N-80-19, N-87-12 and N-87-8 (Table 1).While the results of comparison revealed that genotype N-87-8 had highest gene expression for α-amylase and high CT than other genotypes. As a result of this it became PHS sensitive.

However the genotype Nai60 had lower levels of α-amylase gene expression and low CT due to this it was tolerant to PHS. The other varieties e.g N-80-19 and N-87-12 were in between the above two genotypes regarding gene expression of α-amylase and CT (Table 2; Fig. 1), hence had shown medium sensitiveness to PHS.

Table 1 Variation analysis of α-amylase activity in wheat genotypes

Source	*df*	Mean Square
Replication	3	1114.619^{ns}
Treatment	3	5330.642**
Error	9	975.698

Table 2 Mean comparison of α-amylase activity in wheat grains of different genotypes.

Source	Gene expression (Real time)
Nai60 (Hn7//sy swm70053-2y-1y-oy-2ap)	0.411^{b}
N-80-19(SW89-3064-STARcmBw91yo1627s-13y)	0.1613^{b}
N-87-12(SW89.2089/BAKHTAWAR94//SW89.3243)	7.3030^{ab}
N-87-8(HP1761//SW89-5124*2/FASAN)	75.1966^{a}

Relative gene expression of α-amylase activity and beta actin gene in different genotypes revealed that the 196 bp fragments of the amylase gene 3 oxone and 180bp fragment of the beta-actin gene was amplified as shown in (Fig.1).Higher CT mainly indicates less amount of template or less gene expression. CT formula for evaluated genes was according to Melt diagram which is depicted in (Fig. 1,2). CT= -0.802 log (concentration) +18.73.

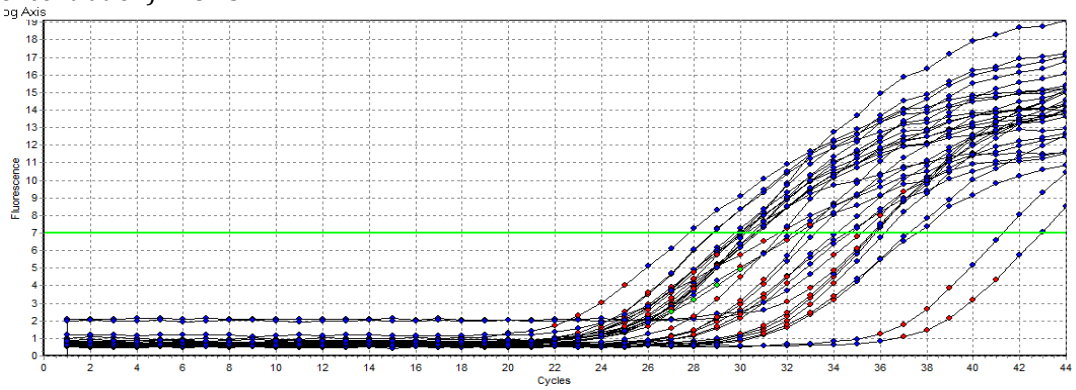

Fig 1 Relative gene expression of α-amylase and beta actin in different wheat genotypes

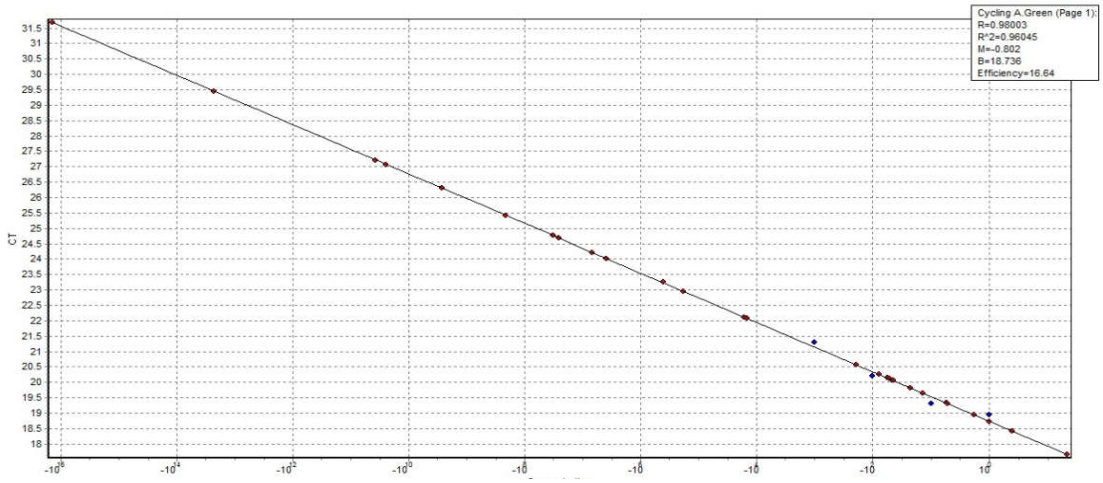

Fig 2 Determination index R^2 fit linear regression for Serial dilutions

The results of mean compression of two, four, six and eight days after germination on gene expression levels of α-amylase revealed that there was no significant difference (Fig. 3).

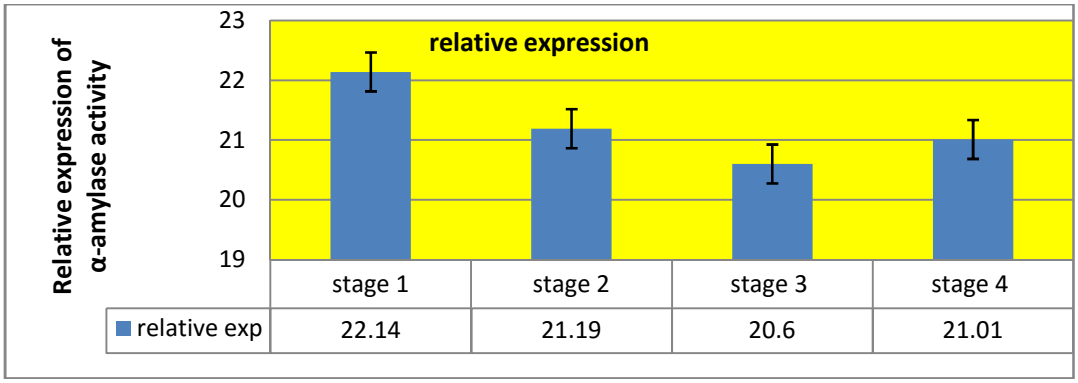

Fig3Mean comparisons of different stages in expression levels of α-amylase activity during eight days of PHS

The results on the effects of PHS indicated that all the five genotypes in third step of MI(after 21 days) showed remarkable reduction in starch, kernel weight and yield as compared to first and second step MI. But in third step of MI α and ß-amylase activities were higher than other two MI.In highly PHS sensitive genotype such as N-87-8, starch(19.0), kernel weight (22) and yield (0.04) were decreased considerably during third step of MI. However α-amylase activity (32.25) was stimulated by PHS after MI. In tolerant genotype such as N-86-12, starch (37.0), kernel weight (39.67) and yield (0.5) were higher during third step of MI(Table 3).

Table 3 Mean comparison of effects of PHS on physiological and biochemical parameters in wheat genotypes

Parameters					
MI	Genotypes	Starch /mg/g	**α-amylase / mg/g**	Kernel weight /g	Yield after MI kgm^2
7	Nai60	140.0 a	1.440e	53.33^a	0.8070a
	N-80-19	182.0 b	1.440e	49.33^a	0.9070a
	N-87-12	140.0 b c d	5.540 cde	32.3 ^{b}	0.8630a
	N-86-12	154.0 b c	3.990 de	46.67^a	0.8030a
	N-87-8	135.0 c d e	3.660 de	46^a	0.8570a
14	Nai60	44.0 de	2.690 de	48.67^a	0.8000a
	N-80-19	34.0 c d e	16.630c d	31.67^{bc}	0.2520c
	N-87-12	32.0 c d e	16.63b	28.9 ^{bc}	0.1600cd
	N-86-12	52.0 cd e	3.990cde	40.33^a	0.7200a
	N-87-8	38.0 d e	23.44b	24.33^{bc}	0.1900cd
21	Nai60	23.0 e	7.130de	38.67^C	0.1500cd
	N-80-19	33.0 de	20.540 c	27.33^{bc}	0.1070cd
	N-87-12	15.0 e	20.72 a	20.67^{bc}	0.06000cd
	N-86-12	37.0de	6.150 e	39.67^a	0.5000b
	N-87-8	19.0 e	32.25 ab	22^{bc}	0.0400d

In each column, means with similar letter(s) had non-significant different according to Tukey test at p=0.05

The results on correlation for selected variables of wheat genotypes indicted that all parameters such as starch (r=0.58*)and kernel weight (0.27) were positively correlated with grain yield after MI. The other parameters such as α-amylase (r=0.82**), and duration of MI (r=0.82**) were negatively correlated with grain yield after MI(Table4).

Table 4 Simple correlation (r) for the selected variables of wheat genotypes during PHS

Variables	Starch	α-amylase	Kernel weight	Duration of MI	Yield under MI
Starch	1				
α-amylase	-0.419*	1			
Kernel weight	0.15*	-0.15	1		
Duration MI	-0.740**	0.566*	-0.02**	1	
Yield	0.58*	-0.82**	0.27*	-0.82**	1

Relationship regression between MI×α-amylase in different genotypes

Preliminary analysis indicated that activity of α-amylase ranged from 34.25 to 1.44 mg/g across genotypes (Table 3). However, the results indicated that relationship between activity of α-amylase and days after MI had linear model depending on genotype. The fitting linear model was prepared for five genotypes such as, Nai60, N-87-8, N-80-19, N-86-12 and N-87-12, but α-amylase in both genotypes, N-86-12 and Nai60 had shown linear decrease as compared to other genotypes. While in N-87-8 genotype, α-amylase activity was very high as compare to other genotypes (2.185 mg/ g per day).

After further investigation the results indicated that all genotypes were approximately similar and there was positive relationship between MI and α-amylase activity. But, α-amylase activity in the both N-86-12 and Nai60 was showing decreased linear model after MI as compared to sensitive wheat genotypes. It might be indicating their increasing tolerance to PHS**.** The results of **r**egression analysis also confirmed the increased activity of α-amylase in N-87-8 (2.185 mg/g) with increased MI, indicating its increasing sensitiveness to PHS (Table 5;Fig. 4).

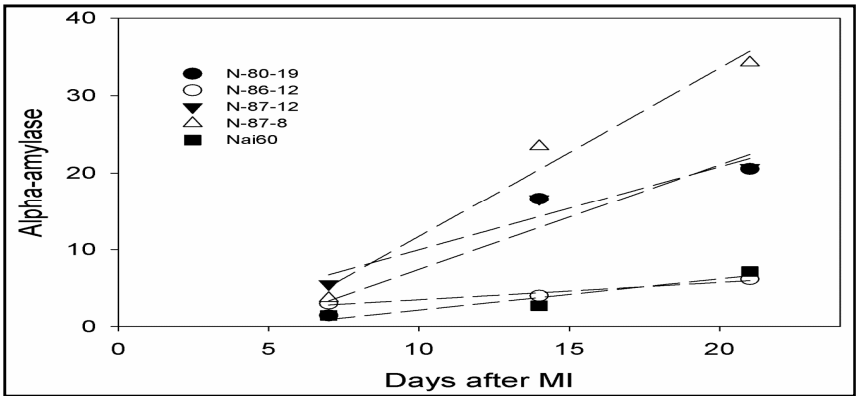

Fig 4 Linear regression between MI × α-amylase in five genotypes of wheat

Table 5 Linear regression between MI x α- amylase in different genotypes of wheat

Traits	Genotypes	a	b	X0	R^2
α-amylase	N-80-19	-6.23	1.3643		0.9
	N-86-12	1.2167	0.2257		0.96
	N-87-12	-0.8833	1.0843		0.93
	N-87-8	-10.14	2.185		0.97
	Nai60	-1.9367	0.4064		0.91

a=Intercept b=Slope X0= In depended variable

Relationship Regression between MI× Kernel Weight and in Different Genotypes

Kernel weight ranged from 53.33 to 20.67 g across genotypes (Table 3), indicating fitting segmented model (dependent on genotypes) relationship between kernel weight and days of MI for five genotypes

including, Nai60, N-87-8, N-80-19, N-86-12 and N-87-12. The results for N-87-12 using the segmented model were 11.11days for turning point between the two phases (x_0) and -1.17 g for reducing slope (b) (namely, at days above x_0, kernel weight reduced linearly with day and before it values of kernel weight was constant). For Nai60, estimates were 13.35 days and -2.57 g, and for N-80-19, estimates were 15.72 days and -2.52 g for x_0 and b, respectively. Therefore, after starting of MI the kernel weight in N-86-12 wheat genotype reduced linearly (-0.91 g per day) with day to about 14.73 days and then the values of kernel weight were constant up to 21 days after MI. There is an exception only for N-87-8 genotype in which kernel weight was mostly reduced as compared to other genotypes (14.75 days for turning point between the two phases (x_0) and -3.1 g per day) (Table 6; Fig. 5).

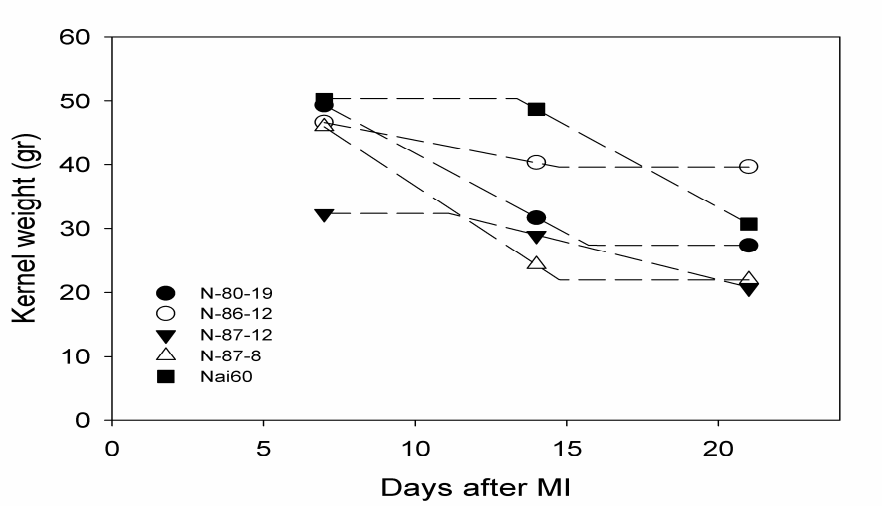

Fig5 Linear regression between MI × kernel weight in five genotypes of wheat

Table 6 Linear regression between MI x kernel weight in different genotypes of wheat

Traits	Genotypes	a	b	X0	R^2
Kernel weight	N-80-19	66.99	-2.52	15.72	0.98
	N-86-12	53.01	-0.91	14.73	0.99
	N-87-12	45.36	-1.17	11.11	0.97
	N-87-8	67.67	-3.1	14.75	0.99
	Nai60	84.67	-2.57	13.35	0.98

DISCUSSION

α- amylase Gene Expression

The results of gene expression of α- amylase during sprouting of grains in all the selected genotypes shown in Table 1 clearly revealed that wheat genotypes such as Nai60, N-80-19, N-87-12 and N-87-8 had significant difference between them. Similarly comparison test also revealed that N-87-8 genotype had the highest relative expression for α-amylase gene and higher CT than other genotypes indicating its sensitivity to sprouting. While genotype Nai60 had lower levels of α- amylase gene expression and less CT showing its PHST (Table2; Fig.1, 2).The enzyme α-amylase was activated during two, four, six and eight days of sprouting period. The 196 bp fragments of the amylase gene 3 oxone and 180bp fragment of the beta-actin gene were amplified. Determination index R2 fit linear regression for serial dilutions and higher CT mainly indicates less amount of template or less gene expression (Fig. 2, 3). De Laethauwer *et al.* [2] noted that molecular genetics tools have helped to understand. The correlation between α-amylase gene expression level and seed dormancy in wild oat and wheat.

Several research workers such as De Laethauwer *et al.* [3]; Zhang *et al.* [13] and Yang *et al.*[14] have focused their studies on use of RT-PCR and QTL analysis involved in seed dormancy and PHST in different crops such as wheat , rice and many other cereals .The results of present study on RT-PCR are in conformity with above findings.

Kondhare *et al.* [15, 16]; Hidalgo *et al.*[17] and Huang *et al.*[18] have studied the correlation between α–amylase activity and its gene expression, seed germination, seed dormancy and PHST or sensitiveness

under different conditions of temperature , moisture / wet conditions and applications of different hormones like GA3 and ABA. Young *et al.* [19] worked in detail on the changes in carbohydrate composition and α-amylase expression during germination and seedling growth of starch-deficient endosperm mutants of maize. Ullrich *et al.* [20] reported the genetic relationships between pre-harvest sprouting and dormancy in barley. Similarly Wilkinson *et al.* [21] noted the use of comparative molecular genetics to study pre harvest sprouting in wheat. The correlation between alpha-amylase and beta-amylase activities in different species of *Triticum* was investigated by Hidalgo *et al*. [17], which helped to screen sensitiveness or tolerance to PHS. Mares and Mrva [10] reported late-maturity α-amylase in wheat in the absence of pre-harvest sprouting.

α-amylase Activity

The results shown in Table 3,5 ; Fig.4 clearly indicated that during third step of MI (21 days) the PHS susceptible genotypes shown very high activity of α, ß and total amylase as compared to the PHST genotypes (N-87-8 and N-87-12). The results of regression analysis between MI×biochemical and physiological traits such as starch and kernel weight (Table 4) indicated negative relationship .This analysis revealed that α-amylase activity was highly stimulated after MI (7, 14 and 21 days) and had caused that hydrolysis of starch, consequently reducing the kernel weight and yield also in PHS sensitive varieties. However, the PHS tolerant variety had shown positive relationship with duration of MI and traits interaction (N-86-12), that is why kernel weight and yield was not decreased (Fig. 4, 5). The results of present investigation are in agreement with DeLaethauwer *et al.* [4], Gao *et al*.[9], Jaiswal *et al*.[22], Singh *et al*.[23] and Ghanbari and Mir [24].They also recorded stimulated activity of α-amylase in different cereals during PHS.

They pursued monitoring of α-amylase activity, both at transcriptional and post-transcriptional levels during kernel development and claimed that damage caused by PHS was associated with increased levels of α-amylase activity in the kernel which negatively affected the nutritional and end-use quality of grains [10]. Highly relationship was reported between the level of seed dormancy and α-amylase activity in wheat and barley [8].

Although different levels of α-amylase activity had been detected in cereals like wheat, rye and triticale, they all showed very specific pattern during kernel development. In many wheat and rye genotypes, α-amylase activity remains low until harvest. Whereas it highly increases at harvest maturity and it is regulated by gene expression [7, 25].

Gao *et al*. [26] suggested that the relationship between α-amylase activity and PHS resistance/sensitiveness was highly remarkable, because α-amylase activity increases very fast, once the seeds absorb enough water and then sprout. The level of α-amylase activity was correlated to resistance and sensitiveness of varieties to PHS in wheat [27]. Singh *et al.* [23] also reported that sprouting in wheat positively influence the level of α- amylase activity, which leads to reduction in grain quality. DePauw *et al*. [28] noted highly significant and positive correlation between PHS and levels of α-amylase in kernels during artificial weathering (MI). One other hand, Singh *et al*. [29] reported that the potential of resistance to PHS in white wheat cultivars was based on harvest-time, seed dormancy and spike morphology. Xing *et al* [30] also recorded that the α-amylase activity in the kernel has direct relationship with seed germination. Potokina *et al* [31] analyzed the α-amylase activity in developing caryopses of sorghum and noted that the susceptibility to PHS was related to α-amylase activity. The results of present study corroborate with above findings.

Effect of PHS on Starch, Kernel Weight and Grain Yield

The results on starch content in selected elite lines of spring wheat indicated significant alterations with duration of MI (Table 3, 6; Fig. 5). The PHST varieties showed more accumulation of starch as compared to sensitive cultivar of wheat [32]. Investigation on changes in starch content may help to avoid its degradation during PHS, as starch is mobilized by the action of hydrolytic enzymes like amylase during germination [33]. In present investigation the wheat cultivar tolerant to PHS (N-86-12) showed less α-amylase activity and more starch. While opposite trend was observed in PHS sensitive variety (N-87-8).

According to Dupont and Altenbach [34] starch is a major determinant of yield, accounting for 65–75% of the grain dry weight and up to 80% of the endosperm dry weight. Hence if starch is reduced kernel weight and yield is also reduced universally during PHS. The genes that encode enzymes required for starch biosynthesis have been sequenced [35]. The information of genes encoding starch biosynthetic enzymes in wheat, barley, rye and maize endosperm is well known [36]. The reduction in starch accumulation due to extreme environmental conditions like humidity, rainfall and temperature accounting for the significant losses in grain yield was well documented [37]. The decline in starch content for Australian wheat varieties exposed to low temperatures during PHS was associated with decrease in rate of conversion of sucrose to starch [38, 39].

Environmental factors mainly rainfall, humidity, low temperature etc are pivotal in PHS of many cereals like wheat, maize, sorghum and barley, because these induces metabolic alterations in starch, sugars and α-amylase activity, which governs the PHST or sensitiveness of the genotypes [40]. The results of present study are in agreement with above findings.

The α-amylase hydrolyzes starch and consequently leads to reduction in kernel weight and grain yield in PHS sensitive wheat genotype which decreases the nutrient values of grain and yield, kernel weight and end use quality [41, 42, 43, 3, and 13]. Many researchers reported significant economic losses due to the reduction in grain yield and kernel weight during PHS [23, 14, 44, 28].

The grain yield and kernel weight was very low in PHS sensitive wheat genotypes, which was due to degradation of starch by the elevated levels of amylase activity [44].The starch is very important biochemical attribute for determining the end-use quality of wheat flour. The degradation of native starch granules negatively affect quality of various products made from wheat flour such as breads, cookies and noodles [3]; Kondhare *et al.*[16]. The losses in grain yield, kernel weight and end use quality as well as grain quality during PHS in wheat grains depend on genotype, the environmental conditions during grain development and the interaction between these factors [3].

CONCLUSION

The accumulation of starch and the level of α-amylase activity in wheat grains are the reliable indicators for knowing tolerance of a variety to PHS. The results of regression analysis indicated that α-amylase activity in both the genotypes e.gN-86-12 and Nai60 have shown lower linear model after MI as compared sensitive wheat genotypes, indicating their higher tolerance to PHS than other genotypes. After MI α-amylase activity in the N-87-8 genotype was highly stimulated with increasing MI, illustrating their more sensitiveness to PHS than other genotypes. The α-amylase gene expression and its level of activity are highly crucial in determining the PHST or sensitiveness of wheat genotypes, providing a pivotal tool to wheat breeder.

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