Grain filling pattern by Measurement of Indolyl-3-acetic acid, Gibberellin and cytokinin content in the grains

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ABSTRACT: Grain filling patterns and their relationships with indole-3-acetic acid (IAA) and gibberellin (GA contents in the grains and roots during grain development were examined in Grain growth rate (GGR), gibberellins, cytokinin and indolyl-3-acetic acid (IAA) levels were studied at different grain type and position within developing grains of wheat (Triticum aestivum L. var. Bahar). Main spikes were divided into three grain positions included proximal, middle, and distal regions, and further into two grain types included basal and apical grains. Three grain filling patterns based on the filling rate of superior and inferior spikelets were observed. All spikelets started filling early and fast at the early filling stage. All spikelets filled slowly at the early filling stage and reached the maximum filling rate late and superior spikelets started filling and reached the maximum filling rate much earlier than the inferior ones. Grain dry matter accumulation, gibberellins including GA_1, GA_3 and GA_4 and IAA levels were determined in ten labeled spikes which sampled five times, seven days interval started from seventh day after anthesis (DAA) up to 30th DAA, and also in maturity. Gibberellins and IAA contents increased until 16th and 23^{st}DAA, respectively. Changes in Z + ZR contents in the superior and inferior spikelets were associated with the grain filling patterns. The maximum level of grain growth rate (GGR) was observed at 16th DAA. Furthermore, the differences in both gibberellins and IAA contents, among spikelets in different regions of the spike, and also among grains within a spikelet were correlated with the differences in dry matter accumulation. Grain filling percentage was significantly correlated with Z + ZR contents in the grains and roots at the early and middle grain filling stages. IAA and GA (GA_l + GA_3 + GA_4) contents in the grains and roots were not significantly correlated with grain filling percentage. The results suggest that cytokinins in the grains and roots during the early phase of grain development play an important role in regulating grain filling pattern and consequently influence grain filling percentage.

Keywords: Gibberellins; IAA; spike; grain development; wheat

INTRODUCTION

Cytokinins play a considerable role in regulating plant growth and development (Sundberg, 1990). In addition to regulating the rate of cell division and cell elongation, cytokinins influence the intensity and direction of assimilate flow (Gutam et al, 2008). In cereals, peas, and beans, high levels of cytokinins are generally found in the endo-sperm of developing seeds, which may be required for active cell division during the early phase of grain setting (Foulkes Morris et al., 2010) reported that zeatin (Z) and zeatin riboside (ZR) in developing wheat grains showed

large transient Increases following pollination, which coincided with the period of seed setting and maximum endosperm cell division. It is generally believed that cytokinins in higher plants are synthesized mainly in the root system and transported via the transpiration system to the aboveground parts where they regulate growth and development (Asthir et al., 1996). Some studies indicate that cytokinins may also be synthesized in developing grains (Aufhammer et al., 1996). It was suggested by (Michael and Seiler-Kelbitsch, 2008) that transient grain cytokinin content is correlated with final grain yield. Increased grain set and grain yield by the application of exogenous cytokinins has been reported in wheat, barley, and maize. Wheat (Triticum aestivum L.) is the most important cereal crop in the food culture of both developed and developing countries in the world. World wheat production increases by approximately 1.5% annually to meet the growing demand for food that results from population growth and economic development (Davies et al., 1995). A Substantial increase in grain yield potential, together with good use of water and fertilizer is required to ensure food security in the future. For improvements in photosynthetic capacity to result in additional wheat yield, extra assimilates must be partitioned to develop grains and/or potential grain weight be increased to accommodate the extra assimilates. The position of grain within a spike to some extent determines its final grain weight which can range from 20 to 60 mg. the grains from spikelet's in the middle region of the spike and from the basal region within each spikelet are more towards the upper level of this rand . Various factors such as assimilate availability and/or transport capacity or the possible role of plant growth regulators (Chen et al., 2008) are offered to explain these differences. Gibberellins play important role in regulating plant growth and development such as intensity and direction of assimilate flow, cell elongation, and cell division rat (Mauseth et al., 1991). In cereals, high levels of gibberellins are generally found in the endosperm of developing seeds, which may be required for active cell division during the early phase of grain setting. The position of grain within a spike to some extend determines its final grain weight which can range from 20 to 60 mg.

The grains from spikelet's in the middle region of the spike and from the basal region within each spikelet are more towards the upper level of this range (Davies et al., 1998) Various explanations such as assimilate availability and/or transport capacity (Chen et al., 2008) or the possible role of plant growth regulators (Banowetz et al., 1998) are offered to explain these differences. Gibberellins (GAs) play an important role in regulating plant growth and development such as stem elongation, germination, dormancy, synthesis of α-amylase, flowering, sex expression, enzyme induction and leaf and fruit senescence. (Asthir et al., 2007) reported that gibberellins act as positive modulators of grain sink activity, whereas, IAA acts as a negative modulator. It was confirmed by Zhang et al., 2010 by exogenous application of gibberellins that resulted in improvement of sink activity due to the GAs role as modulators of sugar metabolism. On the other hand, indolyl-3-acetic acid (IAA) is another plant growth regulator which plays an important role in some of the physiological responses such as stimulation of the closure of stomata, inhibition of shoot growth; synthesize storage proteins of seed, inhibition of the effect of gibberellins on stimulating de novo synthesis of α-amylase, and maintenance of dormancy. (Ahmadi and Baker ,2002) observed a reduction in sucrose transportation into the grains with lowered the ability of starch synthesis in intact grains. (Ober et al., 2009) demonstrated that IAA could be translocated from leaf tissue to grains and acts as a sensory link between developing reproductive structures and maternal tissues deprived of water. Furthermore, IAA also may influence early establishment of sink size through regulation of cell number (King et al., 2003). Both field and pot trials indicated that IAA content in the grain increased up to the start of grain ripening and then decreased gradually with the cessation of dry matter accumulation and rapidly later as the moisture content of the grain decreased. Wang et al., 2003]suggested that the poor grain filling of rice was associated with low grain doses of both IAA and IAA. Evaluation the relation of dry matter accumulation, gibberellins and IAA levels at different grain type and position could be important to identifying the role of plant growth regulators on differences in dry matter accumulation of grains in a spike, which could be the key in developing wheat with higher grain yield potential. Hence, the objective of this study was to evaluate the GAs and IAA levels along with dry matter accumulation at different grain type and position within a spike of Bahar wheat. Abscise acid (ABA), gibberellin (GA) and indole-3-acetic acid (IAA) are also involved in regulating grain development. (Bail et al., 1999) reported that ABA content in the grains was positively correlated with grain filling rate in the early stage of wheat grain filling, while at later stage it was negatively correlated. Application of ABA increased the percentage of ripened grains, particularly in the inferior grains . (Awan and Alizai ,2006) observed that application of GA and IAA significantly reduced spikelet sterility in rice. (Yang et al., 2010 and Wang et al., 2009) suggested that the poor grain filling was associated with low IAA and ABA contents in the grains.

Increasing panicle size (spikelets per panicle) is a common approach for the rice breeders to enhance the crop sink size (spikelets per m^2), and consequently Improve yield potential (Mauseth et al., 1996) The new plant type lines developed at the International Rice Research Institute using tropical japonica germ plasma have large panicles and therefore increased sink size compared with the indicia type. Inter sub specific hybrid rice developed

with indicia and japonica rice have larger biomass and bigger panicles than conventional inbred rice. However, these new cultivars with larger panicle size usually have lower grain filling percentage. Therefore, improving grain filling is the key In developing new plant type and hybrid cultivars with higher yield potential.

Spikelets can be classified into superior and inferior spikelets according to their position within a panicle. In general, superior spikelets are located in the top primary branches while inferior ones are in low secondary branches. Grain filling rate of superior and inferior spikelets during grain development is measured to determine the grain filling pattern of a cultivar (Wang et al., 2008). So far little is known whether cytokinins, IAA and GA contents in the grain and roots are related to grain filling pattern and percentage in rice. The purposes of this study were to (I) determine the grain filling patterns of various types of rice genotypes, determine the changes in cytokinin, IAA, and GA contents in the superior and inferior spikelets during grain filling period, and establish the relationship between cytokinin, IAA, and GA contents in grains and roots and grain filling percentage.

MATERIALS AND METHODS

2-1 Experimental Setup and Plant Sampling

Single plants of the wheat (Triticum aestivum L. var. Bahar-138) were grown in plastic containers with a diameter of 4.5 cm and depth of 20 cm. The pots were filled with a pasteurized soil which classified as a clay loam with 30.1% sand, 25.7% clay and 46.2% silt, an electrical conductivity (EC_e) of 1.2 dS m^{-1}, a pH of 7.1 (saturated paste), and organic C of 0.62%. The plants were grown in a screen covered hall under otherwise natural conditions. The pots were watered as described by Houshmandfar et al. 2008, and fertilized once a week with half strength Peter's solution (NPK = 10:10:10) (Salisbury et al., 2009). The secondary tillers were removed as they appeared. Ten labeled spikes were sampled five times, seven days interval started from seventh day after anthesis (DAA) up to 30th DAA, and also in maturity. Spikes were divided into three grain positions included proximal (spikelet number 1 to 5), middle (spikelet number 6 to 15), and distal (spikelet number 16 to 20) regions, and further into two grain types included basal (bold) (grain No. 1 and 2) and apical (small) (grain No. 3 upward). All samples were divided into two parts, one was dried in an oven at 70 °C for 72 h, and then weighed for dry matter accumulation, and another was frozen in liquid N_2 for one min and kept in a freezer at -70 °C for gibberellins included GA_1 and GA_4, and also IAA analysis. Grain growth rate (GGR) was calculated using the following equation (Salisbury et al., 2008):

$$GGR(mgd^{-1}) = \frac{W_2 - W_1}{T_2 - T_1}$$

Where,
W_1 = Total dry matter of grain at time t_1
W_2 = Total dry matter of grain at time t_2
T_1 = Time of first observation
T_2 = Time of second observation

Gibberellins and IAA contents were expressed on fresh weight. Linear regression was used to evaluate the relationships between traits. The data were analyzed statistically using analysis of variance and critical differences (CD) at 5 percent level were computed.

2-2 Hormone extraction and purification

The methods for extraction and purification of Z, ZR, IAA and GAs were modified from those described by (Bollmark et al., 2007). Samples corresponding to 50-80 dehulled and frozen grains or 3 to -4 g frozen roots were ground in a mortar (on ice) in 10 ml 80% (v/v) methanol extraction medium containing 1 mmol L-I butylated hydroxy toluene (BHT) as an antioxidant. The methanolic extracts were incubated at 4 °C for 4 h and centrifuged at 4000 rpm for IS min at the same temperature. The supernatants were passed through Chromosep C 18 columns (C 18 SepPark Cartridge, Waters Corp., Millford, MA, USA), prewashed with 10 ml 100% and 5 ml 80% methanol, respectively. The columns were then washed and eluted with 10 ml 100% methanol and 10 ml ether, respectively. The hormone fractions eluted from the columns were dried in a freeze dryer (Labconco, England), and dissolved in

2 ml phosphate buffer saline (PBS) containing 0.1 % (v/v) Tween 20 and 0.1 % (w/v) gelatin (pH 7.5) for the analysis by an enzyme-linked immunosorbent assay (ELISA).

2-3 Quantification of hormones by ELISA

ELISA was performed with a 96-well microtitration plate. Each well on the plate was coated with 100 fLl coating buffer (1.5 g L -l Na2C03, 2.93 g L-l NaI-lC03, 0.02 g L -l NaN3, pH 9.6) containing 0.25 fLg ml-l antigens against Z, ZR, IAA and GA (GAl + GA3 + GA4), respectively, and incubated for 4 h at 37°C for Z, ZR and GA, and overnight at 4 °C for IAA, and then kept at room temperature for 30 to 40 min. After washing four times with PBS + Tween 20 (0.1 %, v/v) buffer (pH 7.4), each well was filled by 2 Plant sample + synthetic compounds of Z, ZR, IAA, and GA (GAl + GA3 + GA4). 500 ng of each compound was added to one gram fresh plant sample before purification. Z, ZR, and IAA were from Sigma Chern Co. and GA was provided by Islamic Azad Agricultural University.

3 Mean ± standard deviation of four replications

With 50 fLl of either extracts or Z, ZR, IAA and GAs standards (0 to 2000 ng ml-l dilution range), and 50 fLl of 20 f-Lgmr ' antibodies against Z, ZR, IAA and GAs, respectively. The plate was incubated for 3 h at 28°C for Z, ZR and GAs, and overnight at 4 °C for IAA, and then washed as above. 100 f-Ll of 1.25 fLg ml-J Ig G-HRP (immunoglobulin G-horse radish peroxidase) substrate was added to each well and incubated for l h at 30°C. The plate was rinsed five times with the above PBS + Tween 20 buffer, and 100 f-Ll color-appearing solution containing 1.5 mg mr ' O-phenylenediamine and 0.008% (v/v) H202 was added to each well. The reaction progress was stopped by adding of 50 f-Ll 6 N H2S04 per well when the standard of 2000 ngmr ' had pale, and 0 ng ml- 1 deep color in the wells. Color development in each wel1 was detected by ELISA Reader (Model EL310, Bio-TEK) at OD490 nm absorbance. Z, ZR, IAA and GA contents were calculated fol1owing (Weiler et al 2007). The results are the mean ± S.D. of at least four replicate incubations.

RESULTS AND DISCUSSION

The methods used in this study for hormone extraction and purification and for quantification of hormones by ELISA recovered 80.6% of Z, 82.4% of ZR, 68.3% of IAA, and 76.2% of GAs (GAl + GA3 + GA4). The recovery was similar between grains and roots (Table 1).Figure 1 demonstrates the GGR levels at different grain type and position within developing grains of wheat. Generally, grain growth rate (GGR) was high during 9th to 30th DAA. GGR improved from 9th DAA to 16th - 23st DAA then decreased from 16th- 23st DAA to maturity. The lowest amount of GGR was observed during 30th DAA to maturity. Furthermore, grain growth rate was diversely affected due to different grain positions. The GGR level of middle region of spike as compare with proximal and distal regions, and GGR level of proximal region as compared distal region, improved at all sampled DAA. According to the grain types, the maximum levels of GGR were obtained in basal grains in the direction of all sampled DAA. The only exception was at maturity, middle position basal grains, which the GGR was slightly lower as compared with middle position apical grains. Table 1 indicates the gibberellins content of different grain type and position at various DAA. The gibberellins level slightly increased from 9th DAA until 16th DAA, and then decreased from 16th DAA until maturity. The maximum levels of grain gibberellins for all different grain type and position were observed at 16th DAA. The differences in gibberellins concentration of grains positively correlated with the differences in related GGR levels at various DAA (r^2=0.9382). The correlation hold true both for comparisons between spikelet's in various regions of the spike, and also between florets within spikelets. Hence, the maximum gibberellins levels of grain were also obtained in middle region of spike and in basal grains at all sampled DAA. The maximum disparities between two types of grains in the contents of GAs was observed at 9th DAA, which were to the tune of 36.2, 31.0 and 50.7 percent in proximal, middle and distal segments of spike, respectively.

Table 1.Recovery test for zeatin (Z), zeatin riboside (ZR), (IAA), and gibberellin (GA)

Plant organ	Hormone	Plant sample (ng g^{-1} FW)	Plant sample + standard^2 (ng g-I FW)	Recovery (%)
Grains	Z	221.9 ± 30.6^3	502.3 ± 41.1	70.1±94
	ZR	2504 ± 534	575.3 ± 50.1	81.0 ± 6.9
	IAA	180.6± 15.7	392.6 ± 188	584 ± 5.8
	GA	463.8 ± 31.3	729.3 ± 39.0	73.1 ±3.3
Roots	Z	507.5 ± 34.9	912.5 ± 66.6	81.0±6.7
	ZR	633.5 ± 39.8	1051.9 ± 74.9	83.7 ± 71
	IAA	54.7 ± 10.0	395.7 ± 15.8	68.2 ± 44
	GA	24.8 ± 5.6	420.9 ± 34.1	79.2 ± 6.7

At maturity, the differences suppressed to 26.9, 22.0 and 42.8 percent lower in apical grain as compared to basal grains at the same position respectively. Table 2 presents the IAA content at different grain type and position within developing grains of wheat. The IAA levels increased with grain development from 9th to 23st DAA, and then decreased from 23st DAA until maturity in all the three segments of spike. The highest and lowest levels of grain IAA for all different grain types and positions were observed at 9th and 23st DAA, respectively. The IAA levels were diversely affected due to different grain positions. The IAA levels of distal region of spike as compare with proximal and middle regions, and IAA level of proximal region as compared middle region, reduced at all sampled DAA. According to grain types, the maximum levels of grain IAA were obtained in apical grains for all determined growth stages. The quantum of disparities between basal and apical grains was maximum at 9th DAA. However, there were no significant differences in the levels of IAA between the two types of grains at 9th DAA in all the three segments of spike. Observations similar to those at 9th DAA were recordable at maturity. The differences in IAA concentration of grains negatively correlated with the differences in GGR levels at different DAA.
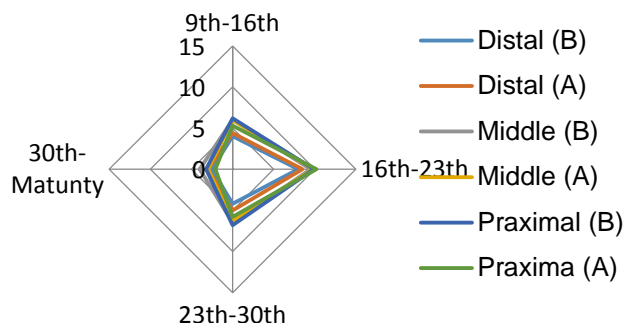

Figure 1. Grain growth rate (GGR) at different grain type and position within developing grains of wheat. B and A are basal and apical grains, respectively

Table 1. Gibberellins (GA$_1$ + GA$_3$ +GA$_4$) content (ng g^{-1} fresh weight) at different grain type and position within developing grains of Wheat

Days after anthesis (DAA)		Proximal			Middle		Distal	
Basal	Apical	Basal			Apical		Basal	Apical
9th	841.4	(– 36.2)	599.5	841.5	(– 30.0)	5719.0	769.4 (– 50.9)	329.0
16th	909.8	(– 33.0)	789.9	1139.1	(– 29.0)	859.1	948.9 (– 45.0)	521.9
23st	605.5	(– 30.8)	320.8	663.1	(– 26.6)	413.3	520.3 (– 40.4)	310.2
30th	282.1	(– 23.0)	240.2	183.9	(– 24.0)	149.2	194.4 (– 46.0)	94.2
Maturity	40.5	(– 26.9)	29.6	42.2	(– 22.0)	32.9	33.6 (– 42.8)	19.2

Values within parenthesis indicate percentage of decrease (-) in small grains over bold grains growing in the same spikelets; CD at 5% level; Age: 41.3; Position: 18.6; Age × Position: 53.5

Table 2. Indolyl-3-acetic acid (IAA) content (ng g^{-1} fresh weight) at different grain type and position within developing grains of wheat

Days after anthesis (DAA)	Proximal			Middle			Distal		
	Basal		Apical	Basal		Apical	Basal		Apical
9th	58.2	(+ 29.0)	79.4	43.6	(+ 26.6)	75.2	46.9	(+ 39.1)	64.3
16th	325.6	(+ 19.9)	490.9	218.0	(+ 19.4)	260.4	228.3	(+ 30.0)	296.8
23st	891.9	(+ 15.5)	560.9	564.9	(+ 9.0)	604.4	599.2	(+ 24.3)	920.2
30th	253.2	(+ 14.2)	195.0	148.9	(+ 9.4)	162.9	159.1	(+ 18.4)	186.1
Maturity	3.9	(+ 9.9)	4.2	3.4	(+ 11.8)	3.8	4.2	(+ 14.3)	4.8

Values within parenthesis indicate percentage of decrease (+) in small grains over bold grains growing in the same spikelets; CD at 5% level; Age: 25.2; Position: 17.6; Age × Position: 32.1

CONCLUSION

Grain filling period is an influential stage of crop life cycle which strongly effected grain yield. Plant growth regulators play an important role in regulating plant growth and development. The differences in dry weight per grain are highly flexible (Yang et al., 2003). Middle region of spike as compare with proximal and distal regions produces the maximum level of grain dry matter accumulation (Zhang et al., 2007). We have investigated the relation between gibberellins and IAA content, along with GGR levels at different grain type and position within developing grains of wheat. Both gibberellins and IAA levels were possessed during middle phase of grain setting while the GGR was high. The differences in gibberellins levels, among spikelets in different regions of the spike, and also among grains within a spikelet were positively correlated with the differences in GGR levels. On the contrary, the differences in the indolyl-3-acetic acid contents, among all aforementioned segments were negatively correlated with the differences in GGR levels. Furthermore, the basal grains were conspicuous in having relatively higher level of gibberellins at subtle stages while the level of indolyl-3-acetic acid was invariably higher in apical grains.

According to previous studies, the increase in gibberellins content at early embryonic stage where a rapid enlargement of embryo takes place implies that gibberellins had signaled the translocation of metabolites to the active sink such as the developing grain(Gebeyehou et al., 1982). The characteristic decrease in gibberellins content at 23st DAA can be explained by the hypothesis put forth by Krishna moorthy, 2005 that at early stage, conjugation might have taken place and it existed in the storage from in the matured grain to be used during germination. There are also reports by earlier workers that the IAA is elevated in the grains during maturation for induction of dormancy. The higher levels of IAA in hard dough stage, along with relatively lower level of gibberellins at approximately middle stages of grain development (Sundberg et al., 2003) suggests that at this stage, maintenance of embryo dormancy appears to be an active process involving IAA. In conclusion, the result suggest that both gibberellins and IAA levels of grains during the middle phase of grain development play an important role in regulating grain filling pattern and grain growth rate of Bahar-138 wheat. Furthermore, it could be possible to improve grain weight by manipulating gibberellins and IAA levels in grain, especially during the filling stage either through breeding or crop management.

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