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COMPARATIVE METABOLITE PROFILING OF TWO WHEAT GENOTYPES AS AFFECTED BY NITROGEN STRESS AT SEEDLING STAGE

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ABSTRACT

Increasing demands for wheat productivity together with environmental concerns about the use of nitrogen-based fertilizers dictate the importance of improving nitrogen use efficiency (NUE). Identifying biological processes responsible for efficient fertilizer use will provide tools for crop improvement under reduced nutrient inputs. Metabolic response under nitrogen (N) stress was investigated at Centre for Carbon, Water and Food (CCWF), The University of Sydney, Australia. GC-MS and LC-MS techniques were used for metabolite and amino acid profiling in N-stress tolerant (Krichauff) and sensitive (Berkut) varieties under with (normal) and without nitrogen (stress) conditions in 28 days old seedlings. Twenty-six metabolites including organic acids, sugars, and amino acids were characterized in both genotypes under stress and normal conditions. Organic acids (citric acid and oxalic acid) and sugars (glucose, sucrose, fructose and mannose) were significantly increased in both varieties under stress conditions, whereas, malic and oxalic acids were increased in tolerant (Krichauff), while decreased in susceptible (Berkut) genotype. Sugar alcohol (pentaerythitol, xylitol and myo-inositol) remained similar in both genotypes under stress and normal conditions. Seven out of twenty amino acids (glycine, cysteine, valine, methionine, isoleucine, leucine and tryptophan) were not detected in both genotypes under both stress and normal conditions. Most of the remaining amino acids were detected under normal condition only, exhibiting the relationship of amino acid with nitrogen applications. Amino acids viz. serine, aspargine, alanine, threonine, glutamine and proline were specifically decreased under stress condition in Krichauff, whereas glutamic acid increases in both genotypes under stress than normal conditions. Compared with Berkut, Krichauff experienced greater increase in both sugars and organic acids, and more pronounced decrease in most of the amino acids under stress condition. L-ascorbic acid, allo-insitol, lysine and tyrosine were unique metabolites found only in tolerant (Krichauff) genotype. Metabolic responses of wheat to nitrogen stress were dynamic and involve many metabolites. Greater N-tolerance and different metabolic expression in Krichauff necessitate further studies to examine various pathways and adaptive reactions at critical stress conditions. Current findings of metabolite profiling might help in unveiling the genetic targets for the improvement of nitrogen use efficiency in wheat.

INTRODUCTION

The challenge of maintaining sustainable agricultural production is one of the primary social scientific problems of the 21st century (Tilman *et al.,* 2002). Population growth is increasingly putting pressure on agricultural production, demanding higher yields from arable land. Both food and energy crops needs fertilizer for higher yield, however higher dose of inorganic nitrogenous fertilizer has detrimental impact on the environment. Only about 30-50% of the applied nitrogen based fertilizer is taken by the crop plants, the remaining deteriorate environment in the form of harmful gases (NOx), denitrification or leaching into terrestrial ecosystems causing eutrophication and contamination of drinking water (Cassman *et al.,* 2003). Therefore, it necessitates improving nutrient use efficiency without affecting yield and quality of crop plants.

Nitrogen (N) is one of the major plant nutrient and key constituent of amino acids, peptides, proteins, chlorophyll, nucleic acids and many other compounds. Most of the higher crop plants take nitrogen from the soil in the form nitrate, as the primary source of inorganic nitrogen. Nitrate is reduced to nitrite, then to ammonium before assimilation into amino acids (Tobin and Yamaya, 2001). Both nitrogen and carbon assimilation interacts with each other in a complex network that regulates the balance between N and C according to the physiological status of the tissue and the environmental conditions (Nunes-Nesi *et al.,* 2010).

Nitrogen mainly remobilized in the form of amino acids from the leaf to the grain during grain filling period. In wheat, free amino acids are major components of both phloem and xylem sap. During vegetative growth phase, phloem amino acid concentrations have been measured eight times greater than the nitrate ions concentration. Aspartate (Asp) and glutamate (Glu) are

the predominant components, comprising 50% of the total amino acids (Hayashi and Chino, 1986). However, Asp and Glu content decrease and glutamine (Gln) becomes the predominant free amino acid in both leaf and phloem extracts at leaf senescence stage (Simpson *et al.,* 1981). This shift in amino acid balance during grain filling stage is a programmed strategy for N remobilization during reproductive development and has potential for exploitation for the improvement of N use efficiency resulting in accumulation of high nitrogen content in grain and ultimately higher grain yield. In the current experiment, GC-MS and LC-MS were used to study metabolic profiling analysis in leaves of two wheat varieties, Berkut and Krichauff, under with and without nitrogen conditions.

MATERIALS AND METHODS

Based on the performance of the genotypes Berkut and Krichauff in earlier genotype × nitrogen contrast trials at Plant Breeding Institute, The University of Sydney, Australia, Berkut and Krichauff were used as N-susceptible and N-tolerant varieties, respectively in this trial. The experiment was conducted in greenhouse at Plant Breeding Institute, The University of Sydney, Australia during December 2015. The experiment was laid out in completely randomized design with three replicates. Six seeds per pot of each variety were planted under added nitrogen (N+) and without nitrogen (N0) condition in each replication. After emergence, four seedlings per pot were kept for further study in the experiment. Pots were filled with 500 g potting mix (80% and 10% 0-8 mm and 0-3 mm composted pine bark and 10% sand). The pots were arranged in separate trays for N+ and N0 treatments. Nitrogen was applied to the trays containing N+ pots @120 kg in the form of urea. The urea was mixed thoroughly in water and was applied @50 ml to each pot, whereas N0 trays were left as such. The average day/night temperature of 18/26 ^{0}C and a photoperiod of 14 h at a relative humidity of ~70 $\pm$ 10% were maintained in the greenhouse throughout the experiment. Twenty-eight days after emergence, leaves were harvested and immediately ground to fine powder in liquid nitrogen using mortar and pestle and were kept at -80^{0}C until extraction. Sub sample from the ground sample were made and were processed for extraction of metabolite and amino acid as follow.

GC-MS based targeted metabolite profiling

Procedure for the extraction of metabolites from leaf tissue: Standard procedure for metabolites extraction was performed as suggested by of Zhao *et al*. (2013). Ground samples were transferred to an Eppendorf tube and weighed on an analytical balance. Methanol/ chloroform/ water (65:25:15) mixture was added to the ground sample at a ratio of 1:3 w/v, followed by 1 mL of internal standard amino acid solution (0.4 M Norvaline and Sarcosine in ddH_2O). The sample was mixed with a vortex three times for 10 seconds each, then centrifuged at 10,000 rpm for 5 min at 4^{0}C. The supernatant (500 mL) was collected into another Eppendorf tube and 100 mL chloroform followed by 150 mL water was added to the sample tube. The sample was mixed again and centrifuged at 10,000 g for 5 min at 4^{0}C. The upper aqueous phase (400 mL) was collected and filtered through a Millex GX 0.22µm disc filter (Millipore, Billerica, USA). The filtrate was then passed through a Biomax 5KNMWL membrane 0.5-mL filter (Millipore). The final filtrate was kept in -80^{0}C until metabolite analysis by GC-MS.

Gas chromatography mass spectrometry (GC-MS) analysis: GC-MS analysis followed the procedure described in Qiu *et al.* (2007). The derivatized extracts were analyzed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer Inc., USA). Extract aliquots of 1µl were injected into a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J and W Scientific, Folsom, CA). The inlet temperature was set at 260°C. After a 6.5 min solvent delay, initial GC oven temperature was set at 60°C; 1 min after injection, the GC oven temperature was raised 5°C min-1, and finally held at 280°C for 15 min. The injection temperature was set to 280°C and the ion source temperature was adjusted to 200°C. The helium carrier gas had a constant flow rate of at 1ml min^{-1}. The measurements were made with electron impact ionization (70 eV) in the full scan mode (m/z 30–550). Turbomass 4.1.1 software (Perkin Elmer Inc., USA) coupled with commercially available compound libraries: NIST 2005, Wiley 7.0 was used to identify the detected metabolites. For GC/MS results, compounds were identified based on retention times and comparison with reference spectra in mass spectral libraries. Peaks areas of metabolites were integrated with the Genesis algorithm, and relative quantities were calculated using the ribitol internal standard.

LC-MS analysis: LC-MS analysis of the underivatized extract was carried out on a 1290 Infinity LC system (Agilent, USA) coupled to a 6520 QTOF Mass selective detector (Agilent, USA). A 3.5 µL sample was injected into a Zorbax SB-C18 column (2.1 x 150 mm, 3.5 µ) and separation was achieved by gradient elution with water and methanol. The QTOF was tuned to operate at the low mass range <1700 AMU and data acquisition was done in scan mode (60-1000 m/z) and ionization was positive ion mode. LC-MS results were identified based on their retention times relative to standards as well as their formula mass. Peaks were integrated and the MassHunter software by Agilent calculated their relative quantities.

Statistical Analysis: Data recorded on various parameters were subjected to analysis of variance appropriate for randomized complete block design following Singh and Chaudhry (1997). Mean graph with standard error bar for different parameters were developed using MS-Excel package for statistics.

RESULTS

The differential metabolic responses of wheat varieties (Berkut and Krichauff) to nitrogen stress may highlight the metabolic processes or pathways associated with the physiological changes in plants under limiting nitrogen condition. A total of 26 metabolites were quantified in leaves of both wheat genotypes under with and without nitrogen condition (Table 1), out of which five were organic acids, eight were sugars or sugar alcohols, and 13 were amino acids. Out of the 26 metabolites, 23 had significantly altered levels under with nitrogen condition (N+) compared with without nitrogen condition (N0) in both varieties. Nitrogen stress (N0) resulted in an increase in the content of 15 metabolites, a decreased content for eight metabolites, and no significant effects on three metabolites compared to the normal nitrogen dose in 28 days old seedlings of both genotypes (Fig 1-3 and Table 2).

Nitrogen stress resulted in significant increases in the content of all five organic acids in 28 days old seedlings. Among organic acids, citric (0.40 vs. 0.76 µg/mL and 0.56 vs. 2.59 µg/mL) and oxalic acid (1.69 vs. 2.59 µg/mL and 1.76 vs. 1.91 µg/mL) content were increased in both Berkut and Krichauff, respectively, while malic (0.41 vs. 2.70 µg/mL), L-Ascorbic (0.75 vs. 1.34 µg/mL) and aminobutyric acid (1.94 vs. 2.03 µg/mL)content were increased only in Krichauff under nitrogen stress than normal condition (Figure 1). Both Berkut and Krichauff experienced increase in fructose (1.06 vs. 1.54 µg/mL and 1.78 vs. 1.96 µg/mL), mannose (2.06 vs. 2.32 µg/mL and 2.42 vs. 2.82 µg/mL), glucose (1.42 vs. 1.84 µg/mL and 2.10 vs. 2.39 µg/mL) and sucrose (5.24 vs. 7.39 µg/mL and 4.89 vs. 9.76 µg/mL), respectively under nitrogen stress than the normal nitrogen dose (Figure 2). Sugar alcohol viz. pentaerythitol, xylitol and myo-Inositol content remained almost similar in both varieties as well as nitrogen treatments, except allo-Inositol, which was found higher in Krichauff only (0.55 vs. 1.10 µg/mL) under nitrogen stress (Figure 3).

Nitrogen application had differential effect on individual amino acids in Berkut and Krichauff varieties (Table 2). Seven out of twenty amino acids distinguished (glycine, cysteine, valine, methionine, isoleucine, leucine and tryptophan) were not detected in both genotypes under both stress and normal conditions. Most of the remaining amino acids were detected under normal condition only, exhibiting the relationship of amino acids with nitrogen applications. Two amino acid viz. lysine and tyrosine were specifically detected in Krichauff only. For tyrosine content, non-significant increase was noticed under nitrogen stress (0.08 vs. 0.14 µg/mL), while lysine was found only under normal condition (0.15 µg/mL) in Krichauff. Similarly, histidine (0.06 and 0.20 µg/mL) and arginine (0.15 and 0.44 µg/mL) were detected in both Berkut and Krichuaff, respectively under normal condition but differences among genotypes were non-significant. Likewise, serine (2.55 µg/mL), asparagine (7.64 µg/mL), alanine (0.39 µg/mL), aspartic acid (0.35 µg/mL), threonine (0.20 µg/mL), glutamine (0.92 µg/mL), proline (0.32 µg/mL) and phenyl alanine (0.16 µg/mL) were detected in Berkut under normal condition only, and under both normal and stress in Krichauff. Significant decrease was noticed in serine (2.10 vs. 1.04 µg/mL), asparagine (5.43 vs. 0.47 µg/mL), glutamine (1.26 vs. 0.27 µg/mL) and proline (0.26 vs. 0.11 µg/mL), whereas non-significant decrease were observed for alanine (0.19 vs. 0.17 µg/mL) and threonine (0.27 vs. 0.20 µg/mL) in Krichauff under stress condition. Furthermore, significant increase were noticed for glutamic acid (0.24 vs. 0.38 µg/mL and 0.21 vs. 0.53 µg/mL) increases in both Berkut and Krichauff, respectively, while non-significant increase were noticed for aspartic acid (0.33 vs. 0.46 µg/mL), tyrosine (0.08 vs.

Table 1. List of 26 metabolites and retention time (RT) identified by GC-MS and LC-MS in 28 days old wheat seedlings under with (N+) and without nitrogen (N0) condition.

S.No	Metabolite	RT
1	Malic acid	13.21
2	Citric acid	17.19
3	L-Ascorbic acid	19.31
4	Oxalic acid	8.36
5	Aminobutyric acid	13.68
6	Pentaerythitol	13.51
7	Xylitol	15.94
8	allo-Inositol	18.40
9	Myo-Inositol	22.71
10	Fructose	17.30
11	Mannose	18.62
12	Glucose	20.12
13	Sucrose	38.86
14	Lysine	1.17
15	Histidine	1.15
16	Arginine	1.15
17	Serine	1.19
18	Aspargine	1.19
19	Alanine	1.17
20	Aspartic acid	1.17
21	Threonine	1.18
22	Glutamic acid	1.21
23	Glutamine	1.20
24	Proline	1.31
25	Thyrosine	3.46
26	Phenylalanine	7.10

0.14 µg/mL) and phenylalanine (0.20 vs. 0.21 µg/mL) in Krichauff under stress than normal conditions (Figure 3 and Table 2).

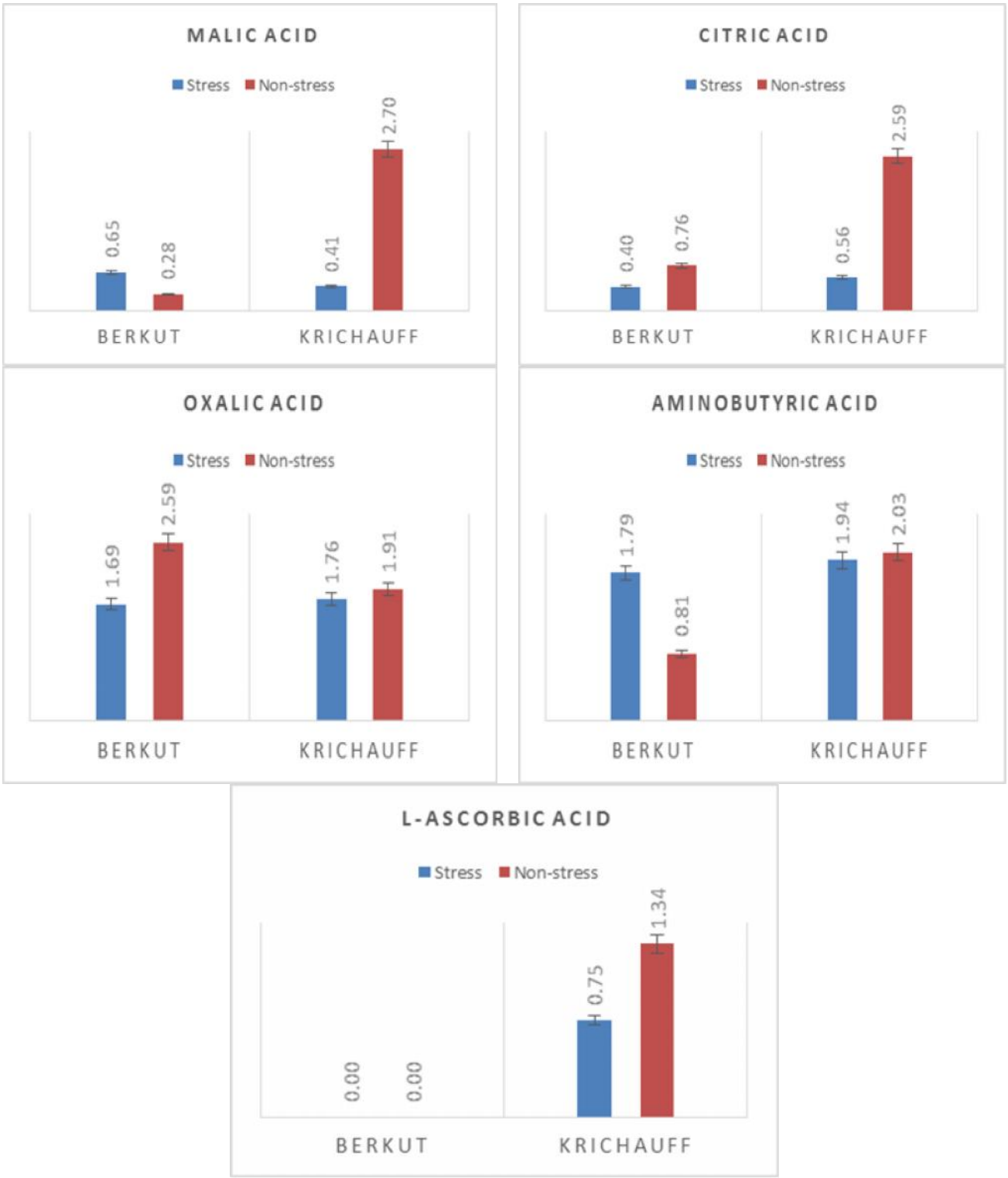


Figure 1. Relative quantities of organic acids in 28-d seedlings of two wheat genotypes under with nitrogen (Non-stress) and without nitrogen (Stress) condition.

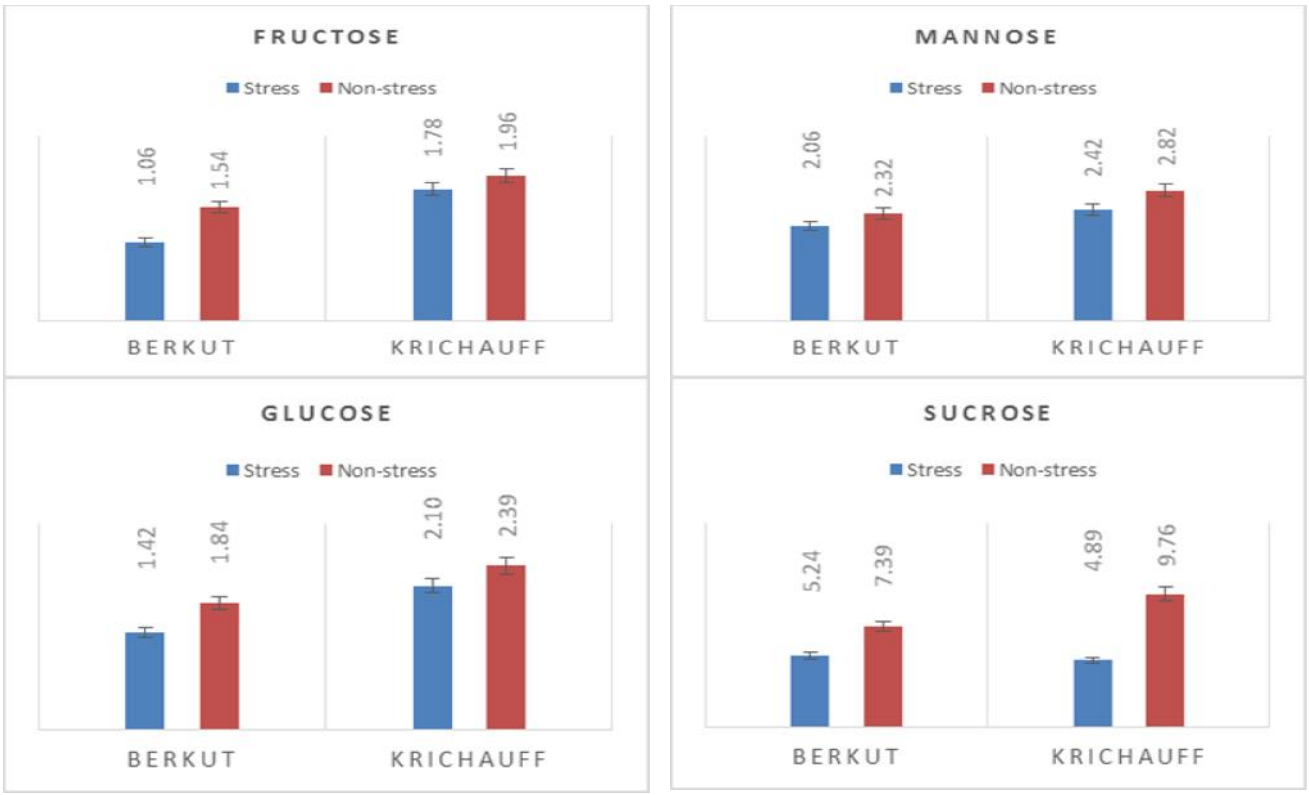


Figure 2. Relative quantities of sugars in 28-d seedlings of two wheat genotypes under with nitrogen (Non-stress) and without nitrogen (Stress)condition.

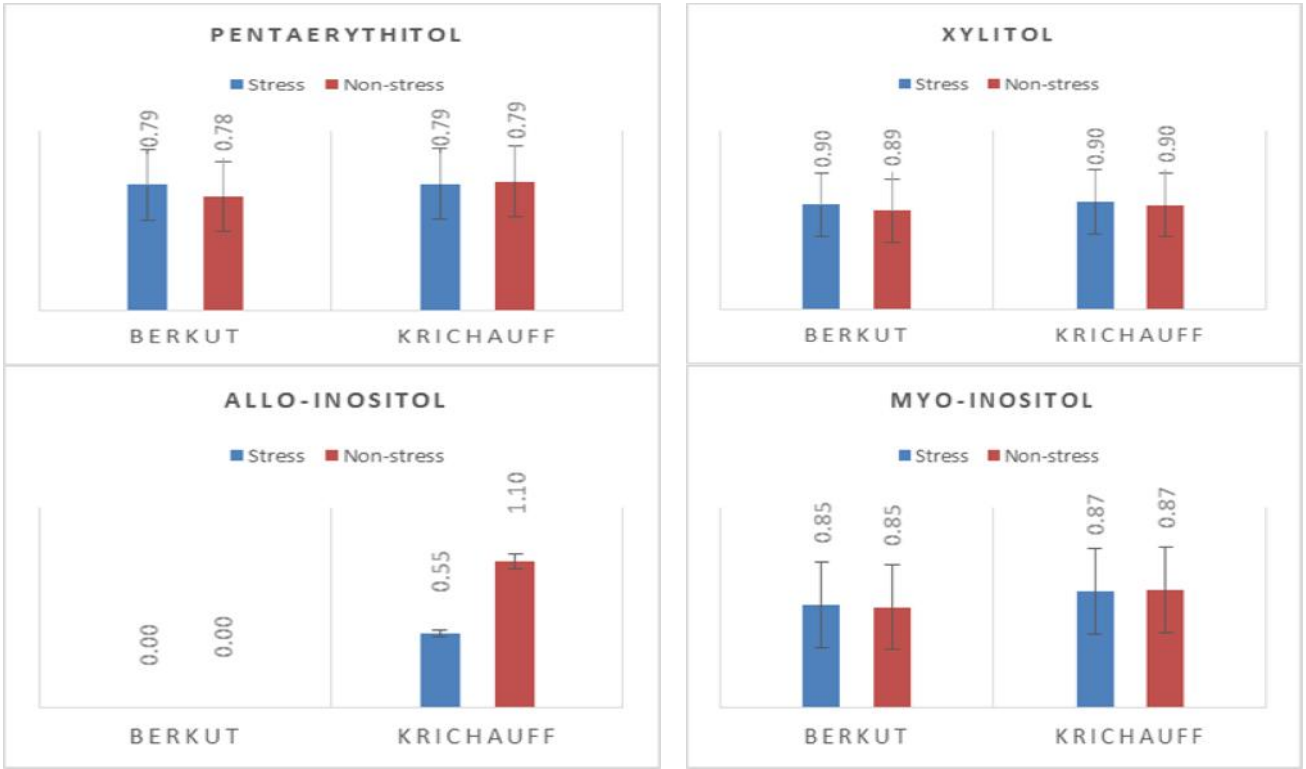
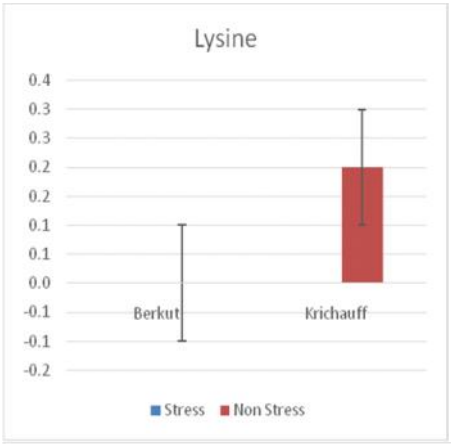
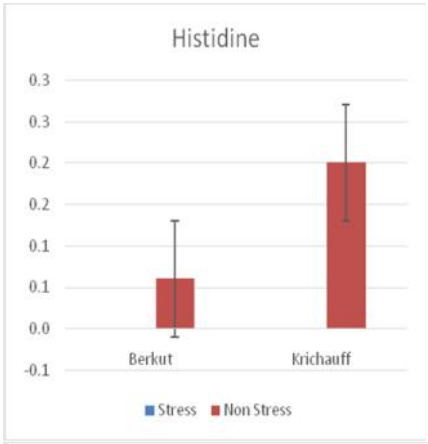


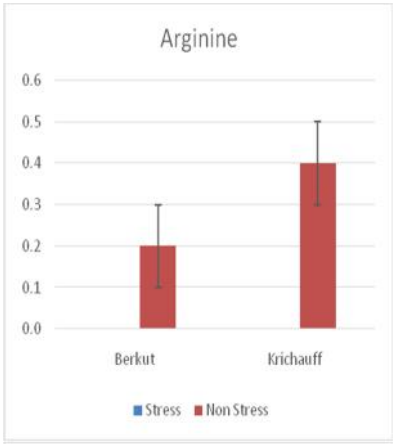
Figure 3. Relative quantities of sugar alcohols in 28-d seedlings of two wheat genotypes under with nitrogen (Non-stress) and without nitrogen(Stress) condition.

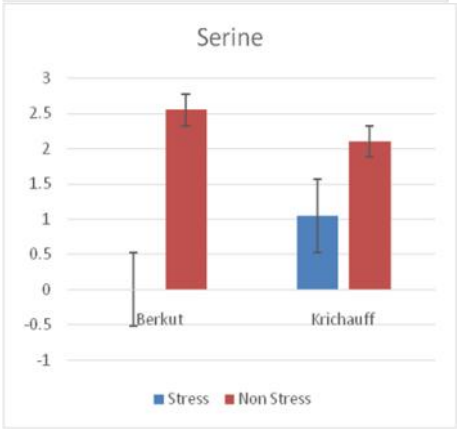
Table 2. Amino acids concentration (µg/mL) in leaves of Berkut and Krichauff under with nitrogen (N+) and without nitrogen (N0) condition.

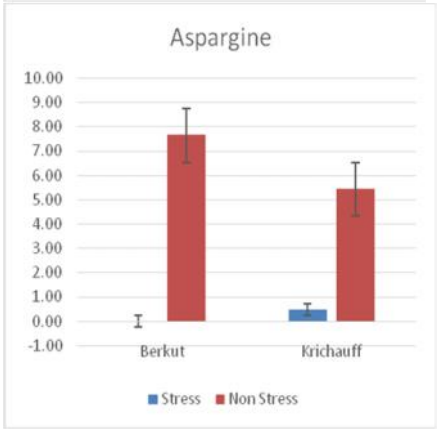
Amino acids	Varieties			
	Berkut		Krichauff	
	N+	N0	N+	N0
Lysine	0.00	0.00	0.15	0.00
Histidine	0.06	0.00	0.20	0.00
Arginine	0.15	0.00	0.44	0.00
Glycine	0.00	0.00	0.00	0.00
Serine	2.55	0.00	2.10	1.04
Aspargine	7.64	0.00	5.43	0.47
Alanine	0.39	0.00	0.19	0.17
Aspartic acid	0.35	0.00	0.33	0.46
Threonine	0.20	0.00	0.27	0.20
Glutamine	0.92	0.00	1.26	0.27
Glutamic acid	0.24	0.38	0.21	0.53
Cysteine	0.00	0.00	0.00	0.00
Proline	0.32	0.00	0.26	0.11
Valine	0.00	0.00	0.00	0.00
Methionine	0.00	0.00	0.00	0.00
Isoleucine	0.00	0.00	0.00	0.00
Leucine	0.00	0.00	0.00	0.00
Thyrosine	0.00	0.00	0.08	0.14
Phenylalanine	0.16	0.00	0.20	0.21
Tryptophan	0.00	0.00	0.00	0.00

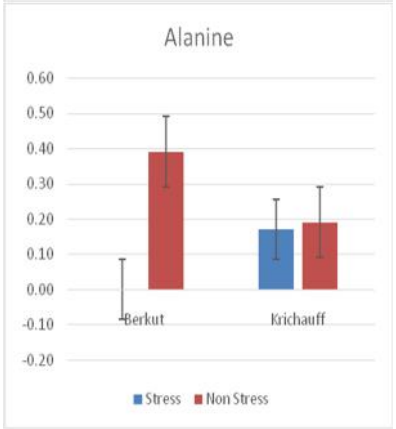












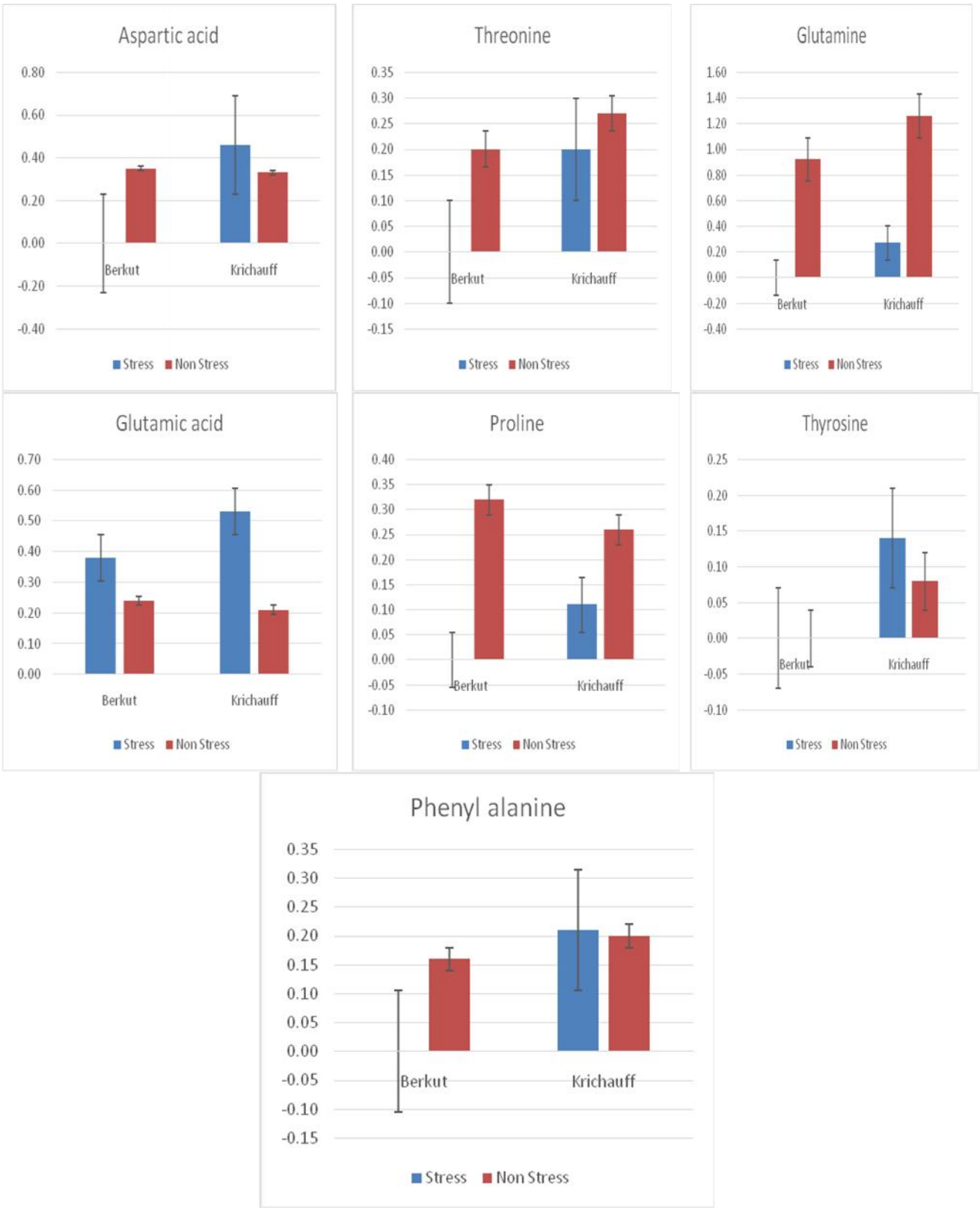


Figure 3. Relative quantities of amino acids (µg/mL) in leaves of two wheat genotypes in 28-d seedlings under with nitrogen (Non-stress) and without nitrogen (Stress) condition.

DISCUSSION

Among sugars, the most abundant disaccharide found in this study was sucrose. Sucrose is a major soluble sugar in plants for the storage and transport of carbon fixed through photosynthesis. Higher levels of sucrose have been associated with enhanced stress tolerance in grass species (Du *et al*., 2011 and Xu *et al*., 2013). Sucrose can also act as a signalling molecule effecting the regulation of other metabolic pathways (Koch, 2004). Increased levels of sucrose reflected active photosynthetic supply of carbohydrates and increased carbohydrate reserves as disaccharides, which could play roles in protecting leaves of wheat from prolonged stress period. Monosaccharides, such as glucose and fructose produced through photosynthesis are readily utilized in respiratory metabolism for energy production, which is critically important for plant survival of stresses (Nilsen and Orcutt, 1996).

Glucose and all other monosaccharides detected increased under nitrogen stress. During stress condition, rate of respiration and respiratory demands for monosaccharides typically increase, which may lead to accumulations of those sugars (Wahid *et al*., 2007 and Lyons *et al.*, 2007). Contrarily, decreases in content of monosaccharides, including glucose and fructose under heat stress have been previously reported in grass species (Du *et al*., 2011; Xu *et al*., 2013 and Yu *et al*., 2012). The maintenance of respiration levels may represent more actively growing tissue, as well as generation of ATP for important stress defence mechanisms such as antioxidative functions, although increased amount of sugars may be consumed or simple sugar content may decline (Couee *et al*., 2006 and Saradadevi and Raghavendra, 1992). The decrease in the content of monosaccharides (fructose and glucose) in nitrogen treated plants compared to the untreated control during heat stress may reflect an increase in carbohydrate consumption or utilization for the maintenance of respiratory metabolism under heat stress (Jespersen *et al*. 2015).

Nitrogen stress resulted in higher accumulations of organic acids (citric acid, malic acid, L-ascorbic acid, oxalic acid and aminobutyric acid) compared to the normal plants. Many of these organic acids with higher accumulation are intermediates of the tricarboxylic acid (TCA) cycle of respiration, including citric acid and malic acid. The increase in those TCA intermediates may represent more active mitochondrial respiration for the generation of ATP, but many of these intermediates can also feed into other metabolic pathways, which are important for regulating various cellular functions, such as nitrogen assimilation and redox balance (Sweetlove *et al*., 2010). The increased accumulation of organic acids corresponded with the decreased content of monosaccharides as substrates in respiration, which together suggested that the nitrogen application could help in maintaining more active energy metabolism and reflected the positive effects on the damages induced by nitrogen stress.

Nitrogen application had differential effects on different amino acids content. Amino acids are major nitrogen containing cellular constituents (Taiz and Zeiger, 2010). The increase in amino acid content with nitrogen applications may be due to higher levels of nitrogen available for the plant to assimilate into amino acids. Nitrogen status has previously been shown to affect free amino acid content (Barneix *et al*., 1984). Higher accumulations of aspartic acid and glutamic acid were observed under nitrogen stress. Both aspartic and glutamic acid is a major precursor to many other amino acids (Azevedo *et al*., 2006) including isolucience and glycine and its accumulation may represent an important shift in amino acid metabolism for enhanced stress tolerance. Additionally glutamic acid plays important roles in nitrogen metabolism as well as chlorophyll biosynthesis (Forde and Lea, 2007). The increase in glutamic acid may improve stress tolerance by improving chlorophyll production, as well as improving the integration of nitrogen into other cellular molecules. Metabolite profiling indicated that the enhanced accumulation of those amino acids associated with nitrogen balance, photorespiration or which are important biosynthetic precursors may represent important shift in metabolism resulting in nitrogen stress tolerance.

Conclusions: Compared with Berkut, Krichauff experienced greater increase in both sugars and organic acids, and more pronounced decrease in most of the amino acids under stress condition. L-ascorbic acid, allo-insitol, lysine and tyrosine were unique metabolites found only in tolerant (Krichauff) genotype. Metabolic responses of wheat to nitrogen stress were dynamic and involve many metabolites. Greater N-tolerance and different metabolic expression in Krichauff necessitate further studies to examine various pathways and adaptive reactions at critical stress conditions. Current findings of metabolite profiling might help in unveiling the genetic targets for the improvement of nitrogen use efficiency in wheat.

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