Metabolomics profiling reveals the detoxification and tolerance behavior of two bread wheat (*Triticum aestivum* L.) varieties under arsenate stress

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Abstract:

The present study conducted metabolomics profiling (targeted and untargeted) in the roots of two wheat varieties (BARANI-70 and NARC-09) under arsenate stress in a hydroponic experiment. The findings indicated a better growth response of BARANI-70 compared to the NARC-09. From amino acid profiling, a total of 26 amino acids (AAs) were quantified in roots. BARANI-70 showed higher induction of stress-responsive AAs compared to the NARC-09. From untargeted metabolomics, a total of 136 metabolites were identified: AAs, fatty acids, purines, carnitines, LysoPCs, and others. The KEGG pathway identified pathways such as linoleic acid metabolism, TCA cycle, glutathione metabolism, and aminoacyl-tRNA biosynthesis that were regulated to improve the defense of tolerant variety. BARANI-70 emerged as a tolerant variety based on the psychological response, As accumulation, and behavior of stress-responsive metabolites. This study should facilitate the breeding of low-As accumulating wheat varieties for future application to ensure sustainable production and food safety.

Keywords:

Amino Acids; Arsenate Reductase; Tolerance; Omics; Glutathione

1. Introduction:

Arsenic (As), a Group 1 carcinogenic metalloid, is pervasively distributed in groundwater and agricultural soils (Saeed et al., 2021). Arsenate is present at a significantly higher level in irrigation water and soils compared to organic forms of As . The intake of As through contaminated crops and drinking water has become a great concern for health safety. Previously, As has been detected in a variety of staple crops i.e., wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (Zea mays L.) (Saeed et al., 2021). Crops are vulnerable to negative impacts on growth, yield potential, and grain quality due to the high accumulation of As when grown in contaminated areas. Even though crops can induce various mechanisms to withstand As toxicity yet its presence can hamper growth, and enzymatic activities, and result in genotoxic and cytological changes (Saeed et al.,2021). The root can experience such impacts at a high level since it is the primary organ that comes into contact with As (Saeed et al.,2021). The conversion of arsenate to arsenite by the arsenate reductase (AR) enzyme in roots plays a significant role in As-mediated oxidative stress (Majumder et al., 2019). Previous reports indicated higher activity of AR enzyme in roots of sensitive varieties compared to tolerant ones (Majumder et al., 2019; Saha et al., 2017). Therefore, the activation of the AR enzyme is a prerequisite for toxicity and sensitivity in crops under As contaminated conditions.

Wheat is one of the top staple crops in the context of global production and consumption. Wheat is contaminated with inorganic species (arsenate and arsenite) which are considered 10^2 times more toxic compared to organic species (Saeed et al., 2021). Recently, a high concentration of As has been detected in wheat grains (Saeed et al.,2022). Moreover, negative impacts of As on wheat have been documented such as a decrease in seed germination, plant biomass, root elongation, photosynthesis, yield, induction of oxidative stress, and inhibition of antioxidant activities (Shi et al., 2020). Previously, our research has identified wheat varieties that can accumulate less As in grains and provide better yields despite high-contaminated conditions (Saeed et al., 2022). However, the mechanisms responsible for As tolerance and detoxification are not extensively explored at the molecular level in wheat.

Recently, molecular approaches have gained great importance to understand growth and tolerance mechanisms in cereal crops. Omics have been employed to understand the impact of both biotic stresses (bacteria and viruses) and environmental stressors (salinity, high temperatures,

and emerging contaminants) in crops (Pérez-Cova et al., 2022). Previous research has pointed toward the potential of metabolomics in understanding the responses of molecular stressors in crops under stress (Nguyen et al., 2022). The metabolomics profiling in crops to understand As behavior has become a point of interest recently since metabolites have relationships with accumulation, transport, tolerance, and detoxification mechanisms (Martínez-Castillo et al., 2022). Pérez-Cova et al., (2022) identified 40 metabolites that were significantly changed due to As exposure in rice. On the other hand, both essential and non-essential amino acids were negatively affected under As exposure in a sensitive variety compared to the tolerant variety of rice (Tripathi et al., 2013). Lu et al., (2021) revealed that the tolerant variety showed higher regulation of stress-responsive amino acids and other metabolites compared with a sensitive variety of wheat under Cd stress. Therefore, the metabolomics profiling can be a useful approach to identifying and understanding mechanisms responsible for tolerance against As in wheat.

However, to our knowledge, no research has been conducted to explore molecular mechanisms responsible for As accumulation, tolerance, and detoxification in wheat. Limited information is available regarding As-mediated toxicity in wheat varieties of Pakistan. Furthermore, no research has been found that explored metabolomics profiling to understand changes occurring at the molecular level in wheat varieties of Pakistan. To significantly contribute in this regard, the present study used metabolomics profiling (targeted and untargeted) in roots of two wheat varieties (BARANI-70 and NARC-09) under arsenate stress to provide extensive knowledge of As tolerance and detoxification pathways. The varieties were selected based on our previous field experiment (Saeed et al., 2022) to investigate their tolerance and response under As stressed conditions. It was hypothesized that the tolerant variety will show better synthesis of As-responsive metabolites and pathways compared with the sensitive variety.

2. Materials and Methods:

2.1. Hydroponic experiment:

In the present study, two wheat varieties (BARANI-70 and NARC-09) were exposed to different arsenate treatments (0, 1, 10, 50, 100, 200, 500, 1000, and 2000 µM) through a hydroponic experiment. The sodium meta-arsenate salt (Sigma-Aldrich, St. Louis, MO, USA) was used to prepare fresh solutions of arsenate treatments. The experiment was conducted in the Department of Ecology, Environment, and Plant Sciences (DEEP), Stockholm University,

Sweden. The experiment was done under controlled conditions in a climate chamber equipped with halogen lamps (Osram Powerstar HQI-R) that provide constant light at a photon flux density (200 µmol.m2/s) for 16h in the daytime (temperature 25^{o}C) and 8h dark time (21^{o}C) right above the plants. Furthermore, the relative humidity was 75% throughout the experiment. In the first week, seeds (n=100) were placed in moist vermiculite for 7 days for germination. In the 2nd week, the plants (n=6) were placed on a single Styrofoam plate floating on a total of 0.9L arsenate and nutrient solution. The solutions were constantly aerated and maintained at 0.9L level with distilled water throughout the experiment. In total, four replicates were used in the present study. On the 28th day of the experiment, samples were harvested. Roots were properly washed for 2-3 minutes in distilled water followed by 3-5 minutes of washing in 20mM EDTA and again in distilled water for 5 minutes to remove any adsorbed As to the root surface. Afterward, shoots were separated from roots while their separate fresh weights and lengths were recorded. The plant materials were milled in liquid nitrogen by hand using a ceramic mortar and the material was preserved at -80^{o}C until further analysis. The Weibull curve distribution was calculated using Spyder Software (Phyton) to find the most suitable treatment for further analysis. Based on findings (Figure S1), treatment 200 µM was found to be effective since both varieties exhibited a significant response in terms of root elongation. Therefore, the treatment of 200 µM was compared with the control for further analysis in this experiment.

2.2. Total arsenic analysis:

Fresh root and shoot samples were dried for 24 hours at 80^{o}C to obtain dried material for wet digestion. Root and shoot-dried materials (0.1g) were digested using nitric acid and hydrogen peroxide through the microwave oven (Speedwave 2, Berghof, Germany) for 35 minutes (Bergqvist & Greger, 2012). With the help of a vapor generator (VGA-77)-atomic absorption spectrophotometer (Varian SpectAA 55B), total As was measured in samples at 193.7 nm. Various solutions: sodium borohydride (3%, Merck), sodium hydroxide (2.5%, EKA chemicals), and hydrochloric acid (6M) were freshly prepared for the instrument. Furthermore, three standards (50, 100, and 150 µg/L) were used to eliminate the interaction effects of the matrix.

2.3. Assay of arsenate reductase enzyme:

The activity of the AR enzyme (EC 1.20.4.1) was examined by using a modified protocol (Das et al., 2018). A root sample of 0.1g was homogenized in 5 mL of extraction buffer solution.

The homogenate was centrifuged at 2000g for 15 minutes and the supernatant was collected. The collected supernatant was centrifuged for 30 minutes at maximum speed. Afterward, the resulting supernatant was centrifuged for one hour at maximum speed and the final supernatant was collected for assay. The assay buffer was prepared containing 50nM glutathione reductase (GR), 1mM glutathione (GSH), 0.1 mg/mL bovine serum albumin (BSA), 300mM sodium chloride (NaCl), 50mM MOPS, and 50mM MES. The 0.5 mL supernatant was mixed with 0.8mL of 250 µM NADPH, 0.5mL of 10mM sodium arsenate, and 2mL of assay buffer. The enzyme activity was detected at 340 nm for 3 minutes as a change in absorbance and expressed as µmol /mg/min. The molar extinction coefficient was 6200 $M^{-1}cm^{-1}$ for the AR assay.

2.4. Extraction of metabolites:

Freeze-dried root samples (5mg) were prepared according to the method (Gullberg et al., 2004) at the Swedish Metabolomics Center for metabolomics analysis. The samples were shaken with extraction buffer (1000 µL) in a mixer will with a tungsten bead at 30 Hz for 3 minutes. The samples were centrifuged at 14,000 rpm for 10 minutes (4 °C) after removing the bead. The supernatant (20 µL for amino acid quantification and 100 µL for LC-MS analysis) was transferred and evaporated to dryness in a speed-vac concentrator. Afterward, the samples were stored at -80 ^{o}C until further analysis. To ensure quality control (QC), a small aliquot of remaining supernatants was pooled and run on MSMS for identification purposes. The extraction buffer contained internal standards for LC-MS such as 13C9-Phenylalanine, 13C3-Caffeine, D4-Cholic acid, and 13C9-Caffeic acid that were obtained from Sigma (St. Louis, MO, USA).

2.5. Amino acid quantification:

2.5.1. Calibration curve and standards

In total, 27 amino acid standards including norvaline internal standard were obtained from Sigma (St. Louis, MO, USA). Stock solutions (500 ng/µL) of every compound were prepared and stored at -80 ºC. A 10-point calibration curve (0.01-100 pmol/µL) was constructed by serial dilutions as well as spiked with internal standards at a final concentration (5 pmol/µL). HPLC grade acetonitrile from Fisher Scientific (Fair Lawn, NJ, USA) and Mass spectrometry grade formic acid was obtained from Sigma-Aldrich (St Louis, MO, USA).

2.5.2. Amino acid derivatization with AccQ-Tag:

Extracted samples were derivatized by AccQ-Tag™ (Waters, Milford, MA, USA) according to the manufacturer's instructions with the following modifications: the dried extract was dissolved in 20mM HCl (20 µL) and diluted with AccQ-Tag Ultra Borate buffer (60µL) spiked with norvaline as internal standard (0.667 pmol/µL). Freshly created AccQ•Tag derivatization solution (20 µL) was mixed with the sample and immediately vortexed (30 seconds). Samples were stored at room temperature for 30 minutes followed by 10 minutes at 55ºC. Procedure blanks and quality control samples were used for every batch. Calibration curves were generated in a similar way as the samples.

2.5.3. Amino acids Quantification by LC-ESI-MSMS:

Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MSMS) was used for the quantification of AAs in the present study. The derivatized samples were examined by a 1290 Infinitely system from Agilent Technologies (Waldbronn, Germany), consisting G1316C thermostated column compartment, G4220A binary pump, and G4226A autosampler with G1330B autosampler thermostat coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with a jet stream electrospray source operating in positive ion mode. Separation was obtained by injecting 1 µL of every sample onto a BEH C18 2.1x100 mm, 1.7 µm column (Waters, Milford, MA, USA) held at 50 ºC in a column oven. The gradient eluents used were acetonitrile 0.1% formic acid (B) and H_2O 0.1% formic acid (A) with a flow rate (500 µL/min). The initial conditions consisted of 0% B, and the following gradient was utilized with linear increments: 0.1-9.1% B (0.54-3.50 minutes), 9.1-17.0% B (3.50-7.0 minutes), 17.0-19.70% B (7.0-8.0 minutes), 19.7% B (8.0-8.5 minutes), 19.7-21.2% B (8.5-9.0) minutes, 21.2-59.6% B (9.0-10.0 minutes), 59.6-95.0% B (10.0-11.0 minutes), 95.0% B (11.0-11.5 minutes), 0% B (11.5-15.0). The flow rate was 800 µL/min from 13.0 minutes to 14.8 minutes for a quicker equilibration of the column. The data were calculated using MassHunter™ Quantitation software B08.00 (Agilent Technologies Inc., Santa Clara, CA, USA), and the content of every amino acid was computed based on the calibration curves. The retention times (rt), MRM-transition stages supervised and collision energies of examined compounds are presented in the supplementary document (Table S1).

2.6. LC-MS analysis for targeted and non-targeted metabolomics:

Before analysis on the LC-MS, the samples were re-suspended in 10 µL (methanol) and 10 µL(water). At the start, samples were estimated in positive mode. Once all samples in a batch were analyzed, the instrument was switched to negative mode and a second injection of all samples was performed. The chromatographic separation was completed on an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, Germany). The sample (2 µL) was injected onto an Acquity UPLC HSS T3, 2.1 x 50 mm, 1.8 µm C18 column in combination with a 2.1 mm x 5 mm, 1.8 µm VanGuard precolumn (Waters Corporation, Milford, MA, USA) held at 40 °C. The gradient elution buffers were B (75/25 acetonitrile:2-propanol, 0.1 % formic acid), and A (H2O, 0.1 % formic acid) at the flow rate (0.5 mL/minute). The compounds were eluted with a linear gradient consisting of 0.1 - 10 % B over 2 minutes, B was enhanced to 99 % over 5 minutes and kept at 99 % for 2 minutes; B was reduced to 0.1 % for 0.3 minutes and the flow rate was enhanced to 0.8 mL min-1 for 0.5 minutes; such conditions were kept for 0.9 minutes, after which the flow-rate was decreased to 0.5 mL/minute for 0.1 minutes before the next injection.

The compounds were measured with an Agilent 6546 Q-TOF mass spectrometer equipped with a jet stream electrospray ion source operating in positive or negative ion mode. The settings were kept identical between the modes except for the capillary voltage. A reference interface was connected for accurate mass measurements. The reference ions purine (4 µM) and HP-0921 (Hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine) (1 µM) were infused directly into the MS at a flow rate of 0.05 mL/min for internal calibration, and the monitored ions were purine m/z 121.05 and m/z 119.03632; HP-0921 m/z 922.0098 and m/z 966.000725 for positive and negative mode respectively. The gas temperature was set to 150°C, the drying gas flow to 8 L/min, and the nebulizer pressure 35 psig. The sheath gas temp was set to 350°C and the sheath gas flow was 11 L/min. The capillary voltage was set to 4000 V in positive ion mode, and to 4000 V in negative ion mode. The nozzle voltage was 300 V. The fragmentor voltage was 120 V, the skimmer 65 V, and the OCT 1 RF Vpp 750 V. The collision energy was set to 0 V. The m/z range was 70 - 1700, and data was collected in centroid mode with an acquisition rate of 4 scans s-1 (1977 transients/spectrum). The samples were analyzed by the following analytical run order: one blank sample, three auto-MSMS samples for the QC sample (10, 20, 40 eV), one QC sample, sixteen root samples, one QC sample, and one blank sample.

The Agilent Masshunter Profinder version B.10.00 (Agilent Technologies Inc., Santa Clara, CA, USA) was used to process all data for both targeted and untargeted analysis. A pre-recognized list of metabolites was examined by the batch-targeted feature extraction in Masshunter Profinder for target processing. An in-house LC-MS, created by reliable standards and run on the same system with the same mass-spec settings and chromatographic, was utilized for the targeted processing. The metabolites' identification was based on MSMS, MS, and retention time information. Such identification was done according to level 1 (confirmed structure), level 2 (probable structure), and level 3 (tentative candidate) as previously reported (Schymanski et al., 2014). The details are added in the supplementary document (Table S5).

2.7. Data Acquisition:

A pre-defined set of metabolites was searched for within the present data. The data was manually curated and putatively annotated by matching accurate retention time and mass with libraries present in the Swedish Metabolomics Center. The LC-MS internal standards showed stable peak areas and good RSDs in general (Figure S3). For QC purpose, PCA was performed to compare blanks, pooled QC extract, and root samples (Figure S4). The extraction blanks were separated from the remaining samples while pooled QC samples were close to each other.

2.8. Bioinformatic analysis:

Metaboanalyst (5.0) was used to compute pathway analysis, partial least squares discriminate analysis (PLS-DA), Pearson correlation, heatmaps, and variable importance in project (VIP) plots. The KEGG pathway database was used to obtain a complete overview of wheat metabolomics for identified pathways. Bioinformatics tool (Venny 2.1) was used to construct Venn diagrams in the present study.

2.9. Statistical analysis:

Statistical software for social sciences (SPSS, version 21; IBM, Armonk, NY, USA) was used to compute significant differences between varieties at $p < 0.05$ by using an independent t-test.

3. Results:

3.1. Physiological analysis and Weibull frequency distribution

The Weibull curve showed that NARC-09 showed more than a 70% decrease in root elongation at 200 µM (Figure S1). However, NARC-09 showed drastic impacts at 500 µM or higher treatments. On the other hand, BARANI-70 showed only a 20% decrease in root elongation at 200 µM and continued to grow even at 1000 µM. To make a proper comparison between the two varieties, 200 µM was found to be an effective treatment for further analysis. In the present study, BARANI-70 showed better growth performance compared to the NARC-09 under arsenate treatment (Table 1). Overall, the BARANI-70 showed less decline in fresh weight and elongation for both roots and shoots under arsenate treatment. BARANI-70 showed 70% root fresh weight under 200 µM while NARC-09 showed only 52% under 200 µM (100%). Similarly, there was more decrease in root length in NARC-09 (66%) compared to BARANI-70 (97%) under 200 µM treatment. Thus, the root was the most affected part of NARC-09 under arsenate exposure. Moreover, t-test results showed a significant difference between the control and 200 µM treatment for both fresh weight and length parameters ($p<0.05$).

3.2. Arsenic accumulation and translocation:

Total As was significantly accumulated in the roots of BARANI-70 compared with NARC-09 (Table 1). Both varieties showed higher accumulation in roots compared with shoots. However, BARANI-70 translocated less arsenic to shoots compared with NARC-09 (Table 1, Figure S2). Since the root accumulated the highest content of As thus it was selected for further analysis.

3.3. Arsenate reductase activity:

In the present study, the BARANI-70 showed no significant difference between the control and 200 µM treatment (Table 1). On the other hand, roots of NARC-09 showed an increase in AR activity when exposed to 200 µM treatment.

3.4. Metabolomics:

From amino acid quantification, a total of 26 amino acids were differentially expressed under As stress. On the other hand, LC-MS analysis revealed a total of 136 differentially expressed metabolites (DEMs) in wheat roots (Figure 2A). The main groups of metabolites were amino acids, fatty acids, carnitines, LysoPCs, purines, carbohydrates, keto acids, and nucleosides in the present study. From LC-MS analysis, a total of 27 amino acids was identified (Table S3) in the present study. The PLS-DA analysis revealed a distinct separation between tolerant (control vs treated)

and sensitive (control vs treated) varieties (Figure 2B) but with relatively low variance (20.2%) by the first component. Overall, the PLS-DA model exhibited Q^2 of 0.63 and R^2 of 0.85, which indicated the accuracy and reliability of the model (Table S2). Venn diagram exhibited unique and shared DEMS between two varieties under control and treated conditions (Figure 2C). Metabolites with VIP scores >1.0 in multivariate statistical analysis and p <0.05 in univariate analysis were termed the most important metabolites (Figure S9). On the other hand, cluster analysis revealed significantly expressed metabolites for BARANI-70 and NARC-09 (Figure S9). Amino acids were found to be highly up-regulated in cluster 2 and cluster 3 for tolerant variety under stress compared to sensitive variety. The pathway analysis revealed the involvement of metabolites in pathways such as the TCA cycle, purine metabolism, glutathione metabolism, aminoacyl-tRNA biosynthesis, nitrogen metabolism, etc. (Table 2; Figure 3). According to the fold change analysis, NARC-09 showed down-regulation in 38 metabolites and up-regulation in 5 metabolites. On the other hand, 14 metabolites were upregulated and 40 metabolites were downregulated in BARANI-70 under stress (Table S4).

3.4.1. Amino Acids quantification:

The essential amino acids (EAAs) i.e., methionine (Met), lysine (Lys), threonine (The), valine (Val), phenylalanine (Phe), isoleucine (Iso), His, histidine (Hisd), leucine (Leu), and tryptophan (Try) were detected in the present study (Figure 1A, Table S3). Under 200 µM treatment, both varieties showed an increase in the content of EAAs except for Val. BARANI-70 showed higher up-regulation of EAAs compared to the NARC-09. The Val content was decreased in NARC-09 but increased in BARANI-70 under arsenate stress.

Non-essential amino acids (NEAAs) i.e., arginine (Arg), alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamine (Glu), glycine (Gyc), serine (Ser), proline (Pro), glutamic acid (Glut), gamma-aminobutyric acid (GABA), asparagine (Aspa), and tyrosine (Tyr) were detected (Figure 1A, Figure S5). All NEAAs were found to be highly synthesized in the roots of BARANI-70 compared to NARC-09 under 200 µM arsenate treatment.

3.4.2. Fatty Acids:

Various fatty acids were detected by LC-MS in roots such as salicylic acid (SA), linolenic acid (LINO), fumaric acid (FA), succinic acid (SU), citraconic acid (CIT), D-leucic acid, malic

317 acid (MA), adipic acid (AA), azelaic acid (AZ), citric acid (CA), isocitric acid (ISO), sebacic acid
318 (SE), suberic acid (SU), linoleic acid (LIN), and arachidonic acid (ARA) (Figure 1B, Figure S5).
319 There was no significant difference between varieties for the content of SA. However, NARC-09
320 showed lower content of FA, SU, ISO, and CIT compared to the BARANI-70. On the other hand,
321 NARC-09 showed higher up-regulation of LINO, CA, and ARA compared to BARANI-70.
322 Furthermore, NARC-09 showed a decrease in the content of ISO compared to BARANI-70.
323 Azelaic acid showed a decrease in both varieties under 200 µM treatment but slightly higher
324 decrease in NARC-09. On the contrary, there was a higher decrease in MA contents for the
325 BARANI-70.

326 **3.4.3. Other metabolites:**

327 Purine compounds were detected in the roots of both varieties in the present study (Figure
328 1D). Purines like xanthine and hypoxanthine were highly upregulated in BARANI-70 compared
329 to NARC-09. Guanine was found to be slightly downregulated in both varieties under As stress.
330 However, Uric acid showed upregulation in the NARC-09 but downregulation in BARANI-70
331 under As exposure.

332 Seven carnitines were detected in roots under As exposure (Figure 1C). BARANI-70
333 showed significant upregulation for L-carnitine, L-acetyl carnitine, hexanol carnitine, and butyryl
334 carnitine under arsenate exposure compared to NARC-09. On the other hand, BARANI-70 showed
335 downregulation of succinyl carnitine and glutaryl carnitine while the NARC-09 showed an
336 increase in these metabolites under stress.

337 Lysophosphatidylcholine (LysoPCs) were detected in the roots of both varieties (Figure
338 S5). Under 200 µM treatment, LysoPCs were significantly increased in NARC-09 compared to
339 the control. On the other hand, BARANI-70 showed a decrease in LysoPCs content under 200 µM
340 treatment compared to the control (Figure S5).

341 **3.4.4. Pathway analysis:**

342 KEGG Pathway analysis showed the regulation of metabolites in 54 metabolic pathways
343 (Table 2, Figure 3). Amino acid metabolism pathways (valine, leucine, and isoleucine
344 biosynthesis, glycine, serine, and threonine metabolism, arginine and proline metabolism, and D-
345 glutamine and D-glutamate), lipid synthesis pathways (biosynthesis of unsaturated fatty acids and

linoleic acid metabolism), TCA cycle pathway, and aminoacyl-tRNA biosynthesis pathway were highlighted in the present study (Table 2, Figure 3).

4. Discussion:

To the best of our knowledge, this is the first published data on wheat metabolomics under As stress. With the help of the LC-MS, a total of 136 metabolites were recorded in root samples of wheat varieties (Figure 2A). The KEGG pathway analysis revealed regulation in different metabolic pathways under As stress such as lipid synthesis, amino acid synthesis, TCA cycle, and vitamin metabolism pathways (Table 2). Previously, Sun et al., (2021) reported regulation of the aforementioned metabolic pathways under As stress in mice. Various amino acid-related pathways were regulated significantly in the present study. Both EAAs and NEAAs were found to be highly regulated in BARANI-70 compared to NARC-09 (Figure 1A). Tripathi et al., (2013) indicated a significant increase in amino acid contents in roots and shoots of BARANI-70 compared to NARC-09 of rice. Less regulation of EAAs and NEAAs in sensitive variety can be linked to the impact of As on amino acid biosynthesis, degradation pathways, and nitrogen metabolism (Kumar et al., 2016). BARANI-70 showed significant upregulation of metabolites compared to the NARC-09 under 200 µM stress (Figure S9).

4.1. Variations in stress-responsive metabolites under arsenate stress:

Various stress-responsive metabolites such as AAs, purines, LysoPCs, carnitines, and fatty acids were significantly regulated in both varieties (Figure 1). Amino acids play a significant role in restricting metal phytotoxicity in crops by regulating defensive pathways. Stress-responsive AAs such as Glu and GABA contents were increased in roots of BARANI-70 under stress compared to NARC-09 (Figure 1A). Das et al., (2022) reported a significant increase in GABA content under As treatments compared with control in rice since it plays a significant function in sulfur and carbon metabolism under As stress. The presence of Glu regulates GABA in crops that can smoothly run the TCA cycle via the GABA shunt pathway (Kumar et al., 2016). Furthermore, GABA can increase the accumulation of amino acids like Val, Ser, Ala, Glut, and Asp. The correlation analysis confirmed the positive impact of GABA on the regulation of such amino acids in the present study (Figure S7). The higher level of GABA in a can be a possible mechanism behind tolerance against As stress for BARANI-70 since it has been previously indicated in rice (Kumar et al., 2016). The findings revealed higher synthesis of Glu, Gly, and Glut in BARANI-70

compared with NARC-09 (Figure 1A). Tolerant varieties of rice showed a similar increase in Gly and Glut content compared with sensitive varieties (Tripathi et al., 2013). The KEGG pathway analysis identified pathways (glycine, serine, and threonine metabolism) that can play an important role under As stress (Table 2). Furthermore, BARANI-70 showed higher up-regulation of Gly, Ser, and amino acids compared with NARC-09 (Figure 1).

Sulfur (S) containing amino acids (Cys, Met, Tau) and Pro were increased highly under As treatment compared to both varieties (Figure 1A, Figure S5). There was also a positive relation between S-containing amino acids in the present study (Figure S7). BARANI-70 showed a high synthesis of Pro, Met, and Tau compared with NARC-09. On the other hand, Cys was highly up-regulated in NARC-09 compared with BARANI-70 (Figure S5). Previously, As exposure increased the content of Pro and Met in rice varieties (Kumar et al., 2016). Majumder et al., (2019) reported an increase in Pro content under As exposure but this increase was significantly higher in tolerant varieties of rice. The higher regulation of Pro and Met in BARANI-70 of wheat protects against As-mediated oxidative stress and scavenges reactive oxygen species (ROS) (Majumder et al., 2019). Previous literature has also indicated the significant role of Cys in thiol metabolism and protects against heavy metal and metalloid stress (Dixit et al., 2015; Kumar et al., 2016; Tripathi et al., 2013). Furthermore, S-containing amino acids play a significant role in the synthesis of GSH-mediated defense in crops under As stress (Dixit et al., 2015). The KEGG analysis showed regulation of the S-containing amino acid metabolic pathway (cysteine and methionine metabolism) under As stress (Table 2). Gai et al., (2019) revealed the positive influence of cysteine and methionine metabolic pathways on ROS detoxification and tolerance in *Alternaria alternata*. Thus, the up-regulation of cysteine and methionine metabolic pathways could contribute towards tolerance against As in wheat.

Histidine was increased under As stress in both varieties but the increase was significantly higher in BARANI-70 (Figure 1A). Tripathi et al.,(2013) indicated a similar trend of Hisd content in tolerant varieties of rice compared to sensitive varieties. The KEGG analysis revealed regulation of the histidine metabolic pathway under As stress (Table 2). Previously, As exposure induced variation in Hisd and Cys-rich RNA-linked heat shock protein (AIRAP) (Sok et al., 2001) that could be involved in the tolerance mechanism against As toxicity. Serine also plays an important role in As detoxification since it is used during Cys biosynthesis (Kumar et al., 2016). In the present

study, a higher increase in Ser content was observed in the BARANI-70 compared to NARC-09 (Figure 1A). Moreover, Ser and Cys showed a strong positive correlation in the present study (Figure S7). In the present study, Ala and Tyr contents were increased in both varieties under As exposure (Figure 1A). It was in accordance with a previous study (Tripathi et al., 2013) where the tolerant variety of rice showed an increase in both Ala and Tyr content compared to the sensitive variety. In the present study, both Ala and Asp were highly up-regulated in BARANI-70 with NARC-09 (Figure 1A) and showed a positive correlation with each other (Figure S7). An important pathway (alanine, aspartate, and glutamate metabolism) was regulated under stress in the present study (Table 2, Figure S8).

The current study indicated As-mediated variations in fatty acids in both varieties (Figure 1B). In the present study, unsaturated fatty acids (UNFAs) such as ARA, LINO, LIN, and FA showed contrasting trends in both varieties. The content of LINO and ARA was found higher in the roots of NARC-09 compared to BARANI-70. However, there was no significant difference between both varieties for LIN and FA under As exposure. Previously, USFAs such as ARA and LINO were reported to be stress-responsive compounds in plants (Upchurch, 2008). Kumar et al., (2019) reported an increase in the level of ARA content under As treatment in rice. Salicylic acid was increased under As stress in both varieties (Figure 1B). BARANI-70 showed a slightly higher level of SA under As stress compared to NARC-09. Salicylic acid has been previously evaluated in plants due to its role in defense against a range of abiotic and biotic stress (Lefevere et al., 2020). Kohli et al., (2017) reported that SA protects plants against heavy metal stress. Previously, SA was found to reduce arsenate-mediated oxidative stress and minimize translocation from roots to shoots in rice (Faizan et al., 2021). In the present study, BARANI-70 showed less translocation and improved growth (Table 1, Figure S2) that could be linked to the role of SA. Azelaic acid showed an arsenate-induced decrease in both tolerant and sensitive varieties (Figure 1B). Even though there is no direct role of AZ in plant defense against metals but it can stimulate the production of SA in plants under stress conditions. Malic acid is an important fatty acid for plant defense against environmental contaminants. Mousavi et al., (2022) found a positive impact of MA on the growth of the Okra plant (*Abelmoschus esculentus* L.) and reduced ROS accumulation under Cd stress. In the present study, MA content was decreased under As treatment in both varieties (Figure 1B). The decrease in MA content could be another potential stress-responsive metabolite to elucidate tolerance mechanisms in wheat

Purine metabolites showed variations under As exposure in both varieties of wheat (Figure 1D). BARANI-70 showed higher upregulation of both xanthine and hypoxanthine compared to NARC-09 in the present study. The accumulation of xanthine triggers xanthine dehydrogenase which plays a role in the oxidation of xanthine to uric acid to eliminate excessive ROS in plants (Sun et al., 2021). Takagi et al., (2016) indicated the role of stress-mediated purine metabolites (allantoin) in stress signaling and homeostasis of stress-related hormones in plants. Uric acid, stress-responsive purine, was significantly upregulated in NARC-09 but downregulated in BARANI-70. Uric acid plays a role in As-mediated toxicity since it was previously correlated with the generation of oxidative stress under stress. Kurajoh et al., (2021) indicated a significant contribution of uric acid towards the generation of ROS and oxidative stress. Therefore, the up-regulation of uric acid could be related to ROS generation in the NARC-09 in the present study.

In the present study, various metabolites of carnitines were identified in the roots (Figure 1C). L-carnitine and acetyl-carnitine were significantly upregulated in roots of BARANI-70 under As treatment compared to NARC-09. L-carnitine has been found to regulate the growth of barely (*Hordeum vulgare*) by mitigating abiotic stress and reducing oxidative stress (Oney-Birol, 2019). Acetyl carnitine mitigates As-mediated oxidative stress and stimulates antioxidant activities (Oney-Birol, 2019). Cereal crops contain L-carnitine and acetyl-carnitine (Oney-Birol, 2019) which can assist tolerant varieties to withstand As-induced oxidative stress while stimulating antioxidants. In the present study, BARANI-70 exhibited a better response to growth compared to NARC-09 (Table 1). The stress-responsive carnitines should be further evaluated to understand their role in As tolerance.

The present study identified 12 metabolites of LysoPCs in the roots of both varieties (Figure S5). LysoPCs were found to be upregulated under As exposure in NARC-09. The cluster analysis revealed that the BARANI-70 showed downregulation of LysoPCs while NARC-09 showed up-regulation under stress (Cluster 1) (Figure S9). In *Arabidopsis thaliana*, degradation of LysoPCs was associated with a tolerance response against Cd-induced oxidative stress (Gao et al., 2010). The synthesis of the AR enzyme can catalyze the transformation of arsenate to arsenite and is responsible for the induction of ROS and oxidative stress (Shi et al., 2016). The study (Majumder et al., 2019) showed higher activity of AR enzyme in sensitive varieties of rice compared to tolerant varieties. A similar trend of AR enzyme activity between tolerant and

sensitive varieties under arsenate exposure was detected in the rice (Saha et al., 2017). In the present study, NARC-09 showed up-regulation of the AR enzyme in the root compared with BARANI-70 (Table 1). The down-regulation of AR enzyme and LysoPCs could be responsible for lower As-induced toxicity in BARANI-70. Previous literature has also detected a negative correlation between the activation of AR enzyme and growth parameters in rice (Majumder et al., 2019; Saha et al., 2017). This was also evident in the present study because BARANI-70 showed a better growth response compared to the NARC-09 (Table 1).

4.2. Behavior of glutathione metabolism under arsenate stress:

Glutathione has a significant role in As detoxification since it can relieve As-mediated ROS and act as a reductant to enhance As excretion. Three NEAAs i.e., Gly, Cys, and Glutamate are required to synthesize GSH under As stress. Previous literature has shown that GSH synthesis is regulated by As stress through Cys and Glutamate (Ran et al., 2020). In the present study, Gly and Cys were regulated significantly under As stress (Figure 1A). The KEGG pathway also identified the regulation of four important metabolites involved in the glutathione metabolism pathway (Figure S8, Table 2). Glutathione disulfide (GSSG) is generated during the reduction of arsenate to arsenite by the AR enzyme and it is reduced to GSH by a process catalyzed by glutathione reductase (GR). Due to the inhibition of GR, there could be an impact on the intracellular GSH: GSSG ratio under As stress (Thomas, 2009). Thus, the reduction of the GSH: GSSG ratio acts as a marker of oxidative stress particularly in sensitive variety. On the other hand, both Glutamate and 5-oxoproline are involved in the GSH synthesis and their up-regulation indicates that GSH could be up-regulated in the tolerant variety (Zhuang et al., 2021). The findings have highlighted the regulation of the glutathione metabolism pathway in wheat varieties that can improve defense against As stress. Furthermore, the present study identified metabolic pathways (D-glutamine and D-glutamate metabolism) under As-stress (Table 2, Figure 3) that are important for GSH stimulation. The higher synthesis of such metabolic pathways and Gly content can improve GSH biosynthesis potential under As stress (Sun et al., 2021) in tolerant varieties and mitigate As-induced toxicity.

4.3. Behavior of citrate cycle (TCA cycle) under arsenate stress:

The citrate cycle (TCA cycle) is the important process required for ATP production and for delivering precursors needed in different biosynthetic pathways such as amino acid

biosynthesis, respiration, and nitrogen metabolism (Zhang et al., 2018). Various metals like Pb, Cd, and As can interfere with the normal functioning of ATP (Saha et al.,2017) but limited literature is available about the connection between As and TCA in wheat. The interlinked pathway of As and phosphorus (P) inhibits ATP production because arsenate replaces P and results in arsenolysis (Saeed et al., 2021). Due to arsenolysis, less energy is available for plant growth and weakens defense mechanisms to cope with stress (Saeed et al., 2021). In the present study, the sensitive variety showed weaker defense mechanisms and low growth compared with the tolerant variety (Table 1) which could be attributed to arsenolysis and inhibition of normal functioning of the TCA cycle. In the present study, the KEGG pathway analysis identified metabolites (6/20) that were DEMs and can stimulate the TCA cycle (Table 2, Figure S8). Succinate is dehydrogenated to fumarate through succinate dehydrogenase enzyme and this enzyme contains a FAD prosthetic group that can be a possible precursor for ROS formation (Tretter et al., 2016). Thus, the regulation of these metabolites in the TCA cycle could link to mediated oxidative stress, particularly in sensitive varieties of wheat. Saha et al., (2017) reported higher levels of citrate in roots of sensitive variety compared to tolerant variety of rice. It was indicated that root apices excrete organic acids due to P deficiency under stress in wheat (Saha et al., 2017). Thus, roots of sensitive variety can regulate organic acids under As stress due to P deficiency and suffer from arsenolysis that can interfere with ATP generation and TCA cycle compared with tolerant variety.

4.4. Behavior of linoleic acid metabolism under arsenate stress:

The KEGG pathway revealed a significant regulation of the linoleic acid metabolism pathway under As stress (1/4) (Figure S8). Linolenic acid is the primary element of the lipid bilayer which could be negatively influenced during oxidative stress by ROS generation in plants (Upchurch, 2008). In the present study, a higher level of LINO was detected in the roots of NARC-09 under As exposure compared to BARANI-70. This could be due to the possible role of LINO in providing protection against ROS induced by As exposure in sensitive variety. Mata-Perez et al., (2015) highlighted the role of LINO to regulate the expression of GST enzyme and methionine sulfoxide reductase enzyme that can protect against As stress. Furthermore, LINO can induce plant response against abiotic stress mediated by the galactinol synthase enzyme (Mata-Perez et al., 2015). Therefore, the regulation of the Linolenic acid metabolism pathway could be the point of interest to understand the tolerance potential of wheat varieties under As stress.

4.5. Behavior of aminoacyl-tRNA biosynthesis under arsenate stress:

The earliest metabolic responses of crops to abiotic stress are to inhibit protein biosynthesis and enhance chaperone levels to regulate protein folding and processing (Baranašić et al., 2021). The aminoacyl-tRNA biosynthesis (AARSs) plays an important role in protein biosynthesis by linking appropriate amino acids with tRNA while hydrolyzing incorrect attached amino acids through editing (Baranašić et al., 2021). The role of AARSs in cellular stress response has been previously well documented. In crops, disruption of AARSs under stress can be lethal or cause severe impacts on growth and development. It was indicated that AARSs are needed for the translation of particular stress-linked mRNAs and the resumption of translation after stress (Baranašić et al., 2021). In the sensitive variety, it is possible that higher induction of oxidative stress reduced the translational fidelity by interfering with the editing activity of threonyl-tRNA synthetase (Ling and Söll, 2010). The KEGG pathway identified 19 out of 46 metabolites of the AARSs pathway in the present study (Figure S8). Therefore, the regulation of AARSs in the present study could be another defense mechanism of wheat against As stress.

5. Conclusion:

The present study provided a detailed psychological and metabolic response of two wheat varieties under arsenate stress. NARC-09 showed significant up-regulation of AR enzyme under stress while depicting negative impacts on growth. On the other hand, BARANI-70 showed better growth response by suppressing the activity of AR enzyme under stress. BARANI-70 depicted better synthesis of stress-responsive amino acids GABA, Met, Glu, Gly, Glut, Ser, and Val,) and S-containing amino acids (Met, Pro, and Tau) under stress to regulate growth and defense mechanisms compared to NARC-09. The KEGG pathway identified the up-regulation of several metabolites involved in amino acid biosynthesis pathways that are regulated to promote defense response under stress. The regulation of UNFAs such as LINO, ARA, FA, and LIN assisted BARANI-70 in defense against As stress by restricting the translocation of As and inhibiting ROS generation. The KEGG pathway identified significant variations in metabolites involved with important metabolic pathways responsible for defense under stress such as glutathione metabolism, linoleic acid metabolism, aminoacyl-tRNA biosynthesis, and TCA cycle. Furthermore, the present study identified a significant role of purines, carnitines, and LysoPCs metabolites in wheat tolerance against As stress. In the literature, such metabolites have not been

557 previously explored regarding their role in As stress for wheat. The results have provided the basis
558 for the hypothesis that the tolerant variety (BARANI-70) showed a better synthesis of As-
559 responsive metabolites and pathways compared with the sensitive variety (NARC-09) (Figure 4).
560 Furthermore, such results have enriched existing knowledge regarding the tolerance and
561 detoxification mechanisms of As in wheat at the molecular level. This report may facilitate the
562 breeding of low-As accumulating varieties for future application to ensure sustainable production
563 and food safety.

564 **6. Authorship contribution statement:**

565 **Muhammad Saeed**: Investigation; Writing – Original Draft; Data Curation; Formal
566 Analysis. **Umar Masood Quraishi**: Methodology; Conceptualization. **Ghazala Mustafa:**
567 Technical Assistance; Writing - Review and Editing. **Abida Farooqi**: Methodology. **Maria**
568 **Greger:** Resources. **Riffat Naseem Malik:** Supervision; Project administration.

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574 **8. Declaration of interest:**

575 The authors declare that there is no conflict of interest in the present study.

576 **9. Reference:**

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