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DIFFERENTIAL PROTEIN EXPRESSION ANALYSIS OF WHEAT CULTIVARS AND GRAIN APHIDS POST-FEEDING

JAVERIA SHAFQAT, MPhil
Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat Pakistan.
Email: 18026160-004@uog.edu.pk

Dr. AMBER AFROZ *, PhD

Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat Pakistan.
*Corresponding Author Email: dramber.afroz@uog.edu.pk,
ORCID ID: https://orcid.org/0000-0002-0023-6107

Abstract

Protein extraction from wheat cultivars (cvs) and aphids was refined through trichloroacetic acid, phenol extraction, and dye-lysis methods. In this study, two wheat cultivars (Galaxy 2013 and NARC 449) were evaluated for their interaction with *Sitobion avenae* and *Rhopalosiphum padi*. Aphids were collected from wheat fields and reared on the wheat cultivars under controlled conditions. Protein extraction from both wheat cultivars and aphids employed by three distinct methods: TCA–acetone extraction, phenol extraction, and the Dye-Lysis buffer method. The Dye-Lysis buffer method demonstrated superior consistency and reproducibility in protein band patterns compared to the other extraction protocols. Protein expression variations in aphids and wheat cultivars pre- and post-aphid feeding were identified using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Densitometry analysis was utilized for the precise quantitation of differentially expressed proteins. Comparative proteomic analysis of wheat cultivars under aphid stress revealed differential expression of proteins related to defence mechanisms, photosynthetic proteins and stress-responsive proteins. Key photosynthetic proteins such as Protoporphyrin IX magnesium chelatase and RuBisCO exhibited downregulation, while stress-responsive proteins like heat shock proteins, as well as defense-related proteins displayed up-regulation following aphids' infestation. Aphids, specifically *S. avenae* and *R. padi*, under infestation conditions, exhibit elevated concentrations of yellow e-3-like protein, peroxidase, trypsin, carbonic anhydrase, apolipophorins, and glyceraldehyde-3 phosphate dehydrogenase in comparison to non-feeding counterparts. These findings contribute to a deeper understanding of the molecular interactions between wheat plants and aphids, providing insights into potential defense mechanisms and vulnerabilities in the host-pathogen relationship.

Keywords: Protein Extraction, Wheat Cultivars, Aphids, Trichloroacetic Acid, Phenol Extraction, Dye-Lysis Buffer Method, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Densitometric Analysis, Differential Expression.

Abbreviations: Trichloroacetic acid (TCA); Ethylenediamine tetraacetic acid (EDTA); Caffeic acid O-methyltransferase (COMT); Mitogen-activated protein kinase (MAPK); Glutathione S transferase (GST); Heat shock protein (HSP); Pathogen related proteins (PR Proteins); Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE).

1. INTRODUCTION

Aphids belonging to the order Hemiptera and family Aphididae constitute a significant agricultural insect pest group on a global scale. Their impact stems from the removal of photosynthetic assimilates and the transmission of various s deleterious plant viruses. Among these, cereal aphids are recognized as particularly destructive pests affecting commercial wheat crops. Their detrimental effects manifest through the extraction of

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photoassimilates and the vectoring of numerous plant viruses, thereby causing substantial damage to the crops [1]. The English grain aphid, *Sitobion avenae*, the bird cherry-oat aphid, *Rhopalosiphum padi*, the greenbug aphid, *Schizaphis graminum*, and *Metopolophium dirhodum*, all belonging to the Homoptera order within the Aphididae family, represent significant pests affecting wheat and various cereals on a global scale [2]. *S. avenae* and *R. padi* emerge as challenging pestiferous aphid species affecting a broad spectrum of wheat cultivars cultivated across diverse geographical regions regions [3-4]. Aphids inflicting harm to wheat plants do so through both direct feeding activities and the transmission of numerous plant-pathogenic viruses [5].

Proteomic methodologies are indispensable as they provide an innovative avenue for investigating species with restricted genetic data. Mass spectrometry facilitates the amalgamation of comparable data acquisition, enabling the swift identification of homologous protein counterparts in closely related organisms [6]. The proteomic approach offers a distinct advantage by providing insights into the steady-state levels of proteins, which result from regulatory mechanisms occurring subsequent to mRNA synthesis. These regulatory processes encompass post-transcriptional, translational, and protein degradation regulations [7]. In comparison to the genome or the transcriptome, the proteome is more proximal to the phenotype. Consequently, it exhibits a more direct responsiveness to natural selection, establishing a close association with the adaptation process [8]. The proteomic analysis proves particularly enlightening in unravelling the intricacies of an insect system, particularly in overcoming the impacts of toxic compounds [9].

Extraction of protein from grain aphids, like other insects, proves challenging. The quantification of proteins, particularly actin and chitin, is complicated because of their similar isoelectric points and molecular weights, presenting a significant obstacle to accurate protein quantification. This similarity in isoelectric points and molecular weights leads to a notable challenge in the quantitation of proteins. Certain exoskeletal proteins, such as actin and chitin, which are not completely solubilized even with substantial chemical treatments, can interfere with polyacrylamide gel electrophoresis by producing undesired bands [10]. To address these challenges, three protein extraction methods documented in the literature, namely TCA-acetone precipitation, phenol extraction, and multi-detergent extraction, as reported in studies focused on cyanobacteria [11-12]. Silver staining is a notably effective and sensitive technique, capable of detecting even minute quantities of proteins separated through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). In contrast to the Coomassie Brilliant Blue R250 staining procedure, silver staining is widely preferred for protein detection owing to its heightened sensitivity. This method enables the detection of proteins at concentrations as low as 0.1 nanograms [13-14].

The commonly employed phenol or TCA-acetone extraction methods exhibit certain limitations for leaf proteome analysis. These drawbacks include lower protein solubility, coextraction of nucleic acids, protein hydrolysis by TCA, and restricted applicability to young tissues [15-16]. Notably, neither of these methods is well-suited for the detection

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of low-abundance proteins in leaf tissues, primarily due to the substantial presence of ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), constituting approximately 50% of the soluble protein, which tends to mask them. Addressing this challenge, the partitioning of the rubisco holoenzyme by polyethylene glycol (PEG) has proven effective in selectively isolating numerous low-abundance proteins from the leaves of various plants, including rice [17-19], sunflower [20], and tomato [21]. Clearly, a singular extraction buffer may not furnish comprehensive information about a specific proteome, and the absence of a universally "perfect protocol" underscores the need for uniquely optimized protocols tailored to each plant species [22].

However, the selection of an optimal protocol is contingent upon the characteristics of the sample tissue and the subsequent applications, such as the scrutiny of protein complexes, membrane proteins, low-abundance proteins, or those with post-translational modifications. In this context, we conducted an assessment of three distinct protein extraction methodologies—namely, Phenol, TCA acetone, and dye lysis method to identify a suitable protein extraction method conducive to one-dimensional (1-D). A straightforward yet efficient protein extraction technique was developed for the examination of differentially expressed proteins in wheat as well as in grain aphids. The utilization of SDS-PAGE methodologies facilitated to distinguish between feeding and non-feeding wheat and aphids. The molecular weight of the identified differentially expressed proteins in wheat and aphids was employed to deduce their potential identities, leading to the nomenclature of these proteins.

2. MATERIALS AND METHODS

2.1 Rearing of Aphids on Wheat Plants

Seeds of two different wheat cultivars, Galaxy 2013 and NARC 449 were procured from the National Agriculture Research Council (NARC) in Islamabad. The seeds were cultivated until reaching the two-leaf stage in pots within a growth room under controlled conditions (25°C temperature, 55-60% humidity, and a photoperiod of 16 hours light and 8 hours dark). Wheat aphids (*S. avenae* and *R. padi*) were collected from wheat fields in the Gujarat, Kharian, and Lala Musa districts. The aphid species were taxonomically identified at the Department of Zoology in Gujarat. *S. avenae* and *R. padi* were cultured on the Galaxy 2013 and NARC 449 wheat cultivars at a temperature of 22°C. Adult aphids were maintained on plants, and their reproduction led to the development of nymphs. Nymphs were allowed to mature on the plants for 8-9 days, after which the aphids were transferred to wheat cultivars at the two-leaf stage. Upon transferring adult aphids to the plants, pots were covered entirely with ventilated plastic bags. The number of nymphs produced and their survival rates were quantified every third day across different wheat varieties, aiming to assess the growth rate and fecundity of *S. avenae* and *R. padi*.

2.2 Extraction of protein from wheat cultivars and aphids

Proteins were extracted from infested and non-infested aphids and wheat cultivars using diverse protocols, including TCA extraction, Phenol extraction, and Dye-Lysis buffer

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extraction. A meticulous sample preparation process was undertaken, and the most appropriate extraction method was selected. Wheat cultivars were cultivated and exposed to *S. avenae* and *R. padi* aphids for 5 days. Aphid samples (*S. avenae* and *R. padi*) were collected from wheat cultivars, with non-feeding aphid samples obtained by transferring them to a sucrose diet for 24 hours before storage at -80°C.

2.3 Protein Extraction Protocols: Strategies for Effective Isolation

2.3.1 TCA–acetone extraction

The TCA–acetone precipitation method, a widely employed procedure for protein isolation from biological samples, offers advantages such as effective recovery, minimal reagent usage, high yield, and compatibility with subsequent analytical processes. The protocol involved grinding 3g of aphids and wheat cultivars into powder with liquid nitrogen, followed by overnight mixing with TCA and mercapto-ethanol [23]. After centrifugation, the precipitates were washed with cold acetone, incubated, and subjected to filtration and centrifugation for purification. The resulting white protein pellet was dissolved in a buffer with urea, thiourea, CHAPS, carrier ampholytes, and *Dithiothreitol* (DTT), followed by storage at -80℃ for further analysis.

2.3.2 Phenol extraction

Aphid powder, weighing 150 mg, was introduced into an extraction buffer composed of 100 mM potassium chloride, 0.1 mM phenyl methyl sulfonyl fluoride, 2% mercaptoethanol, 0.7 M sucrose, 500 mM Tris (pH 7.5), 50 mM EDTA, and 1% polyvinyl poly pyrrolidone. The mixture was then combined with an equal volume of Tris-buffered phenol (pH 7.5) and maintained at 4°C for duration of 30 minutes [24]. Subsequently, centrifugation was executed at 16,000g for 10 minutes, leading to the removal of the upper layer (phenol). The extracted phenol layer underwent a secondary extraction with an equivalent volume of the extraction buffer. The phenol phase obtained was combined with a solution containing 0.1 M ammonium acetate and methanol. Protein precipitation was induced by storing the mixture at -20°C for a period of 12 hours. The resulting protein pellets were subjected to washing with ice-cold methanol and subsequently stored at -80°C for future utilization.

2.3.3 Dye-Lysis buffer extraction method

The extraction process involved creating an extraction buffer to disrupt cellular structures in the samples, aiding in the release of proteins for analysis. The formulation of the lysis buffer was modified in accordance with the procedures described in a prior research study [25].In the Dye-Lysis buffer, aphid and wheat leaves were ground in a mixture of 0.05M Tris-HCl (pH 8.0), 0.02% SDS, 5M Urea, 1% 2-mercaptoethanol, and 0.05% bromophenol-blue. After grinding samples were centrifuged at at 15,000 rpm for 10 min at room temperature. After centrifugation, the supernatant was collected for further analysis and pellet was discarded. Denatured samples were subjected to SDS-Polyacrylamide gel electrophoresis for protein analysis.

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2.4 Quantification of protein

The Bradford assay, as described by Bradford in 1976, was employed to quantify protein content in the samples. Bovine serum albumin (BSA) served as the standard for generating a calibration curve, and absorbance readings were obtained using a spectrophotometer at a wavelength of 595 nm [26]. The recorded absorbance values for *T. aestivum* cultivars, *S. avenae*, and *R. padi* were 0.99, 0.687, and 0.823, respectively. Subsequently, protein concentrations were calculated as 50, 30, and 40 µg mL-1 for the respective samples.

2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was conducted using the Mini-Protean II apparatus MV-10DSYS (Major Science UK) (W x L=3.4"x3.2") from Bio-Rad Laboratories, employing 15% separating gels and 5% stacking gels. Prior to electrophoresis, extracted protein samples were subjected to boiling for 10-15 minutes and subsequently applied to the gels in uniform amounts of 10 µl per lane. 15% separating gel was formulated with the following composition: 4 mL of distilled water, 5 mL of acrylamide, 2.5 mL of 1.5M Tris (pH 8.8), 50 µL of SDS (20%), 100 µL of APS (10%), and 10 µL of TEMED. Following the addition of APS and TEMED, the solution was promptly poured between glass plates to initiate gel polymerization. The polymerization process took a few minutes, influenced by factors such as temperature, catalyst quantity, and radical concentrations within the solution. To prevent bubble formation, a few drops of distilled water were applied to the solution after it was poured between the glass plates. Subsequently, after gel polymerization, the excess water was removed using a pipette.

For the formulation of the stacking gel, 4 mL of distilled water was combined with 1 mL of acrylamide, 500 µL of Tris base (1M, pH 6.8), 30 µL of SDS (20%), 60 µL of APS (10%), and 6 µL of TEMED. In the stacking gel preparation, APS and TEMED were introduced in the final step to initiate gel polymerization. Following the removal of water from the separating gel, the stacking gel was poured atop the solidified separating gel. Subsequently, suitable combs were delicately inserted between the glass plates without inducing bubble formation. Upon complete polymerization of the stacking gel, the combs were gently removed, creating wells for loading samples. The glass plates were then positioned in the SDS electrophoresis tank.

2.6 Loading and running of protein samples

Running buffer was prepared by combining 3g of Tris base, 14g of Glycine, and 1g of SDS to achieve a final volume of 1mL, with pH adjusted to 8.8. Following sample loading, electrophoresis was conducted at 80V and 100 mA. The electrophoresis process spanned two to three hours, contingent upon the applied voltage and gel length. Notably, smaller proteins exhibited faster migration compared to larger ones, resulting in the separation of proteins based on their molecular size.

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2.7 Protein Band Visualization and Analysis

After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 and silver staining.

2.7.1 Coomassie staining (CBB)

For Coomassie staining, gels were fixed in a staining solution for 2 hours, consisting of 50 mL acetic acid, 225 mL distilled water, 1.25g Coomassie dye (R-250), and 225 mL methanol. Following the fixation period, the gel was then immersed in a de-staining solution for 24 hours. The de-staining solution was prepared by combining 25 mL methanol, 35 mL acetic acid, and 440 mL distilled water.

2.7.2 Silver staining

After electrophoresis for silver staining, gels underwent fixation in a solution containing 30% ethanol and 10% acetic acid for a duration of 24 hours. The fixation solution was then discarded, and the gels were transferred to a sensitizing solution composed of 0.5M sodium acetate, 2% glutaraldehyde, and 0.2% sodium thiosulfate, undergoing agitation for 30 minutes. Subsequently, the solution was discarded, and the gels underwent eight cycles of washing with cold water, each cycle lasting 15 minutes with agitation. After the washing steps, the gels were immersed in a staining solution comprising 4g silver nitrate, 10mL NaOH (1N), and 6.5mL ammonia solution (30%) for 30 minutes with continuous shaking. Following this, the solution was discarded, and the gel underwent four cycles of washing with cold water for 4 minutes with agitation. The gels were then transferred to a color developing solution containing 0.025g citric acid and 0.5mL formaldehyde per 500mL. The gels were agitated until color development, after which the solution was discarded, and an immediate addition of a stop solution (5% acetic acid) was made.

2.8 Gel image analyses by GS-900 densitometer

A densitometer is an instrument designed for the measurement of optical density (OD) in semitransparent materials or reflective surfaces. Its principle involves the utilization of a light source directed towards a photoelectric cell. Following the gel electrophoresis process, the GS-900 Calibrated Densitometer, equipped with Image LabTM Software version 5.1, is employed for the precise analysis of band intensities within the gel. SDS gel images were acquired using a GS-900 calibrated densitometer scanner manufactured by Bio-Rad. The optical density and molecular mass of each protein were ascertained using a SDS standard marker provided by Bio-Rad. Various parameters were employed in the analysis of these bands, such as molecular weight analysis, volume, optical density and report generation.

3. RESULTS

3.1 Comparative Proteomic Analysis of Different Protein Extraction Strategies

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Different protein extraction protocols were implemented to evaluate proteins with differing molecular weights in both wheat cultivars and aphids. Protein extraction from wheat cultivars and aphids involved the utilization of three distinct methods: Trichloroacetic acid (TCA)/Acetone, phenol extraction, and the dye-lysis buffer method. Notably, TCA-acetone and phenol extraction procedures exhibited inconsistent outcomes when compared to the dye-lysis extraction method, as illustrated in Figure 1. The dye-lysis buffer method displayed notable consistency and reproducibility in band patterns, particularly in the context of gel-based quantitative-comparative analysis. This method effectively extracted major protein bands that remained undetected by other extraction protocols.

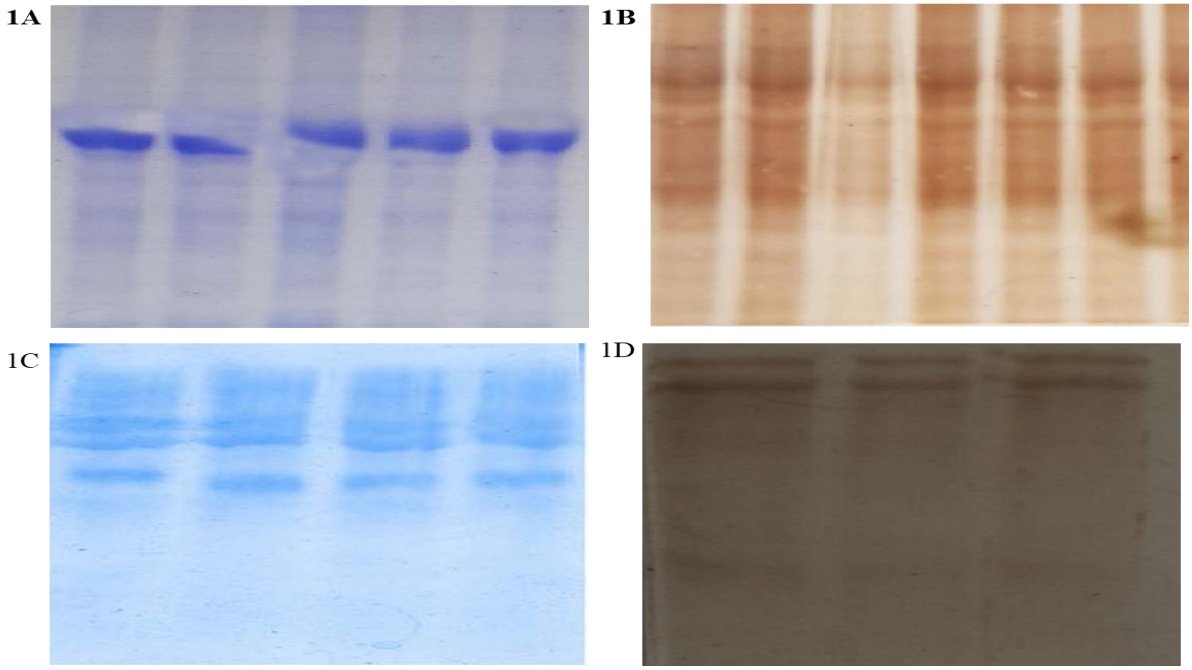

Figure 1: TCA extraction (1A) *T. aestivum* cultivars SDS-PAGE (1B) (Silver Staining). Phenol extraction (1C) *S. avenae* SDS-PAGE (CBB), (1D) (Silver staining).

3.2 Protein Profiling of Wheat under Aphid Stress and Control Conditions

From the Dye-lysis buffer method, protein extraction was performed, followed by SDS-PAGE to examine the protein profile. The resulting SDS-PAGE gels were analyzed using GS-900 Image Lab software, enabling the comparison of molecular weight and intensity of each protein band with the non-infested wheat variety. The identification of resistance or susceptibility in wheat cultivars was seen by the presence or absence of lighter or darker protein bands and the variations in intensity, signifying the alterations in protein abundance before and after infestation (Figure 2). Differential expression of proteins was observed in control wheat cultivars compared to those exposed to feeding conditions. The proteins identified were heat shock protein (HSP), Caffeic acid O-methyl transferase (COMT), Protoporphyrin IX magnesium chelatase, Chlorophyll a-b binding protein 8,

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glycine decarboxylase subunit, and Rubisco large and small subunit. Specifically, HSP was wiped out in Galaxy 2013, while its levels were reduced in NARC 449 as outlined in Table 1.

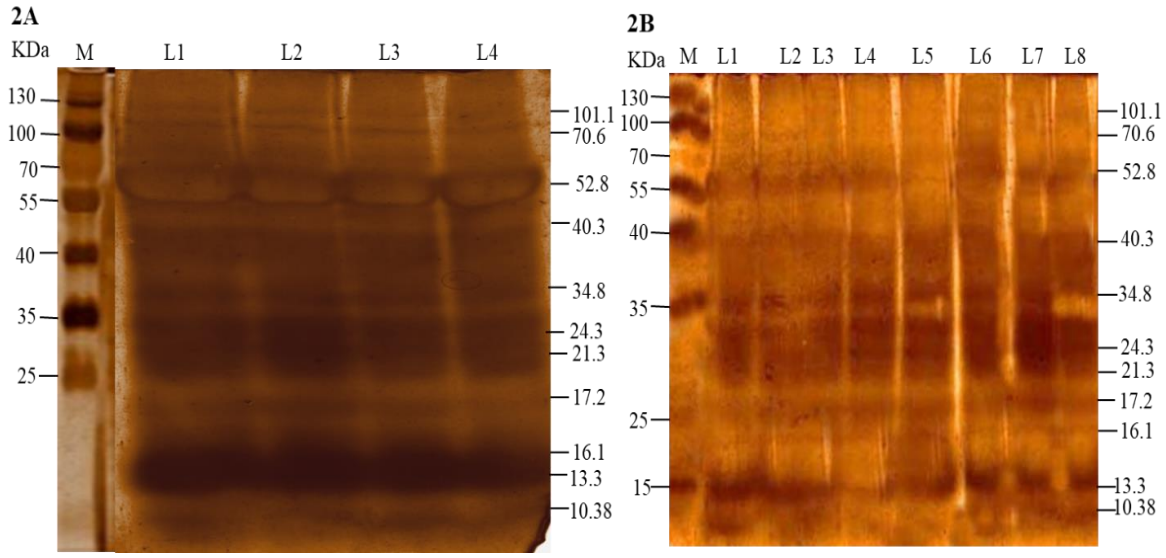

Figure 2: *T. aestivum* cultivars SDS-PAGE (Silver Staining). (3A) Non- Feeding M-Marker, L1-L2 Galaxy 2013, L3-L4 NARC 449 (3B) Feeding Wheat cultivar: L1-L4 Galaxy 2013, L5-L8 NARC 449.

After the aphid infestation, both Rubisco large (52.8KDa) and small subunits (13.3KDa) exhibited a decrease in both wheat cultivars. Additionally, the 130 KDa protein was absent in Galaxy 2013. Gibberellin 20-dioxygenase, with a molecular weight of 40.3 kDa, was identified in both wheat varieties. The presence of Gibberellin 20-dioxygenase exhibited a reduction in abundance in infested wheat cultivars, with a relatively lesser decrease observed in NARC (449). In non-infested wheat cultivars, the intensity of Gibberellin 20-dioxygenase was notably higher in Galaxy (2013) as compared to NARC 449. The expression of COMT (38.8KDa) was markedly reduced in wheat cultivars subjected to infestation. Protoporphyrin IX magnesium chelatase is detected in elevated concentrations across non-infested wheat cultivars due to its dependence on chlorophyll levels in plants. Following aphid infestation, Protoporphyrin IX magnesium chelatase (36.6KDa) is reduced in both wheat cultivars, coinciding with a reduction in chlorophyll levels. This decline in chlorophyll results in the yellowing of wheat leaves in response to the aphid attack. Glutathione S-transferases exhibit a molecular weight of 24.3 kDa. The expression of Glutathione S transferase (GST) showed a decline in wheat cultivars Galaxy (2013), when infested with aphids. In contrast, wheat cultivars, such as NARC (449), maintained elevated GST levels even following aphid infestation compared to control wheat cultivars. Pathogen-related proteins with a molecular weight of 17.2 kDa exhibited an augmentation in infested NARC 449 wheat cultivar, albeit to a lesser extent in Galaxy 2013 cultivar. Calmodulin (CaM) (16.1KDa) was reduced in infested wheat

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cultivars. Pyruvate phosphate dikinase 1 (10.38 kDa) exhibited a reduction or elimination in infested wheat varieties (Figure 3).

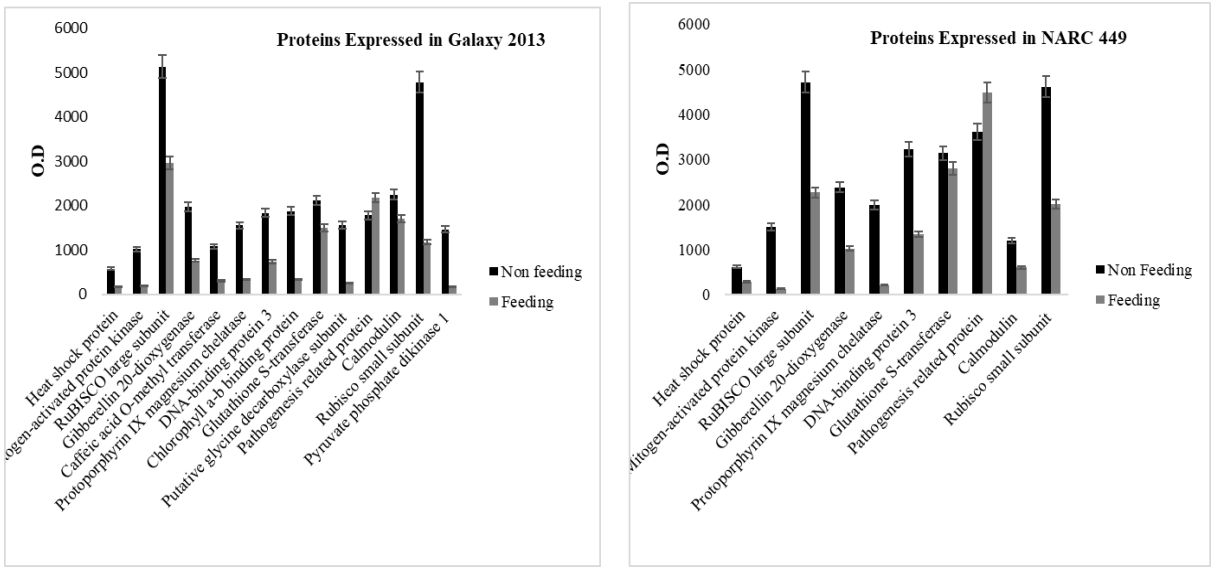

Figure 3: Protein differentially expressed in Galaxy 2013, and NARC 449 post-feeding find by optical density change in spots determined by densitometer.

The DNA-binding protein exhibited the highest density in NARC 449, followed by Galaxy 2013The overall density of the DNA-binding protein decreased across infested cultivars. Pathogen-related proteins, specifically those with a molecular weight of 17.2 kDa; PR Proteins (PR), exhibited an increase in abundance in infested wheat cultivar NARC 449, while their presence was comparatively lighter in Galaxy 2013. In the silver-stained SDS-PAGE analysis of Galaxy 2013, the presence of Mitogen-activated protein kinase (MAPK) (70.6 kDa), Glycine decarboxylase subunit (21.1 kDa) and Pyruvate phosphate dikinase 1 (10.38 kDa) exhibited decreased or complete absence in infested wheat varieties. Notably, NARC 449 displayed an increased intensity in Glutathione S-transferase (24.0 kDa) following aphid infestation.

Ribulose-1,5-bisphosphate carboxylase-oxygenase, commonly known as RuBisCo, represents a significant protein in plants. The photosynthetic reactions within plants rely fundamentally on the enzymatic activity of RuBisCo [27-28]. Higher expression of Gibberellin 20-dioxygenase aligns with the initiation of the plant's intrinsic defense mechanisms in response to aphid infestation. Aphids, upon inflicting damage to plants, instigate the release of a variety of defensive compounds, encompassing jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS), and gibberallic acid (GA) [29]. *Variations were observed in COMT expression within resistant wheat cultivars, and this variation was linked to increased lignin content. The heightened lignin levels, in turn, played a role in reinforcing stem strength [30-31]*. Protoporphyrin IX magnesium chelatase serves as a crucial enzyme in the transfer of Protoporphyrin IX for chlorophyll synthesis and heme biosynthesis [32]. Glutathione S-transferases represent crucial plant

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enzymes with significant roles in various metabolic processes, including the metabolism of xenobiotic, engagement in antioxidant activities, and the elimination of toxic substances [33]. Pathogen-related (PR) proteins demonstrated a significant elevation in NARC 449 wheat cultivar following pathogen feeding, indicative of the activation of the plant's defense mechanisms against the invading pathogen [34]. The elevated expression level of Gibberellin 20-dioxygenase is indicative of the activation of the plant's intrinsic defense mechanisms in response to aphid infestation [35].

Table 1: Molecular weight, accession number/annotation, and optical density of wheat proteins identified through SDS-PAGE in four distinct wheat cultivars.

S. No.	Proteins name/Mol. Weight	Accession No./Molecular function	Galaxy 2013 Non-Feeding	Galaxy 2013 Feeding	NARC 443 Non-Feeding	NARC 443 Feeding
1	Higher Mol. weight protein (130KDa)	0006468/Protein phosphorylation	-----	------	------	-------
2	Heat shock protein/101.1/ gi|4558484	0016887/**0034605-**ATP hydrolysis activity/C**ellular response to heat** Reference: [36]	578	Wiped out	614	279
3	Mitogen-activated protein kinase/70.6/ AAO16559	0004674/0035556 -Protein serine/threonine kinase activity/Intracellular signal transduction Reference**:** [71]	1018	Wiped out	1500	Wiped out
4	RuBISCO large subunit/ 52.8/ BAB47042	0004497/0009853-Monooxygenase activity/P**hotorespiration** Reference: [37]	5133	2961	4713	2262
5	Gibberellin 20-dioxygenase/ 40.3/ O04706	0045544/0009686-Gibberellin 20-oxidase activity/Gibberellin biosynthetic process Reference: [66]	1972	768	2380	1015
6	Caffeic acid O-methyl transferase/38.8/ AAQ07451	0008171/0032259-O-methyl transferase activity/ Methylation Reference: [38]	1087	Wiped out	-----	-----
7	Protoporphyrin IX magnesium chelatase/36.6/ A0A3B6KS74	0016851/0015995-Magnesium chelatase activity/Chlorophyll biosynthetic process Reference: [38]	1557	341	1988	Wiped out
8	DNA-binding protein 3/34.8 /NP188178	0003700/0009738-DNA-binding transcription factor activity/ Abscisic acid-activated signaling pathway Reference: [39]	1834	737	3234	1338
9	Chlorophyll a-b binding protein 8/29.3/ gi|474121685	0046872/0009416-Metal ion binding/Response to light. Reference: [40]	1876	Wiped out	------	-----
10	Glutathione S-transferase/ 24.3/ O04437	0004364/0006749-Glutathione transferase/Glutathione	2114	1500	3136	2800

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		metabolic process Reference: [41]				
11	Putative glycine decarboxylase subunit (21.1KDa)	0019464/Glycine decarboxylation Reference: [38]	1567	Wiped out	-----	------
12	Pathogenesis related protein (17KDa)	Q96458 Reference: [41]	1787	2182	3614	4485
13	Calmodulin (16.1KDa)	0009408/0005509 Response to heat/calcium ion binding Reference: [42]	2252	1706		
14	Rubisco small subunit (13.3KDa)	0000287/0009853 Magnesium ion binding/ Photorespiration Reference: [42, 37]	4787	1182	4614	2005
15	Pyruvate phosphate dikinase 1 (10.38KDa)	0005524/0042866 ATP binding/pyruvate biosynthetic process Reference: [42]	1470	Wiped out	-----	-----

3.3 Differential Proteomic Profiling of Aphids in Response to Wheat Cultivar Infestation

The dye-lysis extraction method yielded the highest total protein content and facilitated the generation of clearer and more reproducible protein bands from *S. avenae* and *R. padi*. Subsequent to extraction, the protein samples underwent separation through SDS-PAGE. A substantial disparity in both the number and intensity of protein bands was evident when comparing aphids subjected to infestation with those that were non-infested. Through comparative analysis of *S. avenae*, distinct bands were observed under infested conditions, while in the non-infested state, certain bands were reduced or wiped out, indicating the disappearance of specific proteins during starvation conditions. Following proteomic analysis of *S. avenae*, the protein profile during feeding revealed the presence of peroxidase (90 kDa), trehalase (68 kDa), yellow e-3 like protein (49 kDa), actin (45 kDa), carbonic anhydrase (30 kDa), trypsin (25 kDa), calmodulin (16.7 kDa), and histone H4 (11 kDa) in comparison to the non-feeding state.

Distinct proteins were identified in *R. padi* during feeding, encompassing Glucose Dehydrogenase (115 kDa), Apolipophorins (80 kDa), Actin (45 kDa), Glyceraldehyde 3-Phosphate Dehydrogenase (37 kDa), Carbonic Anhydrase (30 kDa), Cytochrome c β Subunit (25 kDa), Calmodulin (16.7 kDa), and Histone H4 (11 kDa). Peroxidases play crucial roles in physiological processes, including the synthesis of hormones, innate immunity, detoxification of xenobiotics, and defense against infectious diseases [43]. Yellow e-3 like protein has been implicated in diverse biological functions, such as involvement in pigmentation [44-45], behavioral traits [46], sex-specific reproductive development [47], and caste determination within honeybee colonies [48]. The existence of trehalase in the saliva of aphids may play a pivotal role in modulating the plant's defense mechanisms associated with trehalose [49-51]. Actin is intricately involved in various biological processes, including muscle contraction, mitosis, meiosis, and cellular mobility [52-53]. Carbonic anhydrase (CA) enzymes, categorized as metalloenzymes,

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serve various biological functions, including participation in biomineralization [54], ossein resorption [55] and regulation of osmotic pressure [56-57]. Trypsin (25kDa) is found in the digestive tract of nearly all insects, and its principal functions include molting and the replacement of exoskeletal structures such as horns, hair, skin, and feathers [58]. Additionally, trypsin is implicated in tissue renewal [59], temporary dormancy or diapause [60], and implantation processes [61]. Histone proteins serve as fundamental components within nucleosomes, playing a vital role in the regulation of genes and the conformational arrangement of chromatin [62].

Table 2: Molecular weight, accession number /Annotation, and optical density of aphids (*S.avenae* and *R. padi*) proteins by SDS-PAGE.

S. avenae	**Protein name/Mol. Wt.**	**Annotation (Accession No)**	**Feeding**	**Non-feeding**
1.	Peroxidase/(90KDa)	ACYPI000817; XP_029342677.1; XP_008181566.1	1469	1347
	Reference: [84, 87]			
2.	Trehalase (68 KDa) **Reference: [87]**	GO.0005993	1130	427
3.	Yellow e-3 like-protein (49 KDa)	ACYPI001857	1420	1122
	Reference: [87]			
4.	Actin/45Kda	ACYPI000064/ XP_009671898.1	1510	Wiped out
	Reference: [87]			
5.	Carbonic anhydrase/(30KDa)	ACYPI23752; XM_008190777.1	1185	331
	Reference: [63, 87,84]			
6.	Trypsin/25Kda	P21902/ XM_001952266.3	553	Wiped out
	Reference: [84]			
7.	Calmodulin (16.7KDa)	XP_008182570.1/ XM_001943408.3	1210	Wiped out
	Reference: [84]			
8.	Histone H4/11KDa	ADD24314.1	1594	Wiped out
	Reference: [84]			
R. padi				
1.	Glucose dehydrogenase (115KDa)	A0A009EMA7	634	434
2.	Apolipophorins /80Kda	XP_008183953.1	376	173
	Reference: [84]			
3.	Actin/45Kda **Reference: [84]**	ACYPI000064/XP_009671898.1	1081	329
4.	Glyceraldehyde 3 phosphate dehydrogenase (38 KDa)	AJA37895.1, gi|328709677	699	Wiped out
	Reference: [63-64]			
5.	Carbonic anhydrase (decreased)/30 Kda	ACYPI23752; XM_008190777.1	943	548
	Reference: [64, 84, 87]			

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6.	PR 6 (28KDa)	P34838	642	Wiped out
7.	Cytochrome c β subunit (25KDa)	A0A009H0F7	808	436
8.	Calmodulin (16.7KDa)/	XP_008182570.1/ XM_001943408.3		Wiped out
	Reference: [84]			
9.	15 KDa	**A0A1D3JVL9**	1049	220
10.	Histone H4 (11KDa)	gi|604788259		Wiped out
	Reference: [64]			
	10 KDa	**A0A061R5H3**	271	Wiped out

4. DISCUSSION

Plants inhabiting arid environments exhibit a substantial accumulation of diverse metabolites, posing challenges in the extraction of proteins. The presence of phenolic constituents in these plants emerges as a significant impediment to achieving protein extracts suitable for SDS gel electrophoresis. The solubility, quantity, and quality of the extracted proteins are markedly influenced by non-protein components within the plant system, introducing potential interference during the extraction process. In this study, we assessed the efficacy of different protein extraction methods based on both protein yield and the quality of subsequent 1-D profiling. Our findings reveal that the dye lysis method outperformed as compared to TCA acetone and phenol methods in terms of protein quantity. In our research work, we implemented various protein extraction and precipitation methodologies, specifically TCA-acetone extraction, phenol extraction, and dye-lysis extraction techniques. Our assessment focused on critical parameters such as protein yield, the quantity of protein bands and resolution. These protocols were applied for extracting proteins from both wheat cultivars and aphids. Subsequently, the protein samples underwent SDS. While both TCA and phenol extraction methods proved effective in minimizing interferences, they were found to be time-consuming and yielded a more limited range of protein bands when applied to wheat leaves and aphids (Figure 1). Conversely, the dye-lysis method yielded a greater number of protein bands and provided reproducible results. Notably, this protocol demonstrated efficiency with less time consumption compared to other methods and was successfully standardized for extracting proteins from both wheat cultivars and aphids.

The Tris buffer incorporated into the Phenol method was fortified with protective agents, including EDTA, which serves to chelate metal ions, thereby inhibiting metalloproteases and polyphenol oxidases. Additionally, PMSF was included to effectively inhibit serine proteases, while β-mercaptoethanol (β-ME) played a crucial role in preventing protein oxidation. The presence of potassium chloride (KCl) in the buffer contributed to the salting-in effect, facilitating protein extraction. A critical aspect of the Phenol method involved maintaining the phenol at pH 8.0, ensuring the preferential partitioning of nucleic acids into the buffer phase. The introduction of sucrose into the Tris buffer induced phase inversion, leading to the positioning of the phenol phase on top, thereby enhancing its recoverability. This multi-faceted approach involving these various factors collectively contributed to the successful extraction of high-quality proteins using the Phenol method

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[65]. Despite the relative simplicity of the TCA acetone method compared to the Phenol method, it proved less effective in eliminating contaminants. The Phenol extraction protocol demonstrated its versatility by successfully extracting proteins from challenging tissues or organs such as wood [66], potato and rapeseed seedlings [67], potato, apple, and banana leaves [68], olive leaves [69], and tomato, avocado, and banana fruits [70]. Notably, Jellouli et al. [71] corroborated the efficiency of the Phenol method in extracting proteins from leaves and roots of Vitis vinifera.

We subsequently examined differentially expressed proteins of wheat cultivars, as detailed in tables 1. Rubisco, known for catalyzing the fixation of carbon dioxide through the carboxylation of RuBisCO in the Calvin-Benson cycle, was among the proteins analyzed [72]. Infestation of wheat cultivars led to decreased levels of Gibberellin 20-dioxygenase and Pathogenesis-Related (PR) proteins across in both wheat cultivars, with comparatively higher abundance observed in the NARC 449. Post-infestation, these proteins exhibited a marginal reduction in the NARC449 wheat cultivar. Gibberellin 20-dioxygenase, a crucial plant protein, plays a vital role in wheat germination and growth, exerting its activity during various developmental stages such as seed germination, root growth, leaf elongation, and anther and pollen development [73-74]. Gibberellins, the plant hormones regulated by Gibberellin 20-dioxygenase, govern numerous aspects of plant growth and development [75]. Conversely, the expression of COMT significantly decreased in the infested cultivars. The differential expression of COMT in lodging-resistant (R) wheat cultivars has been linked to an increase in the lignin component, a factor associated with enhanced stem strength [76].

Protoporphyrin IX magnesium chelatase is a pivotal photosynthetic enzyme crucial for magnesium incorporation into chlorophyll, an essential component for photosynthesis. This enzyme modulates diverse biological functions encompassing ATPases, protein kinases, glutathione synthase, phosphatases, and RNA polymerases [77]. In contrast, chlorophyll stands as the primary pigment responsible for capturing light and converting it into energy during photosynthesis. Under stress conditions, the accumulation of magnesium-protoporphyrin IX within chloroplasts acts as a signaling molecule, contributing to a reduction in photosynthetic activity [78]. MAPK was absent in infested cultivars, aligning with its documented role in mitigating stress responses [79]. MAPK has demonstrated a decrease in response to various abiotic stimuli, including heat, UV radiation, ozone exposure, drought, heavy metal exposure, physical injury, and biotic challenges like pathogen infections. According to Jagodzik et al. (2018), the signaling cascade involving MAPK often entails the phosphorylation of target molecules, including kinases. The absence of Protein, as a significant element, determines the vulnerability of wheat cultivars under stress signaling pathways [80].

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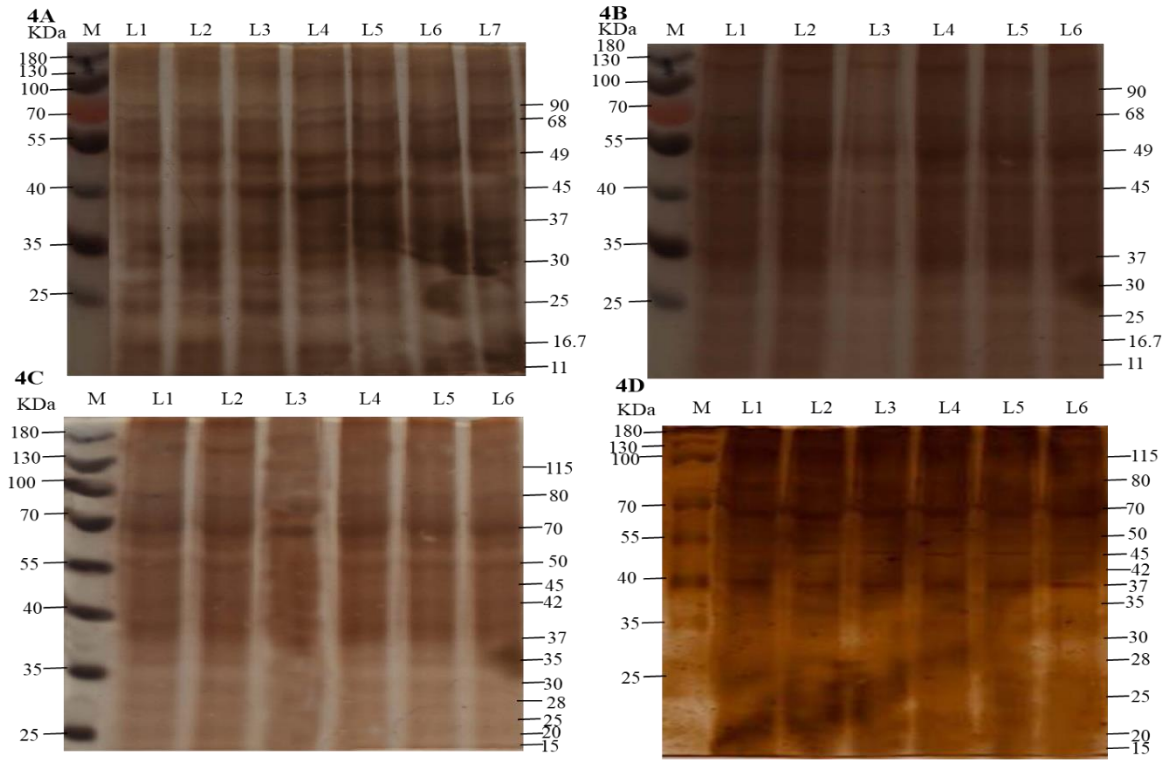

Figure 4: SDS-PAGE (Silver staining) (5A) Feeding *S. avenae* L1-L7 (5B) Non-Feeding *S. avenae* L1-L6 (5C) *R. padi* L1-L6 Feeding (5D) Non- Feeding *R. padi* L1-L6.

Divergences in the protein composition between aphid species *S. avenae,* and *R. padi* predominantly involve structural, energy-related, and defense proteins (Figure 4; Table 2). According to the research conducted by Hayashi and Chino, the phloem of wheat plants is notably rich in compounds such as sucrose, glutamic acid, aspartic acid, and serine [81]. Following feeding by *S. avenae*, enzymes implicated in sugar degradation and proteases, including maltase, beta-glucuronidase, serine protease, trypsin, and cathepsin, were identified in their salivary glands, consistent with our study findings (Figure 5). Upon attacking wheat plants, aphids exhibit an increased production of respiratory enzymes. Carbonic anhydrase, glucose dehydrogenase, and the Cytochrome c subunit were among the respiratory enzymes that demonstrated heightened activity in *S. avenae* in response to feeding [82-83]. Notably, Tian et al. (2021) demonstrated that glucose dehydrogenase contributes to the improved cold tolerance of winter wheat [84].

In addition, Xie et al. (2020) documented that aphid feeding led to elevated concentrations of glucose and sucrose in wheat plants [85]. Cytochrome c oxidase, functioning as the terminal complex in the electron transfer chain, plays a pivotal role in catalyzing the transfer of electrons from ferrocytochrome c to molecular oxygen, ultimately resulting in the production of water [86].

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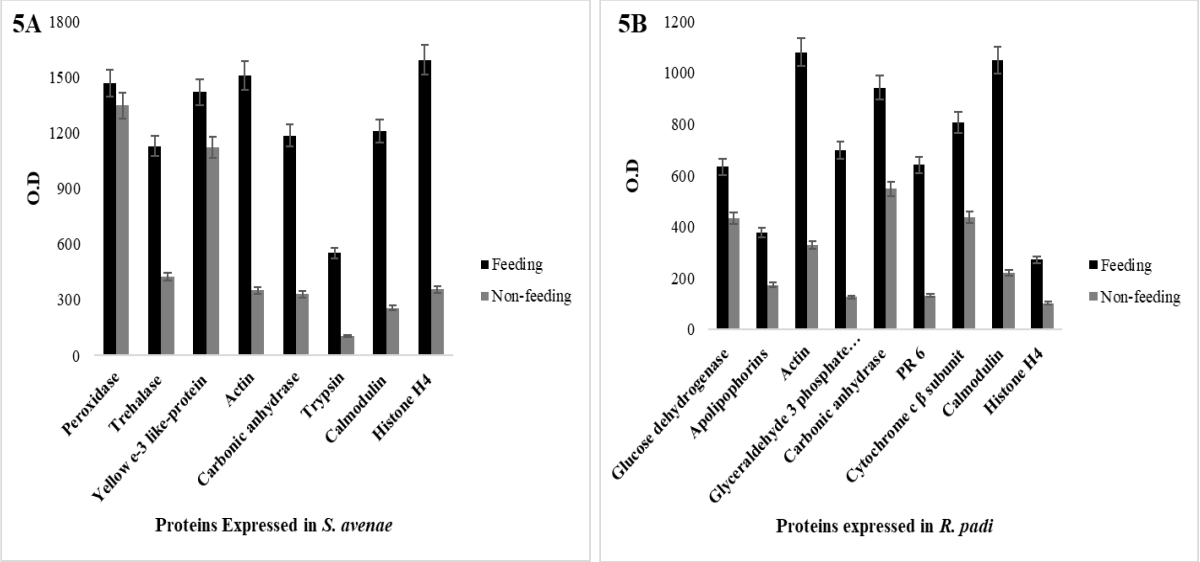

Figure 5: Protein differentially expressed in *S. avenae*, and *R. padi* post-feeding find by optical density change in spots determined by densitometer.

Carbonic anhydrases, a class of zinc-containing enzymes, contribute significantly to pH regulation by facilitating the movement and accumulation of H+ and HCO3- ions in various organisms, spanning bacteria, plants, vertebrates, and invertebrates. Alterations in cellular pH exert an influence on aphid feeding preferences, particularly in neutral to alkaline phloem conditions [87]. Actin, a highly conserved structural protein, is distributed in various cellular components, including cytoskeletons, muscles, and exo/endocytotic structures. Additionally, it plays a role in maintaining the structural integrity of the salivary sheath [88].0

5. CONCLUSION

Based on the quantitative and qualitative outcomes obtained through 1-D gel electrophoresis in the current study, it is advisable to employ the Dye lysis extraction method for the extraction of a substantial quantity of high-quality proteins from the wheat and aphids. The Dye-Lysis extraction methods demonstrated superior performance with respect to protein yield, gel quality, spot numbers, and quantities, considering these pivotal parameters. Protein profiles of *S. avenae* and *R. padi* were scrutinized subsequent to their transfer to various wheat cultivars, with a focus on optimizing protein extraction methods. The investigation revealed an augmented expression of several key proteins from wheat cultivars, including HSP, COMT, Protoporphyrin IX magnesium chelatase, Chlorophyll a-b binding protein, glycine decarboxylase subunit, RuBisCO large and small subunits, Gibberellin 20-dioxygenase, DNA-binding protein, Pathogenesis-Related (PR) Proteins, MAPK, pyruvate phosphate dikinase, and GST following infestation. In the case of *S. avenae* infestation on NARC 449, proteins such as peroxidase, trehalase, and yellow e-3 like protein, actin, carbonic anhydrase, trypsin, calmodulin, and histone H4 exhibited heightened expression levels. Similarly, infested *R. padi* displayed differential expression

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of Glucose dehydrogenase, Apolipophorins, Actin, glyceraldehyde 3-phosphate dehydrogenase, Carbonic anhydrase, cytochrome c β subunit, Calmodulin, and Histone H4. These findings contribute to a deeper comprehension of the molecular interactions between wheat plants and aphids, shedding light on the dynamic alterations in protein expression indicative of the host-pathogen relationship.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Conflict of interest statement

The authors declare no conflicts of interest.

Author Contributions

Experimental Design, Conceptualization, Funding source, Draft edition, Supervision, Validation, and Project administration [Amber Afroz]; Data creation, Experimentation, Formal analysis [Javeria Shafqat].

Consent for publication

Corresponding author had taken consent from all co-authors to submission and publication of their data in Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/Journal of Tianjin University Science and Technology

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