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Comprehensive Insights into *Sitobion avenae* Preferences and Performance on Pakistan's Wheat Cultivars Leading to Identification of Potential RNAi Targets

(Wawasan Komprehensif tentang Keutamaan dan Prestasi *Sitobion avenae* pada Kultivar Gandum Pakistan yang Membawa kepada Pengenalpastian Sasaran Berpotensi RNAi)

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ABSTRACT

Sitobion avenae, a notable hemipteran pest, poses a significant economic threat to *Triticum aestivum* due to its short generation times and high reproductive rates. Challenges like the development of insecticide resistance, the limited impact of insecticidal genes, and associated risks led to seeking a more precise approach like RNA interference. This study evaluated *S. avenae* response on seven different local cultivars (Anaj-2021, Subhani-2022, Fakhar-e-Bhakkar-2021, Akbar-2019, Mexi-Pak-2022, Barani-2022, & Dilkash-2022) through aphid preference test, aphid choice assay, and aphid performance test. Further, differential proteomics of *S. avenae* (pre- and post-feeding on susceptible and resistant wheat cultivars) was performed using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis. Among the local wheat cultivars, Anaj-2021 was regarded as the most susceptible cultivar while Barani-2022 was declared the most resistant. The differential proteome analysis of Anaj-2021 (S), and Barani-2022 (R) show 11 upregulated proteins including Glutathione S- transferase, Cathepsin, Carbonic anhydrases, Ecdysone induced protein, Odorant binding protein 3, Heat shock protein, Salivary effector protein, SID1-like protein, Sodium channel protein, chemosensory protein, and trypsin were upregulated in *S. avenae* on wheat feeding as compared to non-feeding. Trypsin, cathepsin-B and carbonic anhydrases are connected to detoxification and digestion. While odorant binding proteins, salivary effector proteins, sodium channel proteins and ecdysone- induced proteins facilitate feeding process in *S. avenae.* The enhanced expression of proteins having detoxification, digestion or defense activity implicates their essential role in the survival of *S. avenae.* Therefore, these proteins have the potential to serve as RNA interference targets, against which double-stranded RNA could be designed and expressed in wheat cultivars to make them resistant to local *S. avenae* infestation and avert yield loss.

Keywords: Phylogenetic analysis; proteome; RNA interference; SDS-PAGE

ABSTRAK

Sitobion avenae, perosak hemiptera yang terkenal menimbulkan ancaman ekonomi yang ketara kepada *Triticum aestivum* kerana masa generasinya yang singkat dan kadar pembiakan yang tinggi. Cabaran seperti pembangunan rintangan racun serangga, kesan terhad gen insektisida dan risiko yang berkaitan membawa kepada mencari pendekatan yang lebih tepat seperti gangguan RNA. Kajian ini menilai tindak balas *S. avenae* pada tujuh kultivar tempatan yang berbeza (Anaj-2021, Subhani-2022, Fakhar-e-Bhakkar-2021, Akbar-2019, Mexi-Pak-2022, Barani-2022 & Dilkash-2022) melalui aphid ujian keutamaan, ujian pilihan aphid dan ujian prestasi aphid. Selanjutnya, proteomik pembezaan *S. avenae* (sebelum dan selepas makan pada kultivar gandum yang mudah terdedah dan tahan) dilakukan menggunakan Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis. Antara kultivar gandum tempatan, Anaj-2021 dianggap sebagai kultivar yang paling mudah terdedah manakala Barani-2022 diisytiharkan paling tahan. Analisis proteom pembezaan Anaj-2021 (S) dan Barani-2022 (R) menunjukkan 11 protein terkawal termasuk *Glutathione S-transferase, Cathepsin, Carbonic anhydrases, Ecdysone induced protein, Odorant binding protein 3, Heat shock protein, Salivary effector protein*, protein seperti SID1, protein saluran Sodium, protein kemoderia dan tripsin telah dikawal selia dalam *S. avenae* pada pemberian makan gandum berbanding dengan tidak diberi makan. Trypsin, cathepsin-B dan anhidrase karbonik disambungkan kepada detoksifikasi dan pencernaan. Manakala protein pengikat bau, protein efektor air liur, protein saluran natrium dan protein yang disebabkan oleh ecdysone memudahkan proses penyusuan di *S. avenae*. Pengekspresan protein yang dipertingkatkan mempunyai aktiviti detoksifikasi, pencernaan atau pertahanan membabitkan peranan pentingnya dalam kemandirian *S. avenae*. Oleh itu, protein ini berpotensi untuk berfungsi sebagai sasaran gangguan RNA yang terhadapnya RNA untai dua boleh direka bentuk dan diekspresikan dalam kultivar gandum untuk menjadikannya tahan terhadap serangan *S. avenae* tempatan dan mengelakkan kehilangan hasil.

Kata kunci: Analisis filogenetik; gangguan RNA; proteome; SDS-PAGE

INTRODUCTION

Sitobion avenae is a phytotoxic and destructive pest, which infests majorly on economically important crops like wheat (*Triticum aestivum*). It damages wheat by removing photoassimilates; acts as main vector for viral disease transmission; resulting in 30-40% yield loss (Zeb et al. 2016). According to the Pakistan Bureau of Statistics, the total wheat production in the country in the year 2021-2022 was 27464.1 tons, cultivated at an area of 9168.2 hectares. However recent stats suggest that wheat production in Pakistan is severely damaged (up to 80%) particularly by *Sitobion avenae*, and *Rhopalosiphum padi.* In contrast to advanced nations, Pakistan's wheat yield is merely half, while the increasing population complicates efforts to meet demand and curtail damage (Afzal et al. 2015; Hussain et al. 2022).

Plants exhibit natural resistance to aphids through three key modules. Antixenosis, the first line of defense, repels or deters aphids from settling on the host plant, crucial for preventing initial attraction (Dembilio, Jacas & Llácer 2009; Gebretsadik, Zhang & Chen 2022). Antibiosis involves physiological changes in aphids' alimentary canal due to plant defenses like trichomes, affecting their behavior and potentially leading to their demise (Kranti, Nivedita & Shindikar 2021; Platková, Skuhrovec & Saska 2020). Tolerance is the plant's ability to recover and grow despite aphid-induced damage, enhancing resilience and minimizing yield loss (Leimu & Koricheva 2006; Sreelatha, Sharma & Gowda 2018). Plants exert these defenses at multiple levels of their interaction with aphids. These modules, collectively, provide insights for plant breeding and strategies to strengthen plant resistance against aphids, ensuring better crop outcomes (Tabari et al. 2017).

To control aphid infestation, the use of pesticides is a prevalent method but impart harmful effects for instance: development of resistant strains including a notable case where a single amino acid substitution in voltage-gated channels of *S. avenae* reduced pyrethroid effectiveness (Foster et al. 2014). Similarly, issues like resurgence, off-target specificities, and environmental hazards also follow up with the use of insecticides (Hu et al. 2016). Breeding of resistant wheat germplasm is an effective strategy to overcome *S. avenae* challenges (Hesler & Tharp 2005).

To precisely combat *S. avenae* infestation, transgenic crops have been synthesized by insertion of *Bacillus thuringiensis* crystal toxins (Cry), and lectins which showed varied aphid control efficacy, with some toxins having a limited impact (Porcar et al. 2009). Challenges exist in cultivating transgenic hemipteran pest-resistant plants and concerns about non-target gene insertion (Guo et al. 2019; Smith & Chuang 2014). Consequently, there exists a requirement to create novel and environmentally sustainable transgenic plant methods to address the issue of phloem-feeding insects like aphids and whiteflies. Thus, RNA interference (RNAi) is explored as an alternative tool in precise pest control management (Deng & Zhao 2014; Feng et al. 2023).

RNAi is a post-transcriptional gene silencing that selectively targets specific mRNA molecules. This is achieved by introducing double-stranded RNA (dsRNA) or short-interfering RNA (siRNA) to initiate the RNAi pathway, resulting in the suppression of a target gene (Kurreck 2009). Over the years, RNAi has also been used for aphid control as well. A few examples include the silencing of catalase gene (Deng & Zhao 2014), cytochrome c oxidase subunit VIIc precursor; laccase gene (Zhang et al. 2018), serine protease 1 DSR48 via feeding and acetylcholinesterase gene SaAce1 through injection in grain aphid *S. avenae* (Yu et al. 2016). Finding the efficient RNAi target is critical to target particular aphid species. Proteome analysis is important to find RNAi targets; as it allows the study of all proteins involving post-translational modifications in comparison to genomics and transcriptomics. Understanding these active proteins is vital for understanding of metabolic pathways under stress (Afroz et al. 2011).

This research work aimed to classify wheat cultivars as resistant or susceptible to *S. avenae* infestation and perform proteome analysis on *S. avenae* pre- and post-feeding using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to identify differentially expressed proteins (DEPs) as RNAi targets. The study involves proteomics to find DEPs as potential RNAi targets to develop insect-resistant transgenic wheat varieties, mitigating crop yield losses due to aphid infestation. DEPs were found in *S. avenae*, and to find its persistence in related species phylogenetic analysis was performed. As it assists in finding the evolutionary relationship among related species through homology that exists in these organisms (Horiike 2016). This understanding helps to design more effective RNAi molecules leading to improved gene silencing across various species (Zhang et al. 2018). The properties of phylogenetic analysis like simple, visually intuitive representation of evolutionary relationships make it a highly reliable and essential bioinformatics tool (Roy, Dasgupta & Bagchi 2014). Molecular analysis along with phylogenetic analysis can prove to be significant in understanding the polymorphism, and association among different stages of development in insects (De Mandal et al. 2014).

MATERIALS AND METHODS

APHIDS REARING AND COLLECTION

Seven local wheat cultivars, including Anaj 2021, Subhani-2022, Fakhar-e-Bhakkar-2021, Akbar-2019, Mexi-Pak-2022, Barani-2022, and Dilkash-2022, were soaked for 24 h to enhance germination. These local

cultivars were procured from vendors in Gujrat and selected based on specific traits such as high yield potential. They are extensively cultivated across the Gujrat region in Punjab, Pakistan. Following soaking, 5-6 seeds from each cultivar were planted per plastic pot and placed in a custom growth chamber with tailored environmental conditions. Once reaching the two-leaf stage, these cultivated plants were used to sustain distinct aphid colonies.

Initially, *S. avenae* adults were collected from local fields and introduced (2-3 adults per pot) to grown wheat cultivars using a brush. Plastic bags were placed over the pots to isolate and maintain individual aphid colonies, ensuring no cross-contamination. These colonies were then cultivated in controlled lab conditions (21± 2 °C, 16:8 h. photoperiod, and 60-70% relative humidity) for 2-3 weeks to meet sample size needs for subsequent experiments.

APHID OVIPOSITIONAL PREFERENCE TEST

Aphid preference tests were conducted to understand the settling, feeding behavior, and host plant choices of *S. avenae*. Seeds from seven local wheat cultivars were soaked, sown in plastic pots, and allowed to sprout in a growth chamber under specified conditions. Each cultivar had three sets of 6-7 seeded pots, with three replicates (the experiment was repeated three times) to ensure experiment validity. Upon reaching the two-leaf stage, each pot was infested with 3 adult aphids and covered with ventilated plastic bags to restrict aphid movement. Nymph counts at intervals (24, 48, 72, & 96 h) were used to determine aphid preference for each cultivar, aiding in evaluating antixenosis-based plant resistance (Akhtar et al. 2007).

APHID CHOICE ASSAY

Aphid choice assays were conducted to assess antixenosis-based resistance in local wheat cultivars. After overnight soaking, single seeds from each cultivar were sown 7 cm apart in a large pot (21 cm diameter, 15 cm height). When seedlings reached the two-leaf stage, 40-45 alate adult aphids were introduced to the pot's center using a fine brush, and the pot was covered with ventilated plastic bags. Aphid counts on each cultivar were recorded at intervals (24, 48, 72, and 96 h). This experiment was replicated three times for accuracy (Castro et al. 2005).

APHID PERFORMANCE ASSAY

The aphid performance test evaluated antibiosis-based resistance in various wheat cultivars against *S. avenae*. Two alate adult aphids were placed on each plant, and nymph counts were conducted 24-48 h later. One nymph per plant was retained for monitoring reproduction, and the daily count of nymphs produced for the next 5 days was recorded. The daily nymph count facilitated the computation of the aphids' intrinsic rate of increase, providing essential insights into the cultivars' resistance profiles against *S. avenae* infestation (Cao et al. 2015). This test was conducted in two different seasons (January and July) to assess the effect of temperature on the growth of *S. avenae* (intrinsic rate). The intrinsic rate (rm) is number of new nymphs minus the number of mortality/ generation time. It was calculated by using the following equation (Wyatt & White 1977).

$$rm = 0.738x\,(lnMd)/T$$

where 0.738 is the correction factor; M_d is the total number of *S. avenae* nymphs produced; T is the total number of days; and rm is the intrinsic rate of increase of *S. avenae* Nymphs. The weights of *S. avenae* colony 3 weeks post-rearing was also weighed on each cultivar to estimate the damage inflicted by aphids on wheat cultivars.

STATISTICAL ANALYSIS

Excel 365 and Minitab-18 were used for statistical analysis, calculating means, and standard deviations, and generating visual representations. This software also facilitated two-way ANOVA and general linear model analysis to assess significance and p-values for result accuracy. Post hoc analysis was carried out using Tukey's test with the α value set at 0.05.

IDENTIFICATION OF RNAI TARGETS THROUGH PROTEOME ANALYSIS

After the categorization of local wheat cultivars into resistant and susceptible cultivars against *S. avenae*. It was followed by comparative proteome analysis with feeding and non-feeding aphid (20% sucrose diet for 18 h) using SDS-PAGE (MS major science, Sr. no: 170822025) for 5 days. 50 mg adult *S. avenae* were subjected to protein extraction. Protein was extracted using lysis buffer (Tris-HCl (Thermo Scientific Invitrogen Ref no: 15506-017): pH=8, SDS (Art-Nr: 2326.2), Urea (5M), β- Mercaptoethanol (Merck; B124860 310) (1%), SDS-0.2%, Bromophenol blue-0.2%) (Akhremko, Vasilevskaya & Fedulova 2020). The quantity of proteins was determined using Bradford assay (He 2011) using a UV/VIS spectrophotometer at 595 nm. Resolving gel having 15% (Acrylamide (Art-Nr: 7871.1)-10 mL, Tris (Ref no: 15504-020) (pH=8.8)-5 mL, SDS-20%, APS-10%, TEMED (Roth; Art-Nr. 2367.1)-20 µL) and stacking gel was 4% concentration (Acrylamide-2 mL, Tris(pH=6.8), SDS-20%, APS-10%, Temed-12 µL) were used. The gels were then subjected to CBB-R250 (Sigma Aldrich; Lot no: MKB4584A) staining. The bands were then analyzed by GS-900™ calibrated densitometer and the optical density provided by Image Lab™ Software

reflected the expression of proteins (GS-900 Calibrated Densitometry System Version 5.1: Bio-Rad: 170–7991). The band size was estimated by protein ladder and the protein names were identified using databases e.g., Uniprot, and previous literature (Precision Plus Protein Standards BioRad).

PHYLOGENETIC ANALYSIS

Proteins that have the potential to serve as RNAi targets were analyzed for phylogenetic analysis in closely related aphid species, which have an identity of 85% and more. The protein database in NCBI was used to download protein sequences followed by a basic local alignment search tool (BLAST) to retrieve homologous sequences. Then, multiple sequence alignment was carried out by MUSCLE, followed by generating a phylogenetic tree.

RESULTS

APHID OVIPOSITIONAL PREFERENCE TEST

The nymphs of *S. avenae* showed significantly higher nymph settling and feeding preference on Anaj-2021 (7.07), followed by Subhani-2021 (6.5). Fakhar e Bhakkar-2021, Akbar-2019, and Mexi-pak-2022 were moderately preferred. *S. avenae* nymphs showed the least preference for Barani-2022. In the previous studies, the aphid population on various local cultivars was estimated and Akbar-19 had been reported to be moderately preferred with a population of (7.6) upon attack by *S. avenae.* The scale of preference levels among the cultivars in the present studies was categorized based on this already-reported local cultivar (Wains et al. 2023). Most of the cultivars used in the study have not been reported in the literature. Statistical analysis with $P< 0.0001$ shows significant differences between variables (Figure 1).

APHID CHOICE ASSAY

The highest number of *S. avenae* adults were settled on Anaj-2021 with an average value of 16.8 followed by Fakhhar-e-bhakkar-2021 (13.6), and Subhani-2021 (13.2) after four days of rearing. Thus, these were categorized as highly preferred, whereas Barani-2022 attracted the least number of adult *S. avenae.* The period of four days was chosen because it allows us to observe both short-term and long-term responses. Aphids are highly reproductive species but also short-lived, their peak activity thus mostly spans over four days which allows us to observe the time required to settle on any variety, feeding behavior, and reproduction rates. Statistical analysis at $P< 0.0001$ shows the significant difference between variables (Figure 2).

APHID PERFORMANCE TEST

The aphid performance test was used to calculate the intrinsic rate of increase for adult *S. avenae* in winter and summer and the data was subjected to comparison. Statistical analysis shows a significant relationship between variables ($P < 0.001$). The r_m of *S. avenae* feeding on Anaj-2021 was highest in both seasons while it was recorded lowest on Barani-2022. The comparison of the two seasons showed that there was no significant difference between the two seasons.

The weight of *S. avenae* colonies was also measured and these results showed a similar pattern recorded through the other conducted tests; where Anaj-2021 was declared as most susceptible wheat cultivar while Barani-2022 was declared as the most resistant local wheat cultivar (Supp. Figures 1 & 2; Supp. Table 1; Figure 3).

IDENTIFICATION OF RNAI TARGETS THROUGH PROTEOME ANALYSIS

S. avenae differentially expressed proteins; before and after feeding on susceptible and resistant wheat cultivars were compared. Comparison of differential proteins after resistant and susceptible cultivars aphid feeding is an effective tool to find the RNAi targets (Shafqat & Afroz 2024b). 35 µg protein was loaded in wells calculated by Bradford assay (Supp. Figure 3). The protein ladder was run alongside the samples in SDS-PAGE to find the molecular weight of proteins. Further, the molecular weights of protein bands were estimated by comparing the migration distances of bands within the sample to those of standards within the protein ladder. Subsequently, relevant protein databases such as Uniprot were searched with molecular weight estimates, enabling the identification of the proteins.

In both resistant and susceptible cultivars, the expression of Glutathione S- transferases (23.6 kDa), Cathepsin-B 2744 (29.2 kDa), Carbonic anhydrases (129 kDa) and trypsin (83.9 kDa) were higher compared to *S. avenae* non-feeding (Figure 4(A), 4(B)). The proteins are predicted for detoxification or digestive activity. The other proteins identified were SID-1-like (27.4 kDa), ecdysone-induced (112 kDa), sodium channel (109 kDa), chemosensory (10-16 kDa), and salivary effector proteins (16.3 kDa). Since the susceptible wheat is more prone to *S. avenae* attack, there are high chance that more proteins will be expressed in the *S. avenae.* However, the proteins expressed in *S. avenae* fed on resistant cultivars were also observed.

PREDICTED RNAI TARGETS

Among the proteins identified by SDS-PAGE, proteins involved in crucial metabolism for aphid survival were

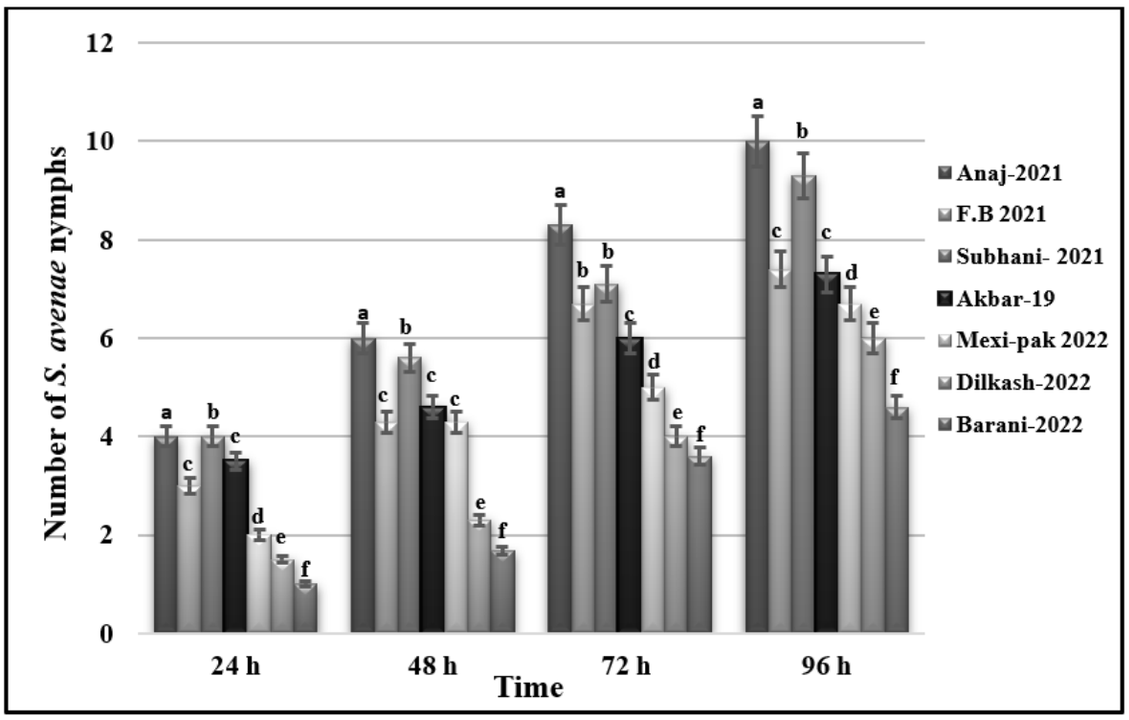

FIGURE 1. *S. avenae nymphs* reproduced on *T. aestivum* local cultivar at 24, 48, 72, & 96 h

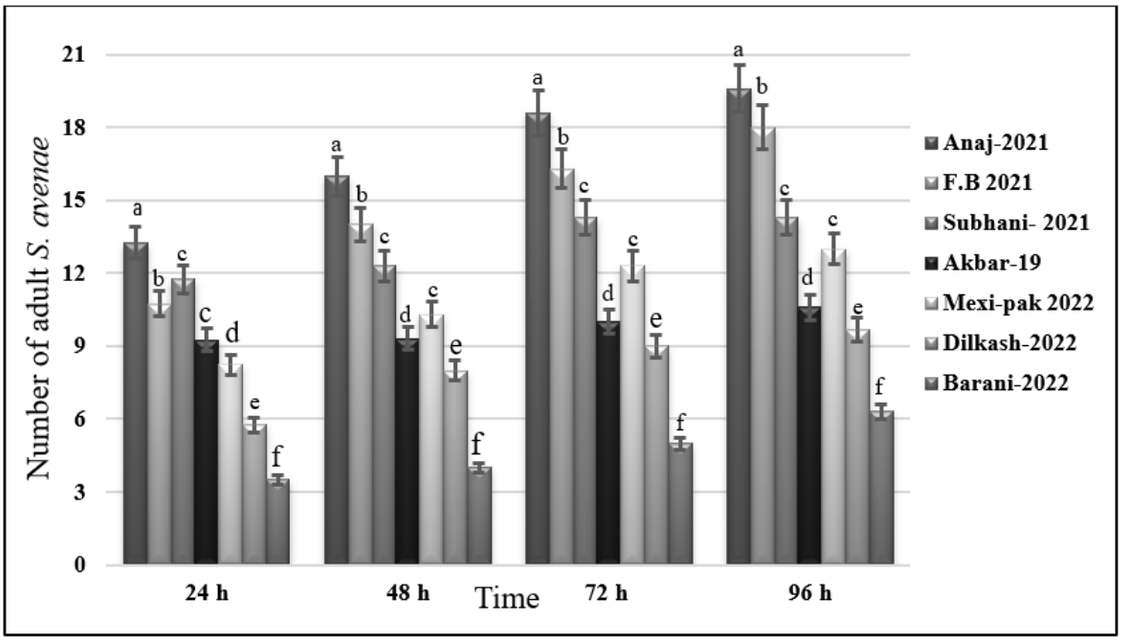

FIGURE 2. Graphical representation of a number of adult *S. avenae* settled on *T. aestivum* local cultivar at 24, 48, 72, & 96 h

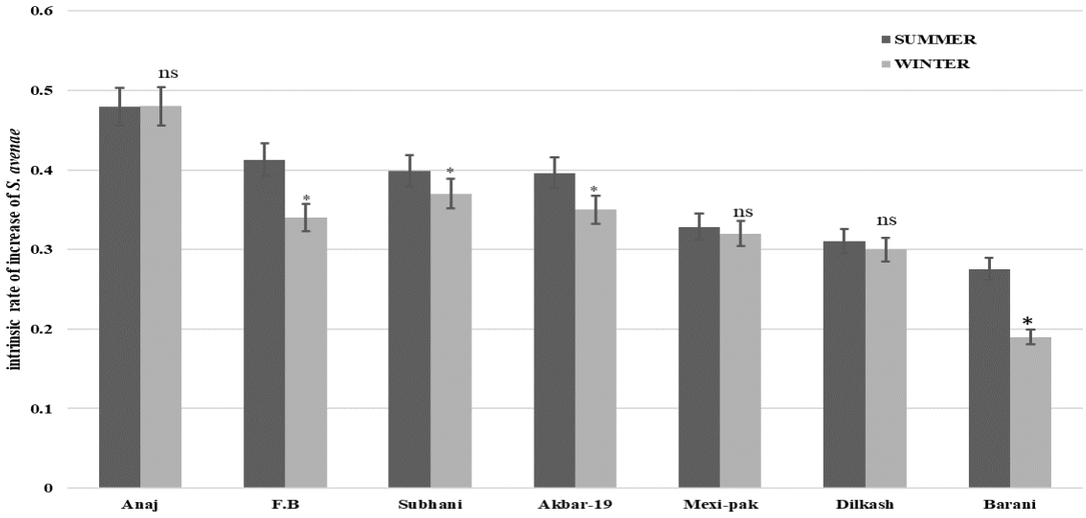

FIGURE 3. *S. avenae* intrinsic rate of increase in winter and summer

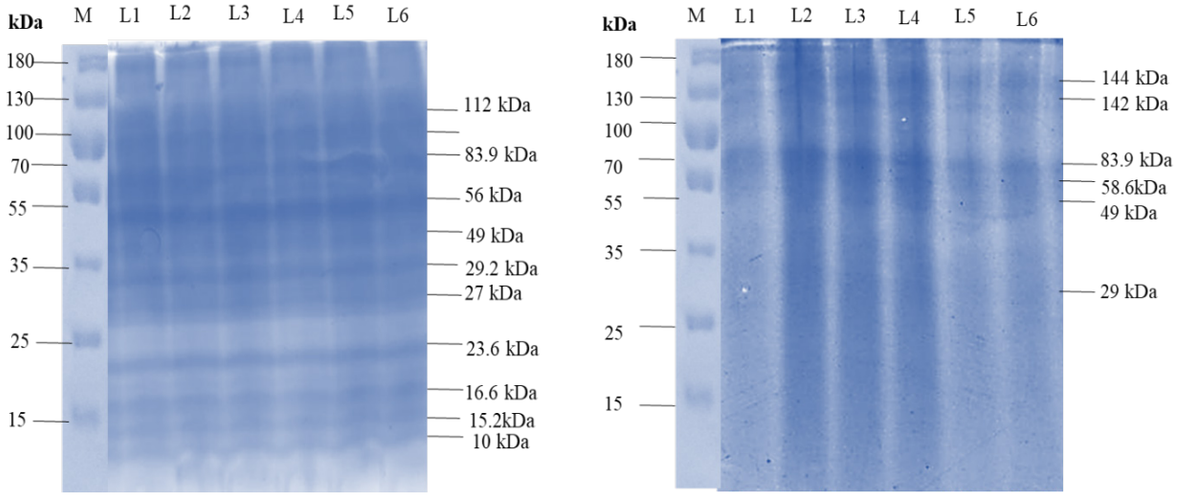

FIGURE 4. Comparison of *S. avenae* proteins in susceptible wheat feeding (A) and non-feeding (20% sucrose diet) (B)

selected and thus predicted as RNAi targets. The proteins enhanced or reduced in response to feeding is presented (Figure 6). Sheath protein, glucose oxidoreductase, chemosensory proteins are reduced. Ecdysone induced proteins, odorant binding protein-3, carbonic anhydrase, HSP, salivary effector proteins, cathepsin-B, SID-1 like proteins, sodium channel proteins, trypsin, and Glutathione S transferase were enhanced (Figure 6). Enhanced proteins in response to feeding can be effective RNAi targets (Shafqat & Afroz 2024a, 2024b). The enhanced protein expression had detoxification, insect probing, and digestive activity implicates their essential role in the survival of *S. avenae* (Mahmood et al. 2022; Vellichirammal et al. 2017). Silencing their expression in *S. avenae* using as RNAi target will have negative effects and can cause the *S. avenae* mortality. Among these potential RNAi targets, proteins of particular interest are ecdysone induced proteins. This protein is known to play crucial role in molting and metamorphosis in insects. It assists in shedding old skin and transform into new phase of their life cycle

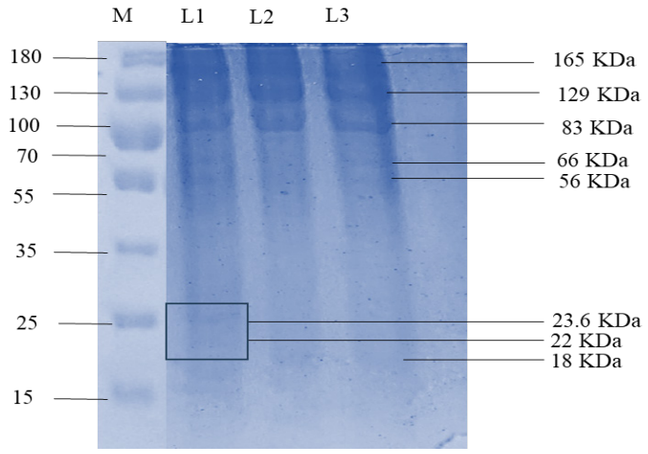

FIGURE 5. L1: *S. avenae* proteins in resistant wheat feeding and L2 and L3 non-feeding

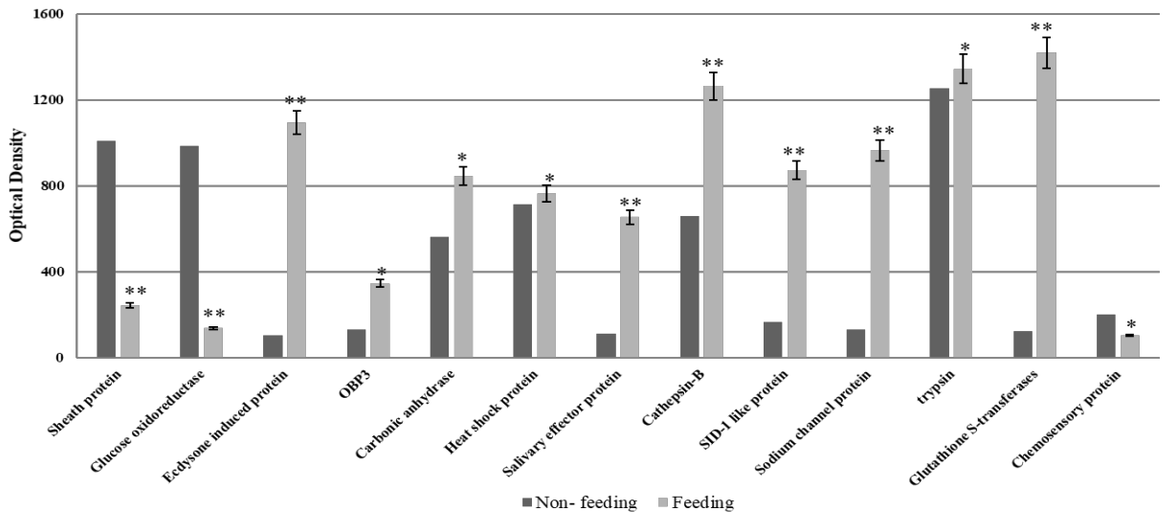

FIGURE 6. Graph showing relative expressions of DEPs in wheat feeding as compared to non-feeding

(Vellichirammal et al. 2017). Similarly, in the previous studies, salivary effector proteins have been shown to promote aphid virulence and suppress plant defense (Mahmood et al. 2022). SID-1 facilitates the uptake of exogenous dsRNA from the environment, and spreads the amplified signal for RNAi (Bansal & Michel 2013). Sodium gated channels are reported for enhanced RNAi activity (Shafqat & Afroz 2024a). Upregulation of both dsRNA channel proteins in response to feeding can be an attractive RNAi target along with some salivary and Odorant target to enhance the process of dsRNA uptake. This process can also make aphids susceptible to RNAi-based pest control strategies, as it enables the delivery of dsRNA that interferes with aphid genes, potentially leading to reduced pest infestations in agriculture.

PHYLOGENETIC ANALYSIS OF SELECTED POTENTIAL RNAI TARGETS

Phylogenetic tree for glutathione S- transferases involved in detoxification pathway show high homology with Cathepsin-B, Glutathione S-Transferase, Trypsin, and Odorant binding protein-3 had close homology to *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis gossypii*, *Rhopalosiphum padi*, *Alternaria solani*, and *Diuraphis noxia* (Figure 7). Cathepsin B involved in protein digestion,

while trypsin, also known as serine proteases, have a significant role in survival of aphids as they contribute in digestion of complex proteins into simpler amino acids for convenient uptake. Odorant binding protein 3 is involved in host selection and feeding process as it detects chemical cues released by possible hosts, thus has the potential to serve as RNAi targets (Mahmood et al. 2022). So, the same predicted RNAi targets can be used for the related species.

DISCUSSION

Aphids feed by puncturing plant cells with a stylet and ingesting their contents. Before reaching the phloem, aphids decide whether to continue feeding or leave (Nam, Powell & Hardie 2013). The phloem contains feeding deterrents, but their concentration might be too low to affect aphid host preference. Host preference depends on various factors, including plant phloem quality, volatiles released by plants and environmental cues along with gustatory cues (Douglas 2006; Powell, Tosh & Hardie 2006; Webster 2012).

Our experiments aimed to record the response of *S. avenae* infestation on local wheat cultivars. There were different experiments conducted to observe the host preference development time from nymph to its maximum reproduction, settling, feeding behavior, and multiplication rates. These tests concluded that Anaj-2021 was most susceptible wheat cultivar towards *S. avenae* infestation and was prone to severe damage which involved early yellowing of leaves, and poor nutritional quality that leads to yield loss. On the contrary, Barani–2022 was declared as the most resistant wheat cultivar as this variety harbored lowest aphid populations and *S. avenae* also inflicted least damage as its leaves showed least discoloration (Supp. Figures 1 & 2). Prior research has indicated that wheat plants exhibiting resistance to *S. avenae* tend to possess higher levels of soluble sugars and lower levels of free amino acids compared to their susceptible counterparts (Cai, Zhang & Cheo 2004). Similarly, presence of non-protein amino acid β-aminobutyric acid is proven to impart toxicity to *S. avenae* that can also attribute towards plant resistance (Cao et al. 2014). This information can provide useful insights to control aphid challenge such as the cultivation of resistant varieties might reduce the need for insecticides, or these varieties can be used in breeding programs to develop new varieties with enhanced resistance. The results are presented in the form of table (Table 1).

Among the local wheat cultivars, *S. avenae* showed the maximum intrinsic rate of increase at Anaj-2021, which further validates its categorization as a most susceptible variety. In both seasons, Barani-2022 showed the least intrinsic rate of increase making it as most resistant wheat cultivar. The varied response of *S. avenae* in terms of nymph reproduction rates over different cvs, and different seasons can be attributed to several factors. For example, the difference in the nutritious quality of phloem sap produced by the different cultivars of *T. aestivum* plays an important role in the selection of host plants by *S. avenae* (Buhler & Schweiger 2023).

In our study, there were morphological differences between leaf surface of resistant and susceptible cultivars. The leaf surfaces of the least preferred cultivars (Barani & Dilkash-2022) were hard and rough as compared to other cultivars indicating that the cell wall surface of these cultivars was probably higher as compared to others (Awmack & Leather 2002). These thick cell walls might have served as a barrier against *S. avenae*, making it difficult for an aphid to reach and suck phloem sap thus depriving the *S. avenae* to feed and reproduce, thus indicating the presence of Antixenosis-based resistance in these cultivars. The presence of trichomes and waxy surfaces also helps in repelling the pest attack. On the other hand, the leaf surfaces of highly preferred (Anaj-2021 & Subhani-2021) were not as hard; imparting that they had lower cell wall densities that allowed aphids to access and suck the phloem sap more easily, making them more susceptible cultivars and allowing adult *S. avenae* to meet their nutritional requirements and reproduce at higher rates.

In our experiment, the differential proteome analysis of *S. avenae* pre-, and post-wheat leaf feeding (Figures 4 & 5) led to the identification of several differentially expressed proteins that were predicted to perform important functions crucial to the survival, host selection, and feeding behavior of *S. avenae.* The DEPs that were identified were sheath protein, glucose oxidoreductase, chemosensory proteins are reduced. While ecdysone induced proteins, odorant binding protein-3, carbonic anhydrase, HSP, salivary effector proteins, cathepsin-B, SID-1 like proteins, sodium channel proteins, trypsin, and Glutathione S transferase were enhanced. Enhanced proteins can be the Systemic RNAi targets; known to be part of machinery that facilitate the uptake and movement of small RNA molecules. The expression of this protein indicates the presence of RNAi, a natural cellular process (Huvenne & Smagghe 2010; Xu & Han 2008; Zhang et al. 2013). The study of this protein can provide insightful knowledge regarding the uptake and systemic movement of short RNA molecules.

RNAi had been applied in research to suppress key genes involved in plant-insect interactions and defense system suppression. Notable applications include dsRNA against Snf 7 to control western corn rootworms in transgenic maize. In wheat, targeting Laccase gene found in the aphid's salivary glands; regulate the glycosyl phosphatidyl inositol-anchor biosynthesis pathway along with lipid biosynthesis reduced wheat aphids (Zhang et al. 2018). RNAi has also been employed in aphid control, silencing genes such as catalase, cytochrome c oxidase subunit VIIc, zinc finger protein, serine protease 1 DSR48, and acetylcholinesterase gene SaAce1. These strategies successfully reduce pest populations (Yu et al. 2016).

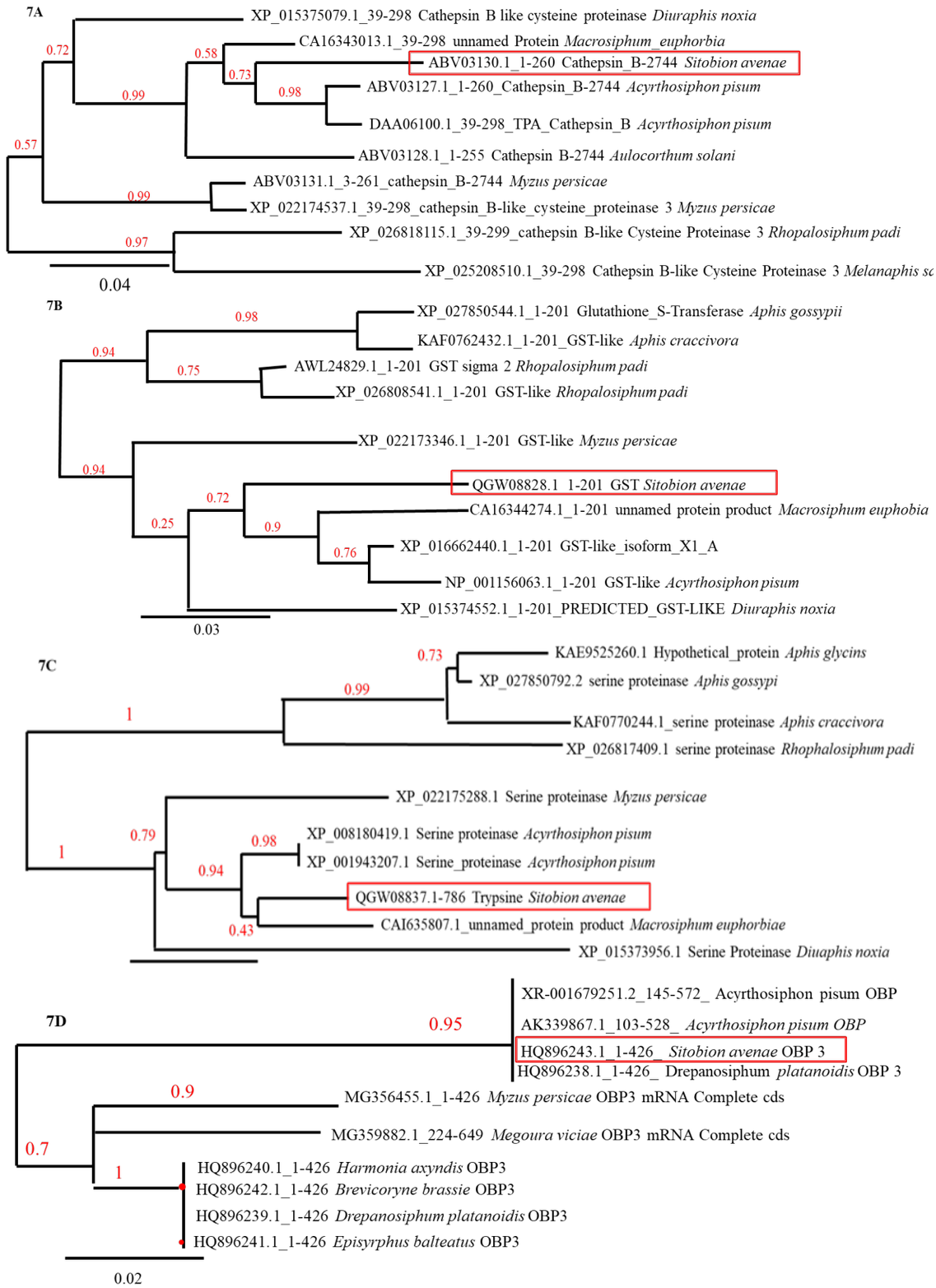

FIGURE 7. Phylogenetic tree of potential RNAi targets in *Sitobion avenae.* 7A: Glutathione S-Transferase (GST); 7B: Cathepsin-B; 7C: Trypsin; 7D: Odorant Binding Protein 3 (OBP3)

TABLE 1. Number of *S. avenae* adult settled and fed on *T. aestivum* local cultivars in aphid performance assay and aphid choice assay & their categorization

S. No	Varieties	Aphid preference test after time in hours				Aphid choice assay after time in hours				Cat
		24	48	72	96	24	48	7	96	
1.	Anaj	4±0.54	6±0.27	8.3±0.47	10 ± 0.27	13.25± 0.72	16 ± 0.47	18.6± 0.98	19.67± 0.98	HP
2.	Fakhar-e-Bhakkar	3± 0.98	4.3±0.81	6.7±0.81	7.4± 0.81	10.75± 0.47	14 ± 0.47	16.3± 1.2	18 ± 0.81	HP
3.	Subhani	4± 0	5.6±0.81	7.1±0.27	9.3± 0.27	11.75± 0.72	12.3± 1.4	14.3 ± 1.6	14.3± 2.12	HP
4.	Akbar	3.5±0.27	4.6±0.98	6±0.81	7.3± 0.27	9.25± 1.18	9.33± 2.4	10 ± 1.2	10.6± 0.98	MP
5.	Mexi-Pak	2± 0.27	4.3±0.27	5± 0.47	6.7± 0.72	8.25± 0.47	10.3± 0.98	12.67± 1.2	13 ± 1.24	MP
6.	Dilkash	1.5±0.27	2.3±0.54	4 ± 0.47	6± 0.54	5.75± 0.81	8 ± 0.47	9± 0.8	9.6 ± 1.18	MP
7.	Barani	1± 0.27	1.7±0.47	3.6 ± 0	4.6± 0.27	3.5± 0.54	4 ± 0.47	5 ± 0.5	6.3 ± 0.72	LP

Similarly, proteins having proteolytic activity such as cathepsin- b and trypsin were also identified. The upregulation of these important proteins in wheat feeding implies that *S. avenae* requires these proteins to extract vital nutrients from phloem sap and manipulate plants to their advantage (Pyati et al. 2011). It can therefore be hypothesized that silencing their expression using a precise approach like RNAi would have negative effects on the survival and reproduction rates of *S. avenae* which will lead to better management of aphid population control and protect the overall grain yield. Cathepsin-B protein enhanced post-wheat feeding is a proteolytic protein that aids in digestion, secretion of saliva, and immune defense. The identified differentially expressed proteins mostly belonged to detoxification and metabolism (Figure 4).

Carbonic anhydrase (50 kDa) band was enhanced in feeding; crucial enzyme that facilitates insects to feed, regulate the pH, cope with environmental stress, and regulate pH (Giordanengo et al. 2010; Guo et al. 2023). Glutathione s-transferase was also differentially expressed in wheat-fed *S. avenae* protein sample; which plays a key role in the detoxification of important secondary metabolites by conjugating toxic compounds with glutathione. They have also been reported to confer resistance to certain insecticides and provide protection against oxidative stress (Zhang et al. 2022). Odorant binding protein proteins was another protein in the wheat-fed *S. avenae* protein sample. It had been reported to play a crucial role in host selection by insects. Similarly, chemosensory proteins also possess the ability to perceive and respond to a wide range of chemical cues in the environment. These chemical cues are crucial in terms of the reproductive behavior, defense response, and feeding behavior of *S. avenae* (Jacquin-Joly et al. 2001; Liu et al. 2014; Xue et al. 2016).

Phylogenetic analysis of predicted RNAi targets proteins involved in detoxification and digestion processes in *S. avenae* like glutathione S-transferases and trypsin, cathepsin B, and odorant-binding protein 3 upregulated on wheat feeding showed sequence homology with *D. noxia*, *A. pisum, M. persicae*, *A. glycines*, and *R. padi* (Figure 7). Proteins are common RNAi targets for all aphids.

CONCLUSION

The present study aimed to predict potential RNAi targets using comparative proteome analysis of *S. avenae*. It was speculated that 11 proteins named: Glutathione S-transferases, Cathepsin-B 2744, Carbonic anhydrases, Ecdysone induced protein, odorant binding protein 3, Heat

shock, Salivary effector, SID1-like, Sodium channel, and chemosensory protein, and trypsin were upregulated within *S. avenae* have important role in detoxification, digestive and insect probing activities. The knockdown of the predicted genes (Glutathione S-transferases, Trypsin, odorant binding protein, and Cathepsin-B) will result in reduced fecundity rates and increased mortality rates within *S. avenae* which will eventually lead to crop yield protection.

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SUPPLEMENTARY TABLE 1. *S. avenae* colony weight on wheat local cultivars along with weights of cultivar 3 weeks post-rearing

Cultivars	Control weight (g)	Infested weight (g)	Weight of Aphids infested (mL)
Anaj-2021	0.21 ± 0.007	0.16 ± 0.014	42 ± 1.44
Fakhar-e-Bhakkar-2021	0.25 ± 0.01	0.16 ± 0.014	32.67 ± 1.41
Subhani-2021	0.32 ± 0.01	0.14 ± 0.01	41.67 ± 1.69
Akbar-2019	0.26 ± 0.007	0.22 ± 0.009	20.67 ± 0.98
Mexi-Pak-2022	0.17 ± 0.014	0.15 ± 0.014	24.33 ± 1.18
Dilkash-2022	0.27 ± 0.011	0.26 ± 0.015	18.33 ± 0.98
Barani-2022	0.35 ± 0.007	0.33 ± 0.007	24 ± 1.19