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The Efficacy of Chitosan As A Natural Component to Combat Aphids and Enhance The Physiologic Response of Barley Productivity

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ABSTRACT

Due to the high environmental risks of chemical pesticides, biological control of plant pests with bio-pesticides is highly encouraged and recommended. Chitosan and oligochitosan are well-known biological control agents for their nontoxic, biodegradable, and biocompatible properties. A field experiment was conducted at Nubaria Research Station during the 2020/2021 and 2021/2022 growing seasons to study the effect of different applications of chitosan on barley plants against aphid infestation under field artificial infestation conditions as well as yield and physiologic related traits. The experiment was laid out in a randomized complete block design (RCBD) with three replications and seven treatments *among spraying, soaking, and combinations between them at two concentrations 250 and 500 mg.* The study results revealed that there were significant variations among the seven treatments for all studied traits. The highest mortality percent was obtained from the treatment T7 (soaking seeds & spraying with 500 mg/L.), which was 99.5 % compared with untreated 2.66%; this treatment induced an increase in the thickness of grains and stem tissues, which caused a reduction in the feeding of aphids, resulting in a clear drop in population number. Significant increases were recorded for total phenolic content, protein content, yield and its components in T7 and T5 treatments.

INTRODUCTION

One of the most important food and feed crops worldwide is barley (Hordeum vulgare L.). According to historical accounts, barley was utilized in ancient Egypt for a variety of medical and therapeutic purposes in addition to being used as a food to create bread and brew. Due to the recently revealed nutritional worth of its grain, employing barley as a staple diet has garnered increased interest in recent years. It was characterized by its ability to resist adverse environmental conditions, i.e., low rain-fed stresses, poor fertility soil status, salinity, etc. On the other hand, barley is subjected to several insect species; aphids, as sap-feeding insects, are a common pest on it. Also, aphids are among the most destructive pests in agriculture, particularly in temperate regions. The damage caused by aphid colonies is mainly aesthetic due to sucking liquid pith, adding to the large amounts of sticky honeydew produced and the resulting black sooty mold that grows on the infested plant portions which bring hazards to normal photosynthesis (Blackman and Eastop 2000). It is able also to transmit several dangerous viral diseases which weaken plant growth and result in yield losses (Davoodi Dehkordi *et al*.2013). Aphids secrete virulence protein "effectors" in their saliva that modulate plant responses which negatively affect the immunity system (Jaouannet *et al.* 2014). Chitosan was effective in controlling lepidopterous and homopterous insects and also considers a new line that has been developed and improved to be a valuable tool in integrated pest management (IPM) programs and as a safe means for human health and the environment for its properties: safe natural cationic biodegradable, biocompatibility, high permeability, and high effectiveness (Archita Sharma 2019). As (Badawy and El-Aswad 2012) pointed out to continue developing insect chitinase as a bioagent for insects and plant pathogens, by introducing its gene into crops plants such as rice, wheat, corn, and sorghum because Insect chitinases appear to possess properties that make them uniquely useful for insect control, (Hanan Alfy *et al.* 2020) revealed the effect of chitosan nanoparticles to the most important enzymes in insect; the activity of the chitinase enzyme was colossal hence the activity of this enzyme was duplicated 6.4 times than control in *S. litoralis* and 5.8 times than control in *L. migratoria.*, the activity of the protease enzyme was also duplicated to 1.9 times than control in *S. litoralis* and 2.1 times than control in *L. migratoria.*, and alkaline phosphatase has recorded a decrease in activity. It has been demonstrated that chitosan improves plant defense responses to biotic and abiotic stresses (Malarla and Cerrana 2016) and induces the expression of genes involved in a variety of physiological processes, such as systemic acquired resistance, the plant immune system, photosynthesis, and hormone metabolism. Chitosan also influences the re-programming of protein metaplasm with an increase in storage proteins (Landi *et al.* 2017 and Xoca-Orozca *et al.* 2017). Moreover, in many plant species treated with chitosan, it can result in the stimulation of plant defense enzymes and the manufacture of secondary metabolites, such as polyphenolics. The fact that insect herbivores significantly reduce plant production is beyond dispute. Plants have developed a range of direct and indirect, constitutive, and induced, chemical, and physical defenses to fend off this constant barrage, and second metabolites are a crucial category that supports these responses. Most of these biologically active secondary metabolites are polyphenols, which are abundant in flowering plants (Singh *et al.* 2021). It has been well documented that different groups of polyphenols protect most plant species against a wide range of attackers and can not only protect plants against a broad spectrum of insect herbivores but also be specific and highly regulated.

Chitosan has been shown to trigger several ways to act as a high defender of the plant. For example, it has a powerful activity of characteristic enzymes like chitinase and β-1,3-glucanases Amborabé *et al.* 2004). (Notsu *et al.* 1994) reported that chitosan caused increased lignification response and lipoxygenase activity in wheat as shown by (Mitchell *et al.* 1999; Mohanta *et al.* 2001) and in cotyledons of the herbal plant *Mimosa pudica* leading to an accumulation of many phenolic compounds (Rossard *et al*. 2006).

Considering the facts stated above, the present study was undertaken to investigate the effect of different applications of chitosan against English Grain Aphid *Sitobion avenae* as an example of a sucking pest on barely under field conditions (with artificial infestation), as well as to investigate its physiological response and productivity.

MATERIALS AND METHODS

1. Chitosan Preparation:

Chitosan was obtained from Sigma Chemical Co. (USA). The degree of deacetylation was about 85% as determined by elemental analysis, and the average molecular weight of the chitosan was 220 kDa. The concentration of the stock chitosan solution was 9000 mg/L in a 5% acetic acid solvent (w/v) and diluted to obtain the concentrations (250 and 500 mg/L), followed by 15‑20 min of constant stirring with the magnetic stirrer (Jaouannet *et al.* 2014).

***2.* field application:**

A field study was carried out during the 2020/2021 and 2021/2022 growing seasons at Nubaria Agriculture Research Station, in North Tahrir, Egypt. The geographical location of the experimental site is (Latitude 30-22N, Longitude 30-21E). The barley genotype used in this study was Giza 137, a six-rowed spring barley (Hordeum vulgare, L) developed by the Barley Research Department, ARC. The sowing dates of the experiments were November in both seasons. The layout of this study was a randomized complete block design (RCBD) with three replicates. Each plot consisted of seven rows, 20 cm apart and 3.5m long, cultural practices were carried out according to the methods being adopted for growing barley in the locality. No pesticides were used during the two seasons. Seven chitosan treatments were applied as below, in addition to different combinations between them:

T1. Spray with water (control).
T2. Soaking seeds in 250 mg/L chitosan solution.
T3. Soaking seeds in 500 mg/L chitosan solution.
T4. Foliar spraying of chitosan, 250 mg/L.
T5. Foliar spraying of chitosan, 500 mg/L.
T6. Combination of T2 and T4.
T7. Combination of T3 and T5.

A foliar application of chitosan was sprayed two times, the first after 30 days from sowing (tillering stage) and the second after 50 days from sowing (before the heading stage). The volume of water was one liter/plot, and a 0.5% wetting agent of Tween 20 was used. The soaking application was incorporated with seeds before sowing.

The recorded data included: the number of spikes per m^2; recorded as the number of tillers bearing fertile spikes (two randomly taken rows in each plot) and the average number of spikes per square meter. Grain yield was determined as the weight of grains harvested from the middle rows and converted to $ard.fad^{-1}$, thousand kernel weights were calculated as the weight of 1000 grains.

3. Physiological and Chemical Studies:

The total phenolic content of leaves (TPC) at 75 DAS was determined using the Folin Ciocatteau (FC) method described by (Shirazi *et al*. 2014). TPC expressed as mg gallic acid equivalents (GAE)\ g sample.

Grain samples from the two growing seasons were subjected to chemical analysis to determine crude protein in grains according to (A.O.A.C. 1990).

4. Artificial Inoculation and Insecticidal Activity of Aphis:

Laboratory-reared of English Grain Aphid *Sitobion avenae* (Fab.) (Homoptera; Aphididae) were used to infest barely plants in the field, they reared in the environmental chambers (photoperiod of 16:8 [light: dark] h at $22c^0$. 20 adult aphids were placed with an artist's brush onto the top of each test plant (after ten days from the first foliar application) which were selected randomly from a zigzag way in each plot by clipping them. Three weeks after infesting, the intensity of infestation was recorded by collecting colonies by shacking plants after putting the strong white paper under infested plants and then putting them in a paper bag for counting in the laboratory and calculating the mortality percent by Adjusted mortality rate P was calculated based on the following formula (Chen 1991):

$$P = \frac{P_1 - P_0}{1 - P_0} \times 100\%$$

P1 is the mortality rate of chitosan solution treatment, P0 is the mortality rate of control.

5. electron Microscope:

Scanning Electron Microscopy (SEM) " Jump up":

Small pieces of fresh specimens of bare plant (stem and grains) and aphids were removed and fixed by immersing them immediately in 4F1G (Fixative, phosphate buffer solution) PH=7.4 at 4 ° C for 3 hours. Specimens were then postfixed in 2% OSo_4 in the same buffer at 4° C for 2 hours. Samples were washed in the buffer and dehydrated at 4C through a graded series of ethanol.

The Samples were dried by means of a critical point method, mounted using carbon paste on an AL- stub and coated with gold up to a thickness of 400 A in a sputter–coating unit (JFC-1100 E).

Observations of plant and insect morphology in the coded specimens were performed in a Jeol JSM- 5300 scanning electron microscope operated between 15 and 20 KeV (Tahmasbi *et al.* 2015).

6.Data Analysis:

The comparison of the mean error squares between the two seasons for all traits was done with the help of Bartlett’s test of homogeneity of variances (Snedecor and cochran 1989) and found not significantly different. Data from the two seasons were combined and statistically analyzed according to (Steel and Torrie 1980). The discussion of the obtained results was carried out based on the combined analysis values.

RESULTS AND DISCUSSION

1. Effect of Different Chitosan Application Levels on The Population Of Aphids:

Aphids that were motionless and exhibited strong chitosan treatment responses were recorded as dead and given a mortality percentage. Scanning micrograph revealed that there was an increase in the thickness of grains and stems by 1.3 and 1.2 times, respectively more than the control as shown in Figures 1, 2. These increases and hardness have led to the prevention of feeding nymphs and adults of aphids; hence they have become longer than the length of the stylet (the main part of mouthparts looks like a needle). Data revealed that treatment T7 affected the number of colonies on the plant being the highest significance which was 30 nymphs after three weeks from artificial occultation and the mortality percent was 99.5 % as shown in Table 1, the flowed treatment was T6 hence it recorded 50 and 97.3 for the number of insects and mortality percent respectively. Treatment by T6 and T5 recorded non-significant in the number of insects because the total amount of chitosan which reached the plant is the same, but these two treatments revealed significant in the mortality percent which means the mortality was happing due to more sickness of plants that occurred due to the method of applying (soaking), the same was for treatments T4 and T3. The lowest treatment was T2 which recorded 76 for the number of insects/plants and 21% for mortality percent compared with untreated control where the number of insects/plants was 112 nymphs, and the mortality percent was 2.7% as shown in Table1.

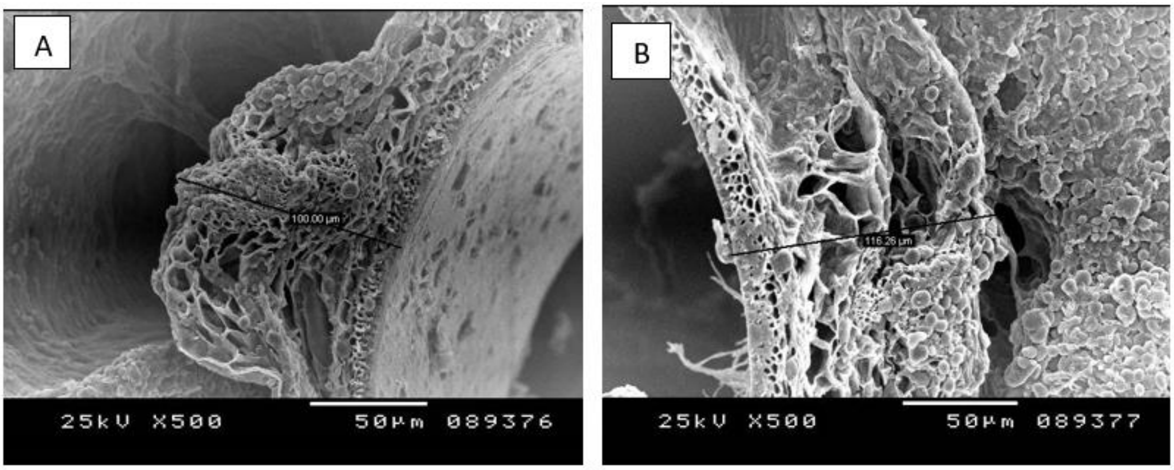

Fig.1: Scanning microscope for the sectors from the two grains; A for control and B for T7.

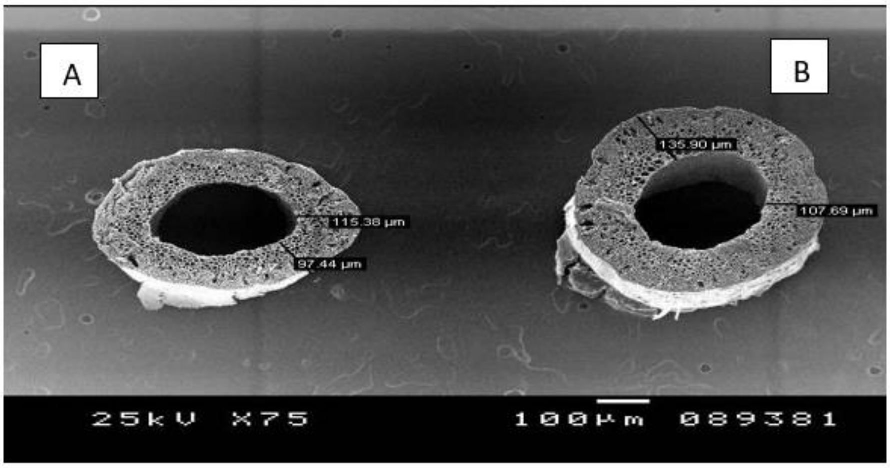

Fig. 2: Scanning microscope for the sectors from the two stems at the same tall; A for control and B for T7.

Table 1. insecticidal activity of chitosan on *Sitobion avenae*

Treatments	N. insect	M%
T1	111.833 a	2.667 g
T2	75.500 b	21.00 f
T3	66.00 c	81.33 d
T4	65.833 c	30.00 e
T5	48.33 d	91.333 c
T6	50.00 d	97.333 b
T7	30.000 e	99.500 a
L.S. D	**2.697**	**1.512**

From the field results obtained, all chitosan applications had a significant effect on antifeeding against aphids. The best application (T7) was chosen through the previous results to evaluate the effect of the treatments on the anatomical traits of infected barley grains and stems. The effect of chitosan on the antifeeding activity of aphids is well documented by a scanning micrograph which confirmed the field observations.

In Figure 3, giving the dead adult appeared with broken stylet and dead nymphs hence aphids possess mouthparts composed of stylets that navigate to the plant vascular system, predominantly the phloem, for long-term feeding. (Piotr *et al.* 2014) Found resemble results when treated with bulk chitosan with low molecular weight which gave 50% of mortality after 7 days from treatment. Lignin substance has been an important factor in biosynthesis and insect control as reported by (Hanan alfy *et al.* 2017). There was an interrelationship among flavonoids, lignin and polyphenols which contribute to insect resistance in the stem of soybean (Chiang and Norris 1984). Increasing lignin contents leads to an increase in the secondary xylem and decreases the pith diameter leading to hampering the growth of larva in the pith (Hanan Alfy 2016). Additionally, the impact of chitosan treatment was higher than salicylic acid treatment in increasing the thickness of the blade (µ), the thickness of the lower and upper epidermis, the thickness of mesophyll tissue, bundle length, and width in the midrib. Chitosan is a biopolymer, is considered a potent inducer of phytoalexin synthesis and aggregation in different host cells, and stimulates callose, lignification formation, and production protease inhibitor (Singh *et al.* 2019). The aphids died due to two reasons one of them is the insecticidal activity of chitosan, and the other is the thickness of phloem which means that oral uptake is essential for aphid control.

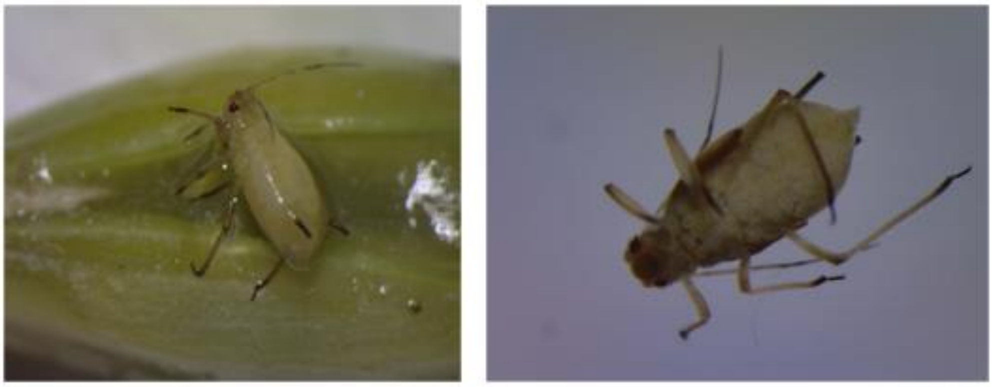

Fig.3. Died adults' aphids and nymphs with broken stylet.

Additionally, as shown by (Bagniewska-Zadworna *et al.* 2014), lignans are synthesized via the phenylpropanoid pathway; as a result, these components have a detrimental effect on insects as evidenced by changes in the activity of various insect enzymes, particularly chitinase, which is the most crucial enzyme in the development of insects(Hanan alfy *et al.* 2020). (Zeng *et al.* 2012) indicated that chitosan coating protected soybean plants against pests efficiently. Similarly, (Faoro *et al.* 2008) demonstrated that using chitosan as a foliar spray on barley reduced both local and systemic infection by the aphid-transmitted powdery mildew pathogen *Blumeria graminis* f. sp. Hordei.

2. The Analysis of Variance of Yield and Its Components Traits:

From a grain production perspective, yield and its indicators are the most important variables. Chitosan has no chance of being widely used in cereals, regardless of its other beneficial attributes, if it does not increase yield.

The influence of chitosan application on yield attributes and grain yield was significant Table 2. Results showed that yield and its component were greater in chitosan-applied plants than in control plants. These results indicate that the application of chitosan increased spike production as well as the increased number of kernels spike^{-1} and 1000 kernel weight which resulted in increased yield attributes and thereby grain yield.

Concerning the effect of chitosan applications on the number of spikes m^{-2}, among the different chitosan treatments, the maximum number of spikes was reported with a combination between soaking seeds and foliar spraying of 500 mg chitosan application (T7) with an average of 319 due to production of a higher number of spike plant^{-1}. In contrast, the lowest number of spike m^{-2} was observed in treatments T4 (Spray with 250 ppm) and T1 (control) with averages of (313.500 and 314.500, respectively.).

Table 2. Yield and yield component of barley plants as affected by the seven chitosan applications**.**

Treatments	Number of tillers m^{-2}	Number of spike m^{-2}	Number of kernels spike^{-1}	1000 kernel weight (g)	Grain yield (Ard. fad^{-1})
T1	316.667 c	314.500 cd	41.167 d	43.172 b	10.293 dc
T2	318.500 abc	314.833 bcd	41.667 dc	43.472 ab	10.417 bcd
T3	319.833 ab	316.833 abc	43.333 ab	44.472 ab	10.790 abc
T4	317.00 bc	313.500 d	41.667 dc	43.192 b	9.975 d
T5	320.167 a	317.667 ab	43.833 ab	45.033 a	11.163 a
T6	319.167 abc	316.50 abcd	43.000 bc	44.388 ab	10.768 abc
T7	321.50 a	319.00 a	44.667 a	44.980 a	11.120 ab
L.S. D	**3.129**	**3.077**	**1.478**	**1.645**	**0.728**

As for the effect of chitosan application on the number tiller number m^{-2}, the results showed that the highest number encountered with T7 treatment followed by T5 treatment with averages of 321.50 and 320.167, respectively. The same trend was observed in the number of kernels spike^{-1}, the highest kernel number obtained from plots was 44.667 for T7 treatment followed by the foliar application of 500 mg chitosan which was 43.833, with an insignificant difference between them. The lowest values were given by the control plots. These outcomes agree with the other studies which showed that the chitosan use significantly improved the number of grains per spike and grain yield as compared to the control (Behboudi *et al.* 2018). Moreover, the maximum 1000-grain weight (g) was recorded when the plant was subjected to T5 and T7 treatments with averages of 45.033 and 44.980 g, respectively, while the lowest 1000-grain weight was seen in the control plants with an average of 43.172g. In this connection, (Zeng and Tu 2012) observed the maximum 1000-grain weight with the foliar treatment of chitosan in the well-watered plant, they also noticed that the chitosan application in soil considerably improved the 1000-grain weight in plants under water stress compared to that of control.

As for the effect of chitosan treatments on grain yield, there were also highly significant differences among the treatments with and without chitosan. The highest values for grain yield were obtained from T5 treatment and T7 with averages of 11.163 and 11.120 ard.fad^{-1}, respectively. While the lowest yield was obtained from the T4 and control with averages of 9.975 and 10.293 ard.fad^{-1}, respectively, without a significant difference between them. (Rahman *et al.* 2018) observed in their study that foliar treatments with chitosan significantly increased plant growth and fruit yield (up to 42% more) when compared to the untreated control in the strawberry plants. (Dzung *et al.* 2017) observed that the enhanced growth and fruit yield following the foliar application of oligochitosan in Capsicum frutescens L. Because of the application of chitosan, plants grew more quickly, namely in terms of fruit weight and total fruit weight per plant, which improved fruit output. This agrees with subsequent research, where an increase in grain weight as well as grain yield, was obtained. In the case of wheat, the application of chitosan resulted in a 13.6% increase in yield per hectare in comparison with a control crop. Furthermore, it had a positive effect on tillers per plant, spikes per plant, and 1000 grains weight (Zeng and Tu 2012). Also, it was demonstrated that exogenous chitosan use on this plant enhances other yield characteristics such as the number of grains/spikes, grain index, and grain yield. Their improvement was also observed during moderate and severe drought circumstances (Farouk

S. *et al*. 2019). Similar encouraging outcomes were attained in a semi-arid trial on barley (Hordeum vulgare L.). When compared to plants lacking chitosan, there was a considerable increase in the number of spikes per plant, the number of grains per spike, and the weight of a single grain (Al-Tawaha A. *et al*. 2013). Chitosan oligosaccharides have also been studied in relation to their application on wheat. It was discovered that the plant stages of tillering and returning green were the most susceptible to this application. Also, it was demonstrated that chitosan oligosaccharide treatment has the potential to raise yield indicators such as spike number and grains per spike (Wang *et al.* 2015). Also, it has been demonstrated that additional multifunctional biologics that contain chitosan improve grain yield and quality (Kolesnikov *et al.* 2019). Also, two wheat types were subjected to salt stress during the research. It was discovered that plants treated with chitosan produced improved outcomes in terms of grain yield, straw yield, biological yield, and 1000 grain weight (Abdallah *et al.* 2020). After soaking the rice seeds before to planting and applying foliar polymeric chitosan four times at a concentration of 20 ppm, the potential for enhanced yields was discovered (Boonlertnirun *et al.*, 2006). Studies done on rice plants in Vietnam also indicated that chitosan led to potential production improvements (Boonlertnirun *et al.* 2008).

3. Physiological and Chemical Studies:

The total phenolic content of leaves (TPC) is an indicator of the plant response under biotic stress conditions. Biochemical analysis of barley leaves for TPC indicated that there are significant differences among treatments Table 3. Data showed that chitosan application treatment T7 gave the highest TPC which was 29.025 compared to the control which was 17.320 mg GAE/g DW. Recent transcriptomic analysis revealed that chitosan induces the expression of genes involved in multiple physiological processes including systemic acquired resistance, plant immune system, photosynthesis and hormone metabolism, (Rahman *et al.* 2018) additionally they found that total phenolic content was enhanced by the application of different rates of chitosan compared to untreated control. Chitosan enhanced defense responses to abiotic stress and it is thought that can lead to the induction of plant defense enzymes and to the synthesis of secondary metabolites, such as polyphenolics, lignin, and flavonoids (Iriti and Varoni 2015 and Malerba; Cerana 2016).

Considering phenolic compounds, polyphenols typically have effects on most insects that are both anti-feeding and anti-deterrent. Via antibiosis, antixenosis, or antisymbiosis mechanisms, it inhibits the growth of herbivores. (Eigenbrode and Trumble 1993). In addition, polyphenols can poison flies and other herbivores by oxidizing the midgut tissue. (Bi and Felton 1995). Insect herbivore fitness is decreased as a result of the binding of polyphenols with the digestive enzymes of insects, as evidenced by the delay in development and molting (Rehman *et al.* 2012, Singh *et al*. 2021).

Table 3. Total phenolic content of leaves and grain protein content of barley plants as affected by different chitosan applications.

Treatments	Total phenols (mg GAE/g Dw)	Protein content (%)
T1	17.320 g	9.813 c
T2	21.230 f	9.835 c
T3	24.540 d	10.292 b
T4	22.685 e	9.855 c
T5	27.450 b	10.697 a
T6	27.070 c	10.348 ab
T7	29.025 a	10.330 ab
L.S. D	**0.3712**	**0.393**

Lignin is deposited in plants through polyphenol oxidase's polymerization of cinnamyl alcohol into lignin, which also provides the plant with resilience. Furthermore, lignans are produced by the phenylpropanoid pathway; these elements are toxic to insects. Lignin and lignan, which make up the majority of plant secondary cell walls in this case, are crucial for plant defense against biotic and abiotic stressors (Schultz *et al.* 1992 and Bagniewska-Zadworna *et al.* 2014). Similar results were seen when wheat seeds were treated with chitosan, showing a rise in hydroxycinnamic (i.e., p-coumaric, caffeic, and ferulic) and benzoic (i.e., benzoic, protocatechuic, and gallic) acid derivatives, resulting in an increase in lignin production and accumulation (Reddy *et al.* 1999).

Results shown in Table 1 elucidated that the average values of protein content were significantly affected by chitosan application. The highest content was detected from T5 followed by T6 and T7 with insignificant differences compared with control. Such findings indicate that the application of chitosan had a tremendous effect on protein content (Landi *et al.* 2017). (Xoca-Orozco *et al.* 2017) noted that chitosan affected heat-shoed protein expression and reprogrammed protein metabolism by increasing storage proteins. In addition, (Khan *et al*. 2002, Chibu 2001, and Górnik *et al.* 2008) stated that the use of chitosan promoted the transportation of nitrogen (N) in the functioning leaves, which boosted plant growth and development, and increased important enzyme activities of nitrogen metabolism (nitrate reductase, glutamine synthetase, and protease). The use of chitosan and its derivatives as possible elicitors to increase plant resistance is widely known (Li *et al.* 2020a). Chitosan can also increase the amount of free amino acids and soluble sugars, as well as the activity of proteases that convert sugar to protein (Huiping and Langlai 2005).

4.Correlation Analysis:

As regard the correlation coefficients under different chitosan applications, there is a strong negative significance between the number of aphids on plants and their percent mortality of them, as well as a strong positive significance between them and the phenol contents, as shown in Figure 4. It affects herbivore growth by antibiosis, antixenosis or antisymbiosis modes of action (Eigenbrode and Trumble 1993). A highly significant positive correlation was shown between physiological traits and yield traits. Regarding the physiological characteristics, a significant positive correlation was revealed between the pair of protein content and phenol content (0.56). Also, the correlation of physiological traits with other measured characters was observed to be significant. For yield traits, highly positive and significant correlations were shown either among yield-related traits or for yield-related traits with other physiological characteristics. While insect number plant-1 correlated significantly but negatively with all measured characters.

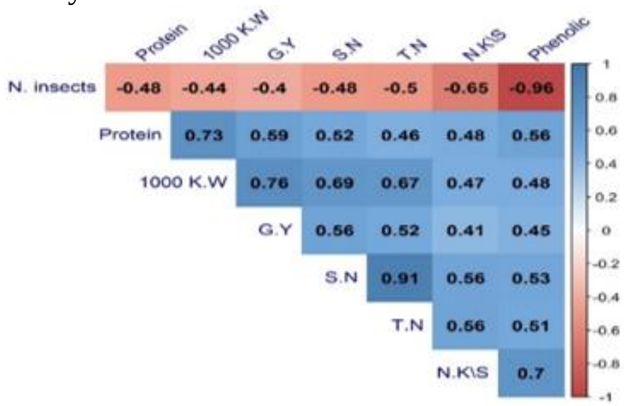

Fig. 4. Correlation matrix of the 7 measured traits of barley plant (Hordeum vulgar) evaluated as affected by different application of chitosan. The increasing color intensities illustrate a higher correlation coefficient.

Conclusion

This study revealed excellent management by applying chitosan as an eco-friendly component before planting by soaking seeds and through the vegetative stage by foliar application as a combination treatment at 500mg/L., especially in the long run for its good efficacy on the second generation of pests, even by suppressing the number of aphids fauna or by increasing the quality and quantity of plant. Hence, applying chitosan in this way resulted in the highest grain yield and its component, furthermore, the chitosan levels increased the protein and phenol content.

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