Increased cell wall activity on Tugela *Dn1* wheat cultivar infested with Russian wheat aphid biotype 2

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Abstract

Wheat plays a major role in food production and security. Infestation of wheat by Russian wheat aphid affects food security as it causes chlorosis on cereal crops. Since plants are sessile, they have developed a range of defense mechanisms against attackers. When aphids feed on the plant nutrients through the stylet they release salivary secretions containing cell-wall modification enzymes that degrade the cell wall. There are enzymes that act on the plant cell wall. These enzymes target the cellulose component of the cell wall. When studying the enzyme activity, increased activity was found on plants that were infested by aphids as compared to the control plants. During cell wall remodeling the esterase activity also increased due to the fact that the ester bond between phenolic acids and hemicellulose is cleaved.

Keywords: Wheat, *Diuraphis noxia,* cell wall, plant-pest interaction, plant defense

INTRODUCTION

Wheat (*Triticum aestivum* L.) are cereal crops of important value that are harvested all over the world. These crops play an important role in food security. Wheat production accounts for more than 20% of the world's food production [1]. In addition of wheat being a source of starch and protein, it also offers many health benefits to humans. Wheat can be used to produce many food products such as bread, noodles, pasta and other ingredients [2]. Infection of wheat by pathogens and infestation by pests (RWA) affects food supply [3]

Russian wheat aphids (*Diuraphis noxia*) are pests that cause chlorosis on cereal crops. These pests have a great impact on mainly wheat and barley crops. Russian wheat aphid (RWA) was first reported as a cereal pest by Mokrzhetski [2]. The first occurrence of Russian wheat aphid in South Africa was recorded in 1978 that was virulent against the *Dn3* resistance gene where it resulted in yield loss of susceptible wheat cultivars [3]. There are currently five Russian wheat aphid biotypes in South Africa (RWASA1-RWASA5) [4]. In South Africa, resistance of *Triticum aestivum* L. to RWA was first observed on Tugela *Dn* wheat cultivar in 1993 [5]. Infestations of wheat carrying the *Dn4* resistance gene were reported in Southern Colorado [6]. Other reports were also made in the Free State on the virulence of RWA on wheat cultivars carrying the *Dn1* resistance gene. This led to the discovery of a second RWA biotype named the Russian wheat aphid biotype 2 (RWASA2) in South Africa [7]. RWASA2 was also virulent to *Dn2*, *3* and *4* resistance genes [6]. This second RWA biotype was distinguished from RWASA1 due to the fact that it was virulent against *Dn1* resistance gene which rendered the plant resistant against RWASA1.

These pests draw up nutrients from the phloem of the host plants and transmit diseases. Aphids have a slender stylet which enables them to feed on the plant nutrients by penetrating the host and probing the tissues through the cell wall [8]. During this feeding, salivary secretions from the aphids are injected into the host plant. These salivary secretions enable the aphids to feed and also infest the plant because as the aphids grow, they multiply [9]. A continuous salivary sheath is formed around the stylet as it probes through the apoplast. This salivary sheath gives the aphid mechanical stability [10].

Studies have shown that there are enzymes and proteins present in the salivary secretions which vary between aphid species and even biotypes, indicating that these enzymes play a role in Infestation and host colonization compatibility [10]. Different cell-wall modification enzymes and factors such as Pectin methylesterase (PME), polygalacturonase (PG) and cellulose-hydrolyzing factors have been identified in the salivary secretions [9].

Since plants are sessile organisms, they need to develop disease resistance mechanisms against pathogens and pests. Pattern-triggered immunity (PTI) and Effector-triggered immunity (ETI) are molecular monitoring mechanisms that that are enabled through the perception of Microbe/Pathogen-associated molecular patterns (MAMPs and PAMPs) or the Damage-associated molecular patterns (DAMPs) by the Pattern recognition receptors (PRR) in the plant [11].

The plant cell wall functions as the first barrier against pathogens [12]. The cell wall plays a role in providing mechanical strength, structure, shape, and rigidity to the cell. Cellulose, hemicellulose and pectin polysaccharides make up the structure of the cell wall. Callose and lignin are also present in the cell walls. Interaction of hemicellulose and cellulose gives the cell wall its rigidity function [13]. Cellulose is composed of β-D-(1-4)-glucan chains. Cellulose is the most abundant and strongest component of the cell wall [14]. Intermolecular hydrogen bonds bind the cellulose chains together to form cellulose microfibrils which wrap the cells together providing a rigid network that give the plant its shape. Cellulose also functions in basal defense against pests and pathogens. Cellobiose which are cellulose dimers act DAMPs that activate mitogen-activated protein kinase (MAPK) signal cascades [13] The functions of callose in the cell walls include formation of sieve plates in the phloem and prevention of penetration of the cell wall by pests and pathogens [13]. Other components such as homogalacturonans (HGs) which are pectins also have a defensive role against pests and pathogens [10]. These HGs can be modified by PMEs, which remove the methyl ester groups [10].

Hemicellulose on the other hand, ties up the cellulose microfibrils together in the apoplast through hydrogen bonds and van der Waals interactions regulating the strength and elasticity

and expansion of the cell walls [10]. The hemicelluloses are made up mainly of xyloglucans, glucans and xylans such as arabinoxylans, glucuronoxylans and glucuronoarabinoxylans [13]. In addition, Ferulic acid is esterified to the arabinosyl residue's C(O)-5-hydroxyl group in glucuronoarabinoxylans and arabinoxylans. Ferulic acid can also cross-link with other polysaccharides, lignin and proteins through ether bonds, giving the cell wall its rigidity [13]. Lastly, proteins from part of the cell wall structure [10]. α-expansins and a family-12-endoglucanase, cel12A play a role in cleaving cellulose and xyloglucans. Cel12A is a fungal enzyme with a cell wall-loosening function. Family-9 endoglucanases also have the wall-loosening action similar to that of cel12A proteins [12]. Enzymes such as β-1,4-and β-1,3-glucanases, pectinases, xylosidases, arabinosidases and glucosidases cleave the carbohydrate components of the cell wall [18].

Once the pests penetrate the cell wall polysaccharides are degraded. During this cell wall degradation, an increase in pectin methylesterases result in de-methylesterififation of homogluoronans and depolymerization by polygalacturonase activity [10]. This polygalacturonase activity increase the production of Oligogalacturonides (OGs) which are recognized by infested plants as DAMPs. These OGs induce defense responses [10].

The aim of this study was to look at the carbohydrate activity of the protein extract of Tugela *Dn1* cultivar of wheat infested with Russian wheat aphid biotype 2. To do so, the total protein concentration in the control and infested leaf tissues harvested at 48 and 72 hours post infestation were determined followed by enzyme assays using Bradford reagent. The crude carbohydrate esterase activity assays were also determined in the tissues

Materials and methods

Planting of the wheat plants

For this study, 30 pre-germinated Tugela Dn1 wheat cultivar were planted on trays in the greenhouse (1:1 soil and peat mass). The growth conditions were set to 18-24 °C in the greenhouse.

Wheat infestation by Russian wheat aphid biotype 2 aphids

Russian wheat aphids that were used for this study were multiplied on PAN 3379 wheat cultivar for a period of 2 weeks. The planted Tugela Dn1 wheat plants were infested with about 20 Russian wheat aphid biotype 2 (RWASA2) aphids per plant. The plants were monitored, and the leaves of the control and infested plants were randomly harvested at 48

and 72 hours post infestation (hpi). Once the leaves were harvested, they were immediately transferred into liquid nitrogen and then stored at -20°C.

Determination of the total protein concentration

A protein standard curve was prepared from Albumin stock solution [10 mg/ml] that was made by dissolving Albumin in phosphate buffer. The stock solution was then diluted to different concentrations as in Table 1 to make the standard curve. The standard curve was constructed in Microsoft excel with the concentration in mg/ml.

Table 1: Albumin (BSA) standard curve preparation

Albumin [mg/ml]	Amount of stocks (µl)	Amount of buffer (µl)
0.1	20	180
0.2	40	160
0.3	60	140
0.4	80	120
0.5	100	100
0.6	120	80
0.7	140	60
0.8	160	40
0.9	180	20
1	200	0

The concentration of proteins present in the crushed control and infested leaf tissues harvested at 48 and 72 hpi were determined using the Bradford Protein Assay Kit based on the method by Bradford, [15]. To do so, 25 µL of the samples were pipetted into microplate wells containing 200 µL of the Bradford reagent. This was followed by incubation of the microplate at room temperature for 10 minutes. The absorbance was measured at 595 nm with a plate reader. All the reactions were done in triplicates.

Protein profiling of control and infested plants

A 12% Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity of the enzymes that were present in the samples. This was a way of finding out how variable the proteins are within the treatments (48C, 48I, 72C and 72I).

Stacking and running gels were prepared as in the protocol [16]. The samples were mixed with the sample buffer (loading buffer) and loaded on the stacking gel. Separation was done at 118 V for 2 hours followed by staining of samples with Coomassie staining solution overnight. A de-staining solution was then used to de-stain the gel. Separation of proteins was based on their molecular weights. The results on the gel demonstrate the expression of proteins.

Protein binding studies

To determine the proteins that bind to the cellulose component of the cell wall. Avicel which is a microcrystalline cellulose model substrate was used for this study. The binding reaction occurred at 4 °C and centrifuged for 15 minutes at 5000 × g. The pellet was then washed with 2 mL distilled water followed by adding the SDS buffer. The sample was then boiled at 100 °C for 5 minutes. The appropriate supernatants for the different treatments were then used to run the gel as in the SDS-PAGE protocol [15]. If there were any carbohydrate binding module (CBM) in the samples, they were bound to the cell wall.

Enzyme activity assays

Preparation of standard curves

To prepare the glucose and xylose standard curves, 10 ml of the glucose/xylose stock solution [10 mg/ml] were made by dissolving 0,1 g glucose/xylose stock in sodium phosphate. The stock solution was then diluted to different concentrations as in Table 2 to make the standard curve. Each standard was 1 ml in the Eppendorf tubes. Each reaction was made up of 300 µL of the glucose/xylose standard and 600 µL of DNS reagent. The reaction was then incubated for 5 minutes at 100 °C then cooled on ice for 5 minutes. All the reactions were done in triplicates. The absorbances were read on a spectrophotometer at a wavelength of 540 nm. Microsoft excel was then used to construct the standard curves.

Table 2: Glucose and Xylose standard curve preparation.

Glucose or Xylose [mg/ml]	Amount of stocks (µl)	Amount of buffer (µl)
0.1	10	990
0.2	20	980
0.3	30	970

0.4	40	960
0.5	50	950
0.6	60	940
0.7	70	930
0.8	80	920
0.9	90	910
1	100	900
1.1	110	890

Cellulolytic and xylanolytic enzyme assays

Cell wall model substrates were used to conduct enzyme assays. Beechwood Xylan and wheat Arabinoxylan were the model substrates that represented the hemicellulose component of the cell wall. CMC and Avicel were used as the substrates representing the cellulose component of the cell wall. Enzyme activity was measured by measuring the concentration of reducing sugars in the samples. This was done using a modified dinitrosalicylic acid (DNS) protocol [17].

Assays were conducted by adding reagents except for the enzyme as in Table 3. All the reactions were done in triplicates. The samples were then incubated for 5 minutes. The assay was then initiated by adding the enzyme and incubating for 1 hour. The tubes were centrifuged at 13000 ×g for 5 minutes. About 350 µL of the supernatant was added to the appropriate tubes. This was followed by adding 500 µL of DNS reagent and incubation for 5 minutes at 100 °C. The samples were then cooled on ice for 5 minutes. Absorbances were read at a wavelength of 540 nm on a spectrophotometer. The glucose and xylose standard curves were then used to calculate the enzyme activity in mg/ml.

Crude carbohydrate esterase activity assays

Activity assays of the crude carbohydrate esterase (CE) were conducted by extracting the enzymes from the leaf tissues. Para-nitrophenyl acetate (pNP-A) and Para-nitrophenyl Butyrate (pNP-B) were used as substrates from the enzyme. About 0.2 mM substrates were added to 50 mM sodium buffer (pH 7). For the assays, 3 µL of pNP-A was added top 20 µL of the extract and 987 µL of the sodium phosphate buffer. The pNP-B reaction was prepared by adding 6 µL of pNP-B, 30 µL of the extract and 984 µL of the sodium phosphate buffer. To add, blanks were also prepared for each substrate. The reactions were initiated by adding the

enzyme followed by incubation at room temperature for 30 minutes. The spectrophotometer was used to measure absorbance at 405 nm.

RESULTS

Total protein content

A protein standard curve was constructed by plotting the Albumin concentration (mg/ml) on the x-axis against the absorbance values (595 nm) on the y-axis. The best line of fit is also plotted on the graph, including its formula and the R^2 value (Figure 1).

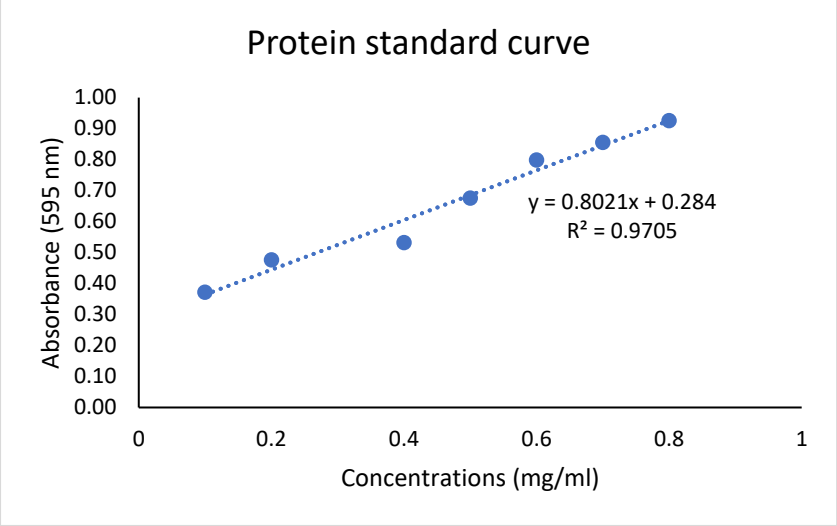

Figure 1: Protein standard curve. Albumin concentration (mg/mL) was plotted on the x-axis against absorbance (595 nm) on the y-axis. The concentrations of BSA range from 0,1 up to 1,0 mg/mL.

The BSA standard curve was used to calculate the total protein content in the leaf tissues. The results demonstrated that there was presence of proteins in all the leaf tissues representing the different treatments. The total protein concentrations for the control plants harvested 48 hpi was more or less equal to that of the infested plants at 48 hpi. The same applies to the control and infested plants harvested at 72 hpi (Figure 2).

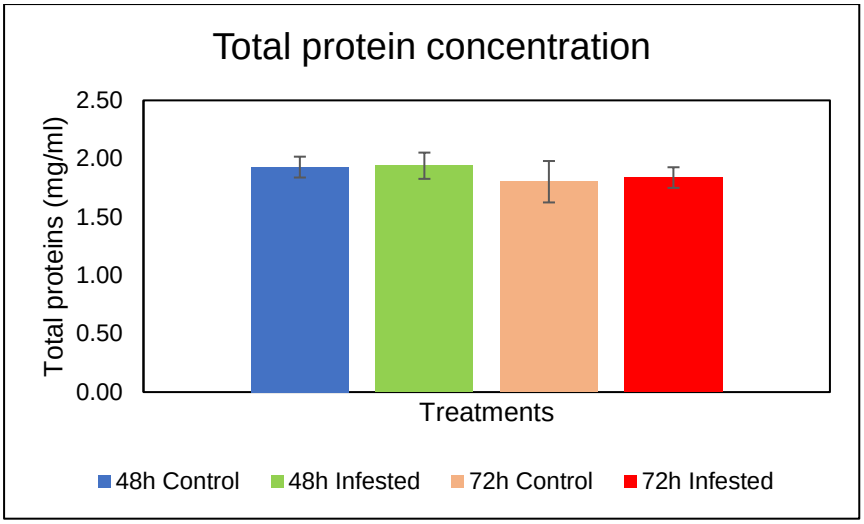

Figure 2: Total protein content in the leaf tissues harvested from control and infested plants 48 and 72 hpi. Standard deviations in the form of error bars were also indicated on the graphs.

Protein purification

The Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) results indicated variation in the expression of proteins within the plants sample (Figure 3). It was observed from the gel that there were four proteins of different lengths that were expressed. The proteins were approximately 3, 14, 18 and 28 kilodalton (kDa). A much highly expressed protein was found to be ~ 18 kDa (Figure 3).

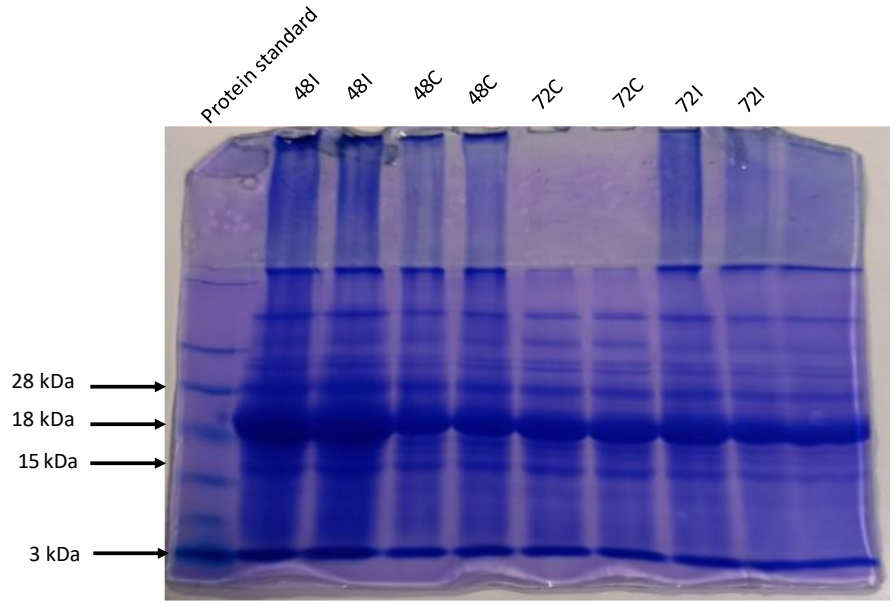

Figure 3: Protein profiling of control and infested leaf harvested at 48 and 72 hours post infestation (hpi). A protein standard curve was included on the first lane of the gel followed by the leaf extracts. 48I and 48C represent the infested and control wheat plants harvested 48 hours post infection, respectively. 72C and 72I represent the control and infested wheat plants harvested 72 hpi.

Protein binding studies

The binding study gel indicates that two proteins bind to the microcrystalline cellulose structure of the cell wall (Figure 4). These proteins had fragment lengths of 3 and 28 kilodalton. The band was much dense in the 48 infested plant as compared to the others.

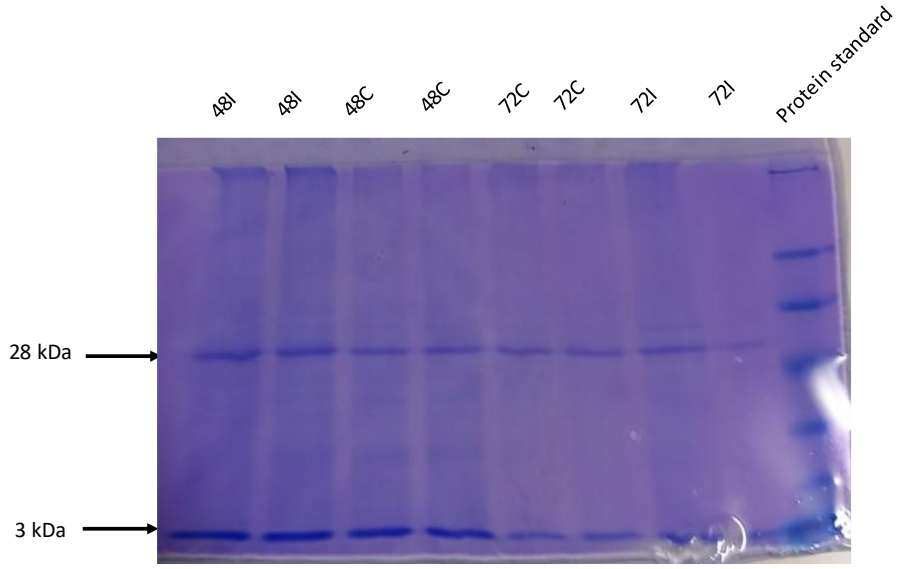

Figure 4: Protein binding studies done with SDS-PAGE. The leaf extracts were loaded on the gel starting with 48I, followed by 48C, 72C and 72I. 48I and 48C represent the infested and control isolates harvested 48 hours post infection, respectively. 72C and 72I represent the control and infested isolates harvested 72 hpi. A protein standard curve was included on the last lane of the gel.

Enzyme activity assays

Figures 5 and 6 are the glucose and xylose standard curves that were constructed by plotting the glucose or xylose concentration on the x-axis against the absorbance values (540 nm) on the y-axis, respectively. The best lines of fit are also plotted on the graphs, including its formula and the R^2 value.

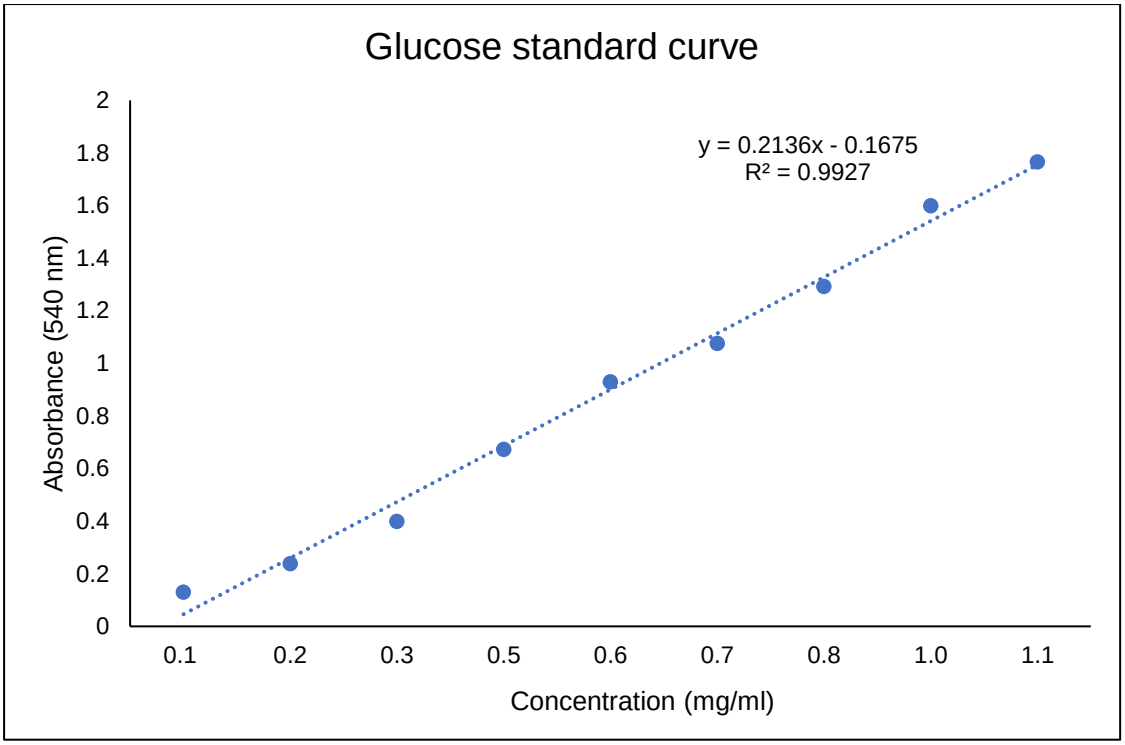

Figure 5: Glucose standard curve. The glucose concentration (mg/mL) was plotted on the x-axis against absorbance (540 nm) on the y-axis. The best line of fit, equation and the R^2 value are also indicated on the graph.

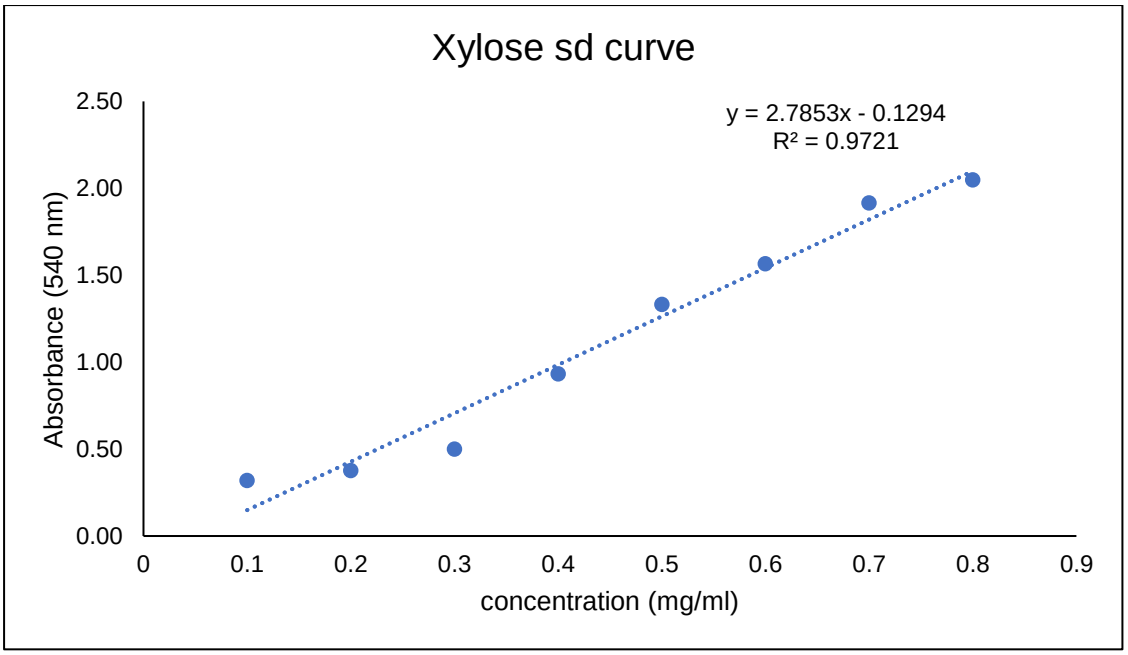

Figure 6: Xylose standard curve. The absorbance (540 nm) values on the y-axis were plotted against the concentration of xylose (mg/ml) on the x-axis. The best line of fit, equation and the R^2 value are also indicated on the graph.

The activity of the enzymes present in the control and infested leaf extracts harvested at 48 and 72 hpi was determine using different cell wall model substrates, namely Beechwood xylan, Arabinoxylan, Avicel and CMC. There was accumulation of enzymes in Avicel in the 72 control plants. Similar concentrations of enzymes were also observed for CMC in 48 hours control and 72 hours control plants. Enzymes were barely present when using Beechwood xylan and Arabinoxylan as substrates. Although little activity was observed in the 48 h Control for beechwood xylan but not at 48 I (Figure 7).

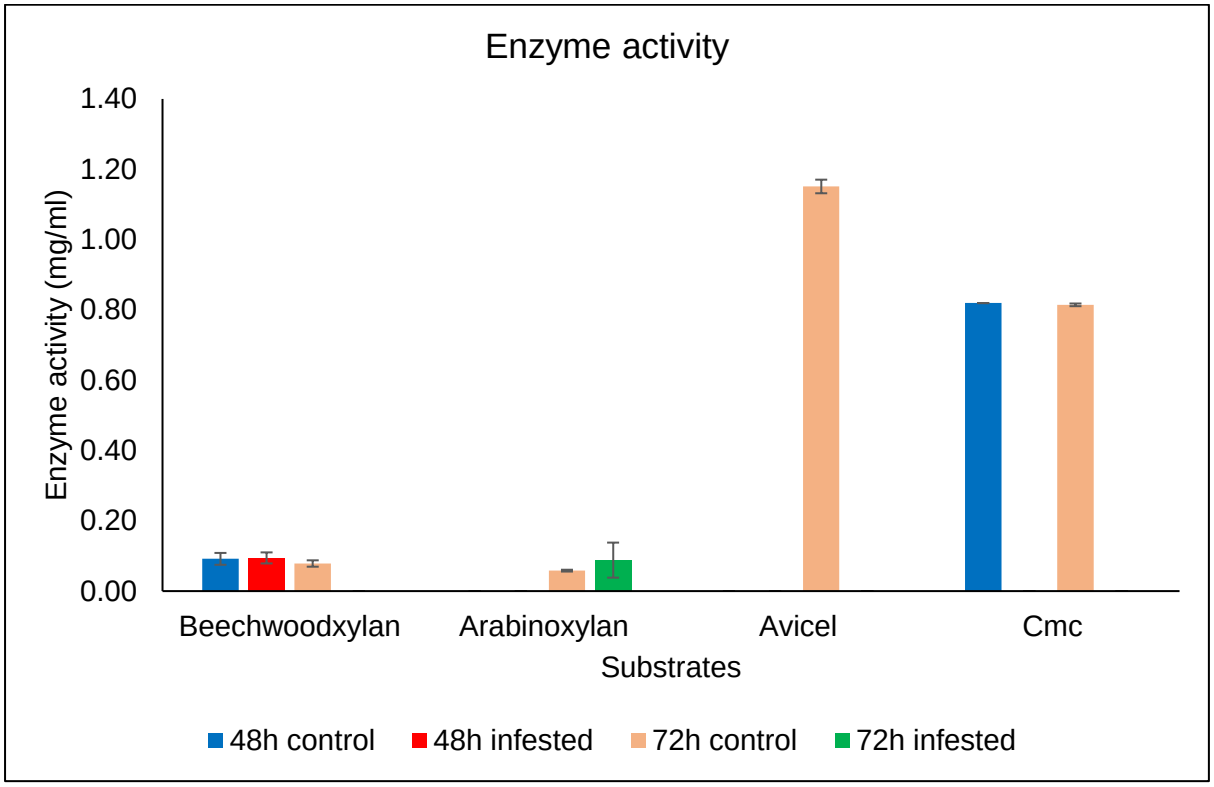

Figure 7: Enzyme activity using Beechwood xylan, Arabinoxylan, Avicel and CMC as model substrates for the enzyme in the control and infested leaf extracts at 48 and 72 hpi. The enzyme activity (mg/ml) was plotted on the y-axis. Standard deviation were presented as error bars on the graph.

Carbohydrate esterase activity assays

Crude carbohydrate esterase assays were conducted using pNP-A and pNP-B as substrates for the enzymes. The pNP-A graph on the left shows that there was activity of acetyl esterases in all the extracts. The highest activity was observed in the 48 hour infested plants and the lowest was found in the 72-hour infested plant. Increased activity was seen when the plants were infested with the aphids 48 hpi. The activity of esterases was much lower in the infested plants 72 hpi as compared to the controls. The pNP-B graph on the right showed increase in

activity of butyl esterases between control and infested plants 48 hpi. The same was observed at 72 hpi, where the activity increased when the plants were infested by the aphids. There was decreased activity within the control plants with time (Figure 8).

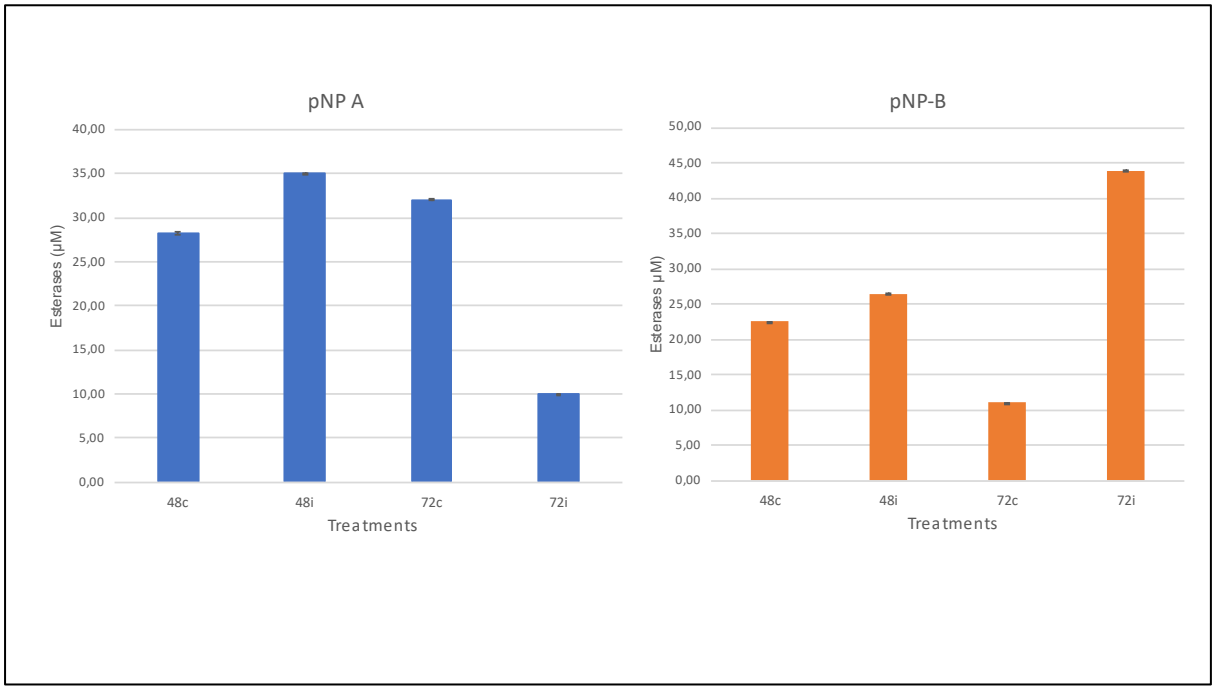

Figure 8: Esterase assay using pNP-A and pNP-B as substrates. The esterase activity was plotted againt the treatments in bopth the graphs. Error bars were also included illustrating the standard deviations.

Discussion

When the total protein content in the control and infested leaf isolates harvested 48 and 72 hours post infestation (hpi) was determined using Bradford assays, the results demonstrated that there were proteins present in all the samples. The protein standard curve was used to calculate the protein concentrations in mg/ml (Figure 1). The proteins concentrations were similar for all the isolates with the error bars indicating that there was not much variation in the data (Figure 2).

The SDS-PAGE results indicated the protein profile between the different treatments (Figure 3). SDS-PAGE is done to determine how variable the proteins are within the treatments. The separation of proteins was based on their molecular weights [15]. Proteins that were between 3 and 198 kDa were separated on the gel. SDS denatures proteins from their dimeric to their monomeric form [18]. The results demonstrated four proteins of lengths 3, 14, 18 and 28 kDa

that were expressed in the cell wall. Glycosidic hydrolases were expected to be found between 35 and 80 kDa in their dimeric form. Glycoside hydrolases are enzymes that play a role in catalyzing the hydrolysis of the glycosidic linkage of glycoside [18]. These enzymes include, β-1,4-and β-1,3-glucanases, pectinases, xylosidases, arabinosidases and glucosidases which cleave the carbohydrate components of the cell wall [14]. Most xylanases have a fragment length of ~22 kDa in their dimeric form. This means that in their monomeric form they will be ~11 kDa [19]. The high expression of the enzymes on the gel means that there are enzymes that can perform activity within the cell wall.

The binding study was done to validate this by using a model microcrystalline cellulose, Avicel as a substrate for the enzymes present in the leaf extracts. The two band that were observed on the gel (Figure 4) represented the Carbohydrate Binding Module (CBM) which bind to the microcrystalline cellulose. These are the proteins that assist cellulases bind to the cell wall [20].

The enzyme activity results indicated that there was accumulation of enzymes in the cellulose components (Avicel and CMC) of the cell wall than in the hemicellulose components (Beechwood xylan and wheat arabinoxylan) (Figure 7). Beechwood xylan is a dicot plant that is mainly composed of glucuronoarabinoxylan, whereas arabinoxylan predominates in wheat arabinoxylan. This just means that enzymes secreted by the aphids did not really affect the hemicellulose of the cell wall. This might be due to the fact that cellulose is the strongest and most abundant component of the cell wall and for this reasons pathogens and pests target it for degradation as a way of facilitating their penetration [14].

In addition, both the 48- and 72-hours control plants showed activity when using Carboxymethylcellulose (CMC). CMC is a non-crystalline cellulose derivative [20]. The fact that these were control plants demonstrates that the enzyme activity was due to the plant defence mechanism [18]. Plants defend themselves by producing peptide inhibitors that neutralize the activities of cell wall degrading enzymes secreted by plants during cell wall remodelling [18]. Another way of doing so is by releasing pathogenesis replated proteins that function as hydrolases that degrade the cell walls of the attacker [14].

Para-nitrophenyl acetate (pNP-A) and Para-nitrophenyl Butyrate (pNP-B) are phenolic acids and were used as substrates from the esterases. These substrates demonstrate cross-linking between xylans and acetate and butyrate, respectively. The crude carbohydrate esterase results on pNP-A demonstrated that there was an increase in the activity of esterases between the control and infested 48-hour plants. This increase just shows that esterases that break down the ester bond between ferulic acid and the arabinosyl residue's C(O)-5-hydroxyl group of glucuronoarabinoxylans and arabinoxylans are deposited by pathogens during infestation

[13]. There was also an increase in esterases from 48 to 72 hours in the control plants, this difference was due to growth and development. There was a decrease in esterase activity between the control and infested 72-hour plants and this was not expected. This reduction in activity might have been due to the fact that plants also produce enzymes such as expansins, during cell wall modification against environmental factors such as temperature [18].

The pNP-B results showed that the esterase activity was higher in the infested plant at 48 hpi as compared to the control. Higher activity was also observed in the infested plant at 72 hpi as compared to the control plant. There weas increased activity within the control plants with time, and this was due to growth and development, These results support the fact that the esterase activity increases when the ester bond between acetate/ butyrate and hemicellulose is cleaved during cell wall degradation or hydrolysis [13].

Conclusion

There are enzymes that perform activity on the cell wall. These enzymes targeted the cellulose component of the cell wall than hemicellulose. The presence of these proteins in both the control and infested plants demonstrated that they are generally plant-based but induced aphid. Carbohydrate binding modules which assist cellulases to bind were found to be bound to the crystalline component of cellulose and this showed that there was cell wall activity within the plant. There was differential activity between 48 and 72 hours. High enzyme activity in the control plants is due to the plant growth during cell wall remodeling, whereas increased activity in the infested plants indicated that the cell wall is being degraded or modified due to the presence of the aphids. The activity of esterases increase as the ester bond between phenolic acids and hemicellulose is cleaved during cell wall remodeling as a result of aphid infestation.

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