NOTE

*Bemisia tabaci* feeding induces pathogenesis-related proteins in cassava (*Manihot esculenta* Crantz)

Binu Antony* and M S Palaniswami
Division of Crop Protection, Central Tuber Crops Research Institute
Thiruvananthapuram 695 017, Kerala, India

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Cassava (*Manihot esculenta* C. Crantz) plants fed upon by whitefly *Bemisia tabaci* showed increased levels of pathogenesis-related (PR) proteins, such as β-1, 3-glucanase, peroxidase and chitinase activities, as compared to uninfested plants. The enzymes increased in specific activities from 2 to 7 fold and protein content in leaf extracts decreased in whitefly-infested plants, compared to uninfested plants. Among the three PR proteins, *B. tabaci* feeding induced significantly higher β-1, 3-glucanase activities, when compared with other two PR proteins. Study also discussed the possible application of PR proteins in whitefly control program.

Keywords: *Bemisia tabaci*, Pathogenesis-related proteins, Cassava

The whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) is considered as an important and ubiquitous pest, attacking over 600 plant species and transmitting more than 60 plant viruses1,2. Recent upsurge in whitefly population has drawn much attention for its worldwide importance as an insect pest and as the vector of emergent begomoviruses (Family: Geminiviridae, Genus: Begomovirus). Begomoviruses are circular, single-stranded DNA viruses, which include cassava mosaic, tomato leaf curl, cotton leaf curl, cucurbit and certain legume viruses. Cassava mosaic disease (CMD) occurs in all cassava growing regions of Africa, India, and Sri Lanka. Annual yield losses in Africa are estimated at $1,200-2,300 million and in India, yields can be reduced by 20-80%3. A specific cassava adapted population of *B. tabaci* is the vector of cassava mosaic virus (CMV) in India4,5.

Pathogenesis-related proteins (PR) induced in the plant by pathogen/insect attack include chitinases, β-1, 3-glucanases, peroxidases, proteinase inhibitors, ribonuclease-like proteins and thaumatin-like proteins6-11. The induction of PR proteins in response to the feeding of the silver-leaf whitefly *Bemisia argentifoli* Bellows and Perring and the leaf miner *Liriomyza trifolii* (Burguss) (Diptera: Agromyzidae) on tomato provides us with an excellent example of host plant response against insect attack12. The PR proteins are thought to play a defensive role against these pests.

Cassava, a Euphorbiaceous plant, is the third most important food crop after cereals and grain legumes. It provides staple food to over 500 million people in tropical countries. It is reported that Euphorbiaceous plants are less susceptible to insect attack, which may be attributing to its milky latex containing high level of chitinases13. In an earlier study, PR protein in cassava was reported in response to CMD14, however, no information is available on PR proteins in cassava against insect attack.

In the present study, an attempt was made to study the induction of PR proteins in cassava plants [both in CMV-infected (diseased) and non-diseased] due to *B. tabaci* attack. As *B. tabaci* is a pest as well as vector of CMV, the present work assumes importance in pest management.

Materials and Methods

Insect colonies

Adult *Bemisia tabaci* were maintained in screen cages [70 cm (height) × 42 cm × 42 cm] in a rearing room (70% relative humidity; 16 h light and 8 h dark; 23-25°C). The original culture of cassava strain of *B. tabaci* was provided by Lisha (Central Tuber Crops Research Institute, Trivandrum, India), and subculture was established on cassava and egg-plant.

Experiments were conducted in CMD-free cassava (raised from true cassava seeds) as well as in CMD-infected cassava plants. For CMD-free cassava plants, seedlings were raised in a net house, as CMV is not transmitted through cassava seeds. Cassava seedlings-raised plants (CSRP) in an insect-proof cage were
taken as CMD-free cassava plants. Cassava seedlings (4 weeks after germination) were placed in insect-proof cages (4 plants per cage, 6 cages). Adult *B. tabaci* (~ 300 nos) were released into four cages and two cages were kept as control (without whitefly) for 16 days. Number of nymphs, pupae and adults in the 3rd and 4th leaves from the upper leaves were counted (data not given) in all the experimental plants in all the six cages and leaf samples were analyzed for studying population build-up. Colorimetric assays for the determination of total protein and peroxidase, β-1, 3-glucanase and chitinase activities were done as described\(^\text{10}\).

**Extraction procedure**

From both *B. tabaci* infested and uninfested plants, 4\(^{th}\) to 7\(^{th}\) leaves from top were collected. Samples were drawn and 1.5 g sample was crushed in chilled acetone with a pre-cooled mortar and pestle in 6 ml 0.1 M phosphate buffer (pH 7.4) and placed into tubes containing 0.3 g of hydrated polyvinyl pyrrolidone. The tubes were capped and mixed for 30 min at 4°C. The samples were centrifuged at 20,000 g for 15 min and then boiled through a layer of Mira cloth into dialysis tubing (Sigma-Aldrich, USA) and dialyzed against sodium acetate buffer (pH 8). The reaction was stopped by addition of 1 ml of arsenomolybdate reagent [45 mM ammonium molybdate (450 ml), conc. H\(_2\)SO\(_4\) (21 ml) 134 mM Na\(_2\)HASO\(_4\) (25 ml)] was added to develop light reddish-brown colour and absorbance at 540 nm was measured, following dilution with distilled water (6.5 ml). The β-1, 3-glucanase activity was defined as the amount of enzyme releasing µmol of glucose equivalent/min/ml of enzyme solution and expressed as µmol glucose equivalents released glucan/min/g fresh wt. Total activity was expressed as mg glucan/min/g dry wt and specific activity was expressed as OD/h/g fresh wt/mg protein g\(^{-1}\) tissue.

**Chitinase activity**

Chitinase activity was assayed using glycol chitin as substrate\(^17\). Briefly, 500 µl of plant extract diluted in 0.05 M sodium phosphate buffer (pH 8) was added to 500 µl of 1% glycol chitin (Sigma-Aldrich, USA) in phosphate buffer (pH 8). The mixture was incubated at 40°C for 2 h. The reaction was stopped by adding 2 ml of Nelson-Somogyi reagent (alkaline copper tartarate reagent) and then boiled for 10 min. An aliquot (1 ml) of arsenomolybdate reagent [45 mM ammonium molybdate (450 ml), conc. H\(_2\)SO\(_4\) (21 ml) 134 mM Na\(_2\)HASO\(_4\) (25 ml)] was added to develop light reddish-brown colour and final mixture was made up to 10 ml with distilled water and the absorbance was measured at 520 nm. Total activity was expressed as OD/h/g dry wt and specific activity as OD/h/g fresh wt/mg protein g\(^{-1}\) tissue.

**Peroxidase activity**

Peroxidase activity was assayed using guaiacol as substrate\(^18\). The plant extract (500 µl) diluted with 3 ml of 0.1 M sodium phosphate buffer (pH 7) was added to 500 µl guaiacol (0.5%) and 500 µl 3% H\(_2\)O\(_2\) in phosphate buffer (pH 7). The mixture was mixed well and kept for 10 min to develop straw colour and absorbance was measured at 460 nm. Total activity was expressed as OD/h/g dry wt and specific activity as OD/h/g fresh wt/mg protein g\(^{-1}\) tissue.

**Total protein determination**

Total protein contents were determined by the method of Bradford\(^15\) using bovine serum albumin (BSA) as the standard. The 200 µl leaf extracts were mixed with 5 ml of protein reagent in tubes for at least 30 min and then absorbance was read at 595 nm. Series of BSA standards (20, 40, 60, 80, 100 and 200 µl) were prepared and 5 ml of protein reagent was added to develop blue colour. Total protein was expressed in mg/g fresh weight.

**Enzyme activity assay**

**β-1, 3-Glucanase activity**

β-1, 3-Glucanase activity was assayed, following the earlier described method\(^16\), which determined the increase in reducing groups, resulting from the hydrolysis of laminarin (Sigma-Aldrich, USA). Typically, 500 µl of plant extract diluted in 100 mM sodium acetate buffer (pH 5) was added to 500 µl of laminarin (20 mg/ml) in sodium acetate buffer (pH 5) and the mixture was incubated at 50°C for 30 min. The reaction was stopped by addition of 1 ml of copper reagent (25 mM Cu\(_2\)SO\(_4\) in 0.91 M Na\(_2\)SO\(_4\)) and then boiled for 20 min. An aliquot (1 ml) of arsenomolybdate reagent [45 mM ammonium molybdate (450 ml), conc. H\(_2\)SO\(_4\) (21 ml) 134 mM Na\(_2\)HASO\(_4\) (25 ml)] was added to develop light reddish-brown colour and absorbance at 540 nm was measured, following dilution with distilled water (6.5 ml). The β-1, 3-glucanase activity was defined as the amount of enzyme releasing µmol of glucose equivalent/min/ml of enzyme solution and expressed as µmol glucose equivalents released glucan/min/g fresh wt. Total activity was expressed as mg glucan/min/g dry wt and specific activity was expressed as OD/h/g fresh wt/mg protein g\(^{-1}\) tissue.
infested cassava plants, when compared to non-infested control plants. The total protein content in whitefly-infested leaf decreased from 23.7 mg/g to 21.94 mg/g in CSRP and from 20.59 mg/g to 16.23 mg/g in CMD-infected plants (Table 1). The decrease in leaf protein content in infested plants suggested that there might be suppression of some plant proteins occurring simultaneously, with the apparent induction of PR proteins.

Enzymes activities for infected and non-infected leaf samples of CSRP and CMD-infected plants (diseased) are given in Table 1. Total as well as specific activities of the enzymes were significantly higher in whitefly-infested leaves, compared to non-infested control plants (Table 1). Specific activities in leaves of CSRP and CMD-infected plants increased significantly with whitefly feeding; the activities of β-1, 3-glucanase, chitinase and peroxidase increased 7-fold, almost 3-fold and 2-fold, respectively in CSRP and approx 4.5-fold, almost 3-fold and 2-fold, respectively in CMD-infected plants. The total protein content in leaves of CSRP and CMD-infected plants increased significantly with whitefly feeding; the activities of β-1, 3-glucanase, chitinase and peroxidase increased 7-fold, almost 3-fold and 2-fold, respectively in CSRP and approx 4.5-fold, almost 3-fold and 2-fold, respectively in CMD-infected plants. Thus, the results indicate the apparent induction of three PR proteins due to whitefly feeding.

The study revealed when compared the activities of β-1, 3-glucanase, chitinase and peroxidase in CMD-infected plants (diseased), CSRP (non-diseased) showed increased response to the B. tabaci feeding. The specific activities increased from 2 to 7 fold in CSRP, compared to CMD-infected plants, indicating whitefly feeding induced lesser enzymatic activities in CMD-infected plants, when compared to CSRP. This may be explained on the basis that CMD-infected plants already had CMV as pathogen that induced PR proteins formation. Among the three PR proteins, B. tabaci feeding induced significantly higher β-1, 3-glucanase activities, when compared with other two PR proteins.

Induction of PR proteins such as chitinases, peroxidases and β-1, 3-glucanase has negative effects on pathogens and insect pests. Chitinase degrades chitin, a major component of insect cell20. It may damage insect peritrophic membrane while in the gut, thereby, increasing susceptibility of the insects to biological control agents/plant chemicals etc. It can also act as α-amylase inhibitor and interfere with digestion of plant parts21. Induction of chitinase activity may interfere with insect development, feeding and growth, facilitate microbial infection, and finally cause death22,23. Citrus root chitinases are capable of digesting peritrophic membrane from larva of Diaprepes abbreviatus24. Thus, chitinase has major role as plant defensive proteins against insect attack.

Peroxidases activity and pattern can be correlated with plants susceptibility to pest attack11. Peroxidases are induced in tomato plants following pathogens and insects damage10. They are involved in production and

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (mg/g tissue)</th>
<th>β-1, 3-Glucanase (mg Glucan/min/g fresh w.)</th>
<th>Specific activity</th>
<th>OD/h/g fresh wt</th>
<th>Total activity (OD/h/g dry wt)</th>
<th>Specific activity</th>
<th>OD/h/g fresh wt</th>
<th>Peroxidase</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infested</td>
<td>21.94±0.68</td>
<td>47.00±9.82</td>
<td>239.80±50.12</td>
<td>2.87±0.53</td>
<td>43.81±13.01</td>
<td>222.97±66.23</td>
<td>2.68±0.72</td>
<td>36.22±7.35</td>
<td>167.70±34.03</td>
</tr>
<tr>
<td>Uninfested</td>
<td>23.70±0.10</td>
<td>8.76±13.35</td>
<td>0.05±0.64</td>
<td>0.43±0.01</td>
<td>16.23±2.92</td>
<td>66.79±12.04</td>
<td>0.30±0.76</td>
<td>14.42±5.33</td>
<td>58.16±21.52</td>
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<td>df</td>
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<tr>
<td>P value</td>
<td>P &lt; 0.001</td>
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<td></td>
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<td>[Values represent as mean ± S D]</td>
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</table>

Table 1—Total protein content and enzyme activities in cassava seedlings-raised (non-diseased) plants (CSRP) and CMD-infected (diseased) cassava from B. tabaci infested and non-infested

In cassava seedlings-raised (non-diseased) plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (mg/g tissue)</th>
<th>β-1, 3-Glucanase (mg Glucan/min/g fresh w.)</th>
<th>Specific activity</th>
<th>OD/h/g fresh wt</th>
<th>Total activity (OD/h/g dry wt)</th>
<th>Specific activity</th>
<th>OD/h/g fresh wt</th>
<th>Peroxidase</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infested</td>
<td>16.23±3.15</td>
<td>38.68±5.91</td>
<td>120.13±18.34</td>
<td>5.99±0.88</td>
<td>31.33±7.53</td>
<td>115.01±27.64</td>
<td>2.64±0.73</td>
<td>40.82±7.01</td>
<td>126.05±19.21</td>
</tr>
<tr>
<td>Uninfested</td>
<td>20.59±0.93</td>
<td>15.68±0.75</td>
<td>34.31±1.63</td>
<td>0.76±0.20</td>
<td>17.28±0.53</td>
<td>71.40±2.17</td>
<td>0.85±0.73</td>
<td>22.62±0.92</td>
<td>49.52±2.01</td>
</tr>
<tr>
<td>df</td>
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</tr>
<tr>
<td>P value</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
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</tbody>
</table>

In CMD-infected (diseased) cassava

Specific activity expressed in OD/h/g fresh wt/mg protein g⁻¹ tissue
Significance determined by student’s t test

-1, 3-glucanase, chitinase and peroxidase in CMD-infected plants. The specific activities increased from 2 to 7 fold in CSRP, compared to CMD-infected plants, indicating whitefly feeding induced lesser enzymatic activities in CMD-infected plants, when compared to CSRP. This may be explained on the basis that CMD-infected plants already had CMV as pathogen that induced PR proteins formation. Among the three PR proteins, B. tabaci feeding induced significantly higher β-1, 3-glucanase activities, when compared with other two PR proteins.
polymerization of phenolics, lignification and hypersensitive responses, limiting the possibility of disease spread\textsuperscript{11}. They also have negative effect on food digestibility and protein availability in herbivorous insects\textsuperscript{25,26}. \(\beta\)-1, 3-Glucanase hydrolyzes \(\beta\)-1, 3-glucons, a major component of the surface structure and cell walls of many microbial and fungal pathogens and insects\textsuperscript{11}.

Insect-induced PR proteins\textsuperscript{7,10,11,24} have been reported in the plants. Feeding by whiteflies and leaf miners has also been found to induce a variety of defensive phytochemicals, including PR proteins such as peroxidases, glucanases and chitinases in the plants\textsuperscript{10,11,27,28}. Similarly, the present study indicated the apparent PR protein induction in cassava due to \textit{B. tabaci} feeding. As PR proteins have negative effects on feeding, food digestibility and protein availability\textsuperscript{10,25,26,28}, elevation of PR proteins levels at appropriate time by application of elicitors, in conjunction with biological control strategies, may possibly find application in whitefly control programme.

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