A METHOD TO PRODUCE INSECT RESISTANCE IN PLANT BY ALTERING AMINO ACID CONTENT IN SAP

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ABSTRACT

Some of the naturally sap sucking insect resistant plant varieties comprise altered amounts of amino acids asparagine, aspartic acid and glutamic acid in their sap. However, role of these amino acids on insect resistance has not been demonstrated. We evaluated impact of asparagine (N), aspartic acid (D) and glutamic acid (E) on survival of Myzus persicae and Bemisia tabaci by altering concentration in artificial diet. We observed that higher concentration of D or/and E caused ~94-100 % mortality of both insects while higher concentration of N did not show any significant mortality. Deletion of such amino acids from normal diet did not show any effect on survival of M. persicae s and B. tabaci. Asparagine synthetase (AS) and asparginase (ASN) mutant of Arabidopsis thaliana plant, which might have altered level of such amino acids were evaluated for insect resistance. AS-mutants of Arabidopsis thaliana showed significant protection against M. persicae and B. tabaci compared with wild type plants while ASN mutants did not affect the survival of both insect. Present study discusses a method of developing sap sucking pest resistant plants by altering amino acid biosynthetic pathway.

KEYWORDS: Sap Sucking Pest Resistance, Aspartic Acid, Glutamic Acid, Bemisia tabaci, Myzus persicae

ABBREVIATIONS

Asparagine synthetase: AS; Asparaginase: ASN; Wild Type: WT
Asparagine: N; Aspartic acid: D; Glutamic acid: E

INTRODUCTION

Bt-Crops provide effective protection against several lepidopteran caterpillars but ineffective against sap sucking insects. During the last few years Bt-transgenic cotton in India has been severely infested by sap sucking pests like leaffoppers, mealy bugs, mirid bug, aphids and whiteflies (ABRO et al. 2004). Although proteins like mannose binding lectins provide some protection against sap sucking pests (UPADHYAY et al. 2010; 2011a; 2012), effective technology is still not available. Therefore, there is a need to explore safer alternatives for the control of sap sucking insects.

Each plant species varies with respect to the extent of damage caused by insect attack. It is due to their characteristics protections like texture, biochemical compositions and others which interfere in feeding, oviposition and development of invading insect species. Nutrient content of host plant also plays significant role in providing protection from insect pests (CAROLINE AND SIMON et al. 2002). Insects with piercing and sucking mouth parts exclusively depend on plant sap for major nutrients. Most of these insects preferred phloem sap only while a few feeds both phloem and xylem sap. Phloem sap contains high-level of sugars, but relatively low amount of amino acids (HAYASHI AND CHINO 1990; AMIARD et al. 2004). Amino acids being the major source of nitrogen their content in sap act as limiting factor in determining the survival of insects (DAS et al.1976; MISHRA et al. 1990). Amino acids compositions in plant sap play critical role in determining susceptibility to sap sucking pests (TSUMUKI et al. 1987). Rice plants with less...
asparagine content show resistance against brown planthopper (SOGAWA and PATHAK 1970). Resistant lines of oats and barley contain less quantity of asparagine and higher amounts of glutamic acid (WEIBULL et al.1987; HEDIN et al. 1990).

Aspartic acid metabolic pathway controls the biosynthesis of amino acids asparagine, aspartic acid and glutamic acid in plants. Aspartic acid is the starting compound for two main pathways, one leads to the synthesis of asparagine and other to aspartate-derived amino acids viz., lysine, threonine, methionine and isoleucine (AZEVEDO et al. 1997). Present study aimed to analyze the effect of altered concentrations of D, E and N on survival of sap sucking insect pests. We have also analysed the survival of insects on AS and ASN mutants of Arabidopsis thaliana plant, which might have altered concentration of such amino acids.

MATERIAL AND METHODS

Insect Culture

Laboratory reared insect cultures of Myzus persicae and Bemisia tabaci were used in the study. B. tabaci were cultured on potted cotton plants, while M. persicae cultured on Arabidopsis thaliana. Insect cultures were maintained at 26 ± 2ºC and 70% relative humidity, and similar conditions maintained for insect bioassay.

Mutants of Arabidopsis thaliana

The Arabidopsis thaliana mutants for AS (SALK_028186 and SALK_144656) and ASN (SALK_064129C, SALK_147134C and SALK_087377C) were selected from TAIR database and obtained from Arabidopsis thaliana Biological Resource Center (ABRC).

Cultivation of Arabidopsis thaliana Mutants

Seeds of mutant were sown on the mixture of perlite and vermiculite (1:1 ratio) in pots, covered with plastic bags and incubated at 4ºC for 72 h. Pots were then transferred to Arabidopsis thaliana growth room maintained at 25±2 ºC, 40-60% relative humidity and 16 h of photo period.

Evaluation of Altered Concentration of Selected Amino Acids in Artificial Diet on Survival of M. Persicae and B. Tabaci

Insect bioassay against B. tabaci was performed as described by UPADHYAY et al. (2011b). The procedure was slightly modified for M. persicae, in which the length of bioassay tube reduced to 3 cm. B. tabaci adults (0-1 day old) and uniform size second instar nymphs of M. persicae were used for bioassay (20 insect/ vial).

Modified artificial diets having varying concentrations of amino acids D, E and N were prepared from normal diet (i.e. Diet A5, Febvay et al. 1988) (Supplementary Table 1) and used for insect bioassay. Insect’s mortality was recorded at different time intervals and compared with control diet. Experiment was performed in triplicates and average value used for calculation of percent mortality.

Survival of M. Persicae on Mutant Arabidopsis Thaliana Plants

One month old mutants and similar size wild type (Col-0) plants were used for insect bioassay. Second instar nymphs of M. persicae (10/ plant) were released on the top youngest rosette leaf and their population counted after one week. Percent decrease in pest population was calculated by using formula of FLEMMING and RETNAKARAN (1985).

\[
(P) = \frac{(\bar{T}_p \times \bar{C}_p) - (\bar{T}_0 \times \bar{C}_0)}{(\bar{T}_0 \times \bar{C}_0)} \times 100
\]
A Method to Produce Insect Resistance in Plant by Altering Amino Acid Content in Sap

\[ P = \% \text{ reduction over control} \]
\[ T_a = \text{population in treatment after} \]
\[ T_b = \text{population in treatment before} \]
\[ C_a = \text{population in control after} \]
\[ C_b = \text{population in control before} \]

The percent reduction in population was further analysed by One way-ANOVA (P <0.05) and compared means using Duncan’s Multiple Range Test (DMRT).

**Survival of B. tabaci on Mutant Arabidopsis thaliana Plants**

*B. tabaci* adults (0-1 day old, 30/pot) were released on separate pots containing similar number of mutant and control plants of *Arabidopsis thaliana*. Number of surviving *B. tabaci* was counted after one week and percent mortality calculated. Experiment was performed in triplicate. Mortality data was further analysed by One way-ANOVA (P <0.05) and compared means using Duncan’s Multiple Range Test (DMRT).

**RESULTS**

**Effect of High Concentration of Aspartic Acid in Artificial Diet**

The aspartic acid concentration in normal diet increased by two (2X) and four times (4X) and used for insect bioassay. 2X concentration caused 28% mortality of whiteflies after two days, which increased to 48% by third day. In case of aphid, 38 and 50% mortality was observed after second and third day respectively. However, 4X concentration caused 94 and 100% mortality of whiteflies and aphids after three days, respectively (Table 1).

**Effect of High Concentration of Glutamic Acid in Artificial Diet**

As in case of aspartic acid, 2X and 4X concentrations of glutamic acids in normal diet was evaluated for toxicity to aphids and whiteflies. 2X concentration caused 28 and 30% mortality of whiteflies and aphids after two days, respectively. Glutamic acid at 4X concentration showed 79 and 75% mortality of whiteflies and aphids, respectively. Mortality of both the insects increased up 94-95% after three day in 4X concentration in normal diet (Table 1).

**Effect of High Concentration of Asparagine in Artificial Diet**

Higher concentration of asparagine (up to 4X) did not cause significant mortality against both insects. Maximum mortality of 18 and 7.5% recorded against whiteflies and aphids, respectively after three days. Synthetic diet without asparagine also did not cause any significant mortality against both the insect (Table 1).

**Effect of High Concentration of Both Glutamic and Aspartic Acid in Artificial Diet**

Combination of both aspartic acid and glutamic acid in diet was studied for toxicity against aphids and whiteflies at 2x and 4x concentrations. Combination at 2x and 4x concentration caused mortality similar to individual amino acids on second and third day of treatment against both the insects (Table 1).

**Insect Bioassay of Mutant Plants**

To evaluate the *in vivo* impact of altered concentration of tested amino acids in plants, we selected two types of mutants; AS-mutants (SALK_028186 and SALK_144656) and ASN- mutants (SALK_064129C, SALK_147134C and SALK_087377C) of *Arabidopsis thaliana*. AS-mutants showed better resistance compared with ASN-mutants against *M. persicae*. Salk_028186 and Salk_144656 showed ~73 and 69% reduction in aphid population after one week, respectively. However, ASN-mutants did not show any resistance (Table 2). Both AS and ASN-mutants showed significant reduction in
whiteflies population over control. Population was reduced by 57-61% on AS-mutants and ~30% on ASN-mutants as compared to control after one week (Table 2).

DISCUSSIONS

Amino acids D and E caused toxicity to aphids and whiteflies when used at higher concentration in artificial diet. It is reported that high concentration of D in culture media causes toxicity to adult nematodes (JACKSON et al. 1973). Alteration in amino acid contents in artificial nectar affects the choice of honeybees; artificial nectar containing proline was preferred over alanine and serine (BERTAZZINI et al. 2010). Resistant lines of oats and barley contains higher amounts of glutamic acid and less of asparagine (WEIBULL et al.1987; HEDIN et al. 1990). These results show that the alteration in amino acid content changes survival as well as behavior of insects.

In plants like Arabidopsis thaliana the amino acids aspartate, asparagine, glutamate and glutamine are major amino acids accounting for 64% of total amino acids content of leaf extract (SCHULTZ et al.1994). These four amino acids also play important role in nitrate assimilation pathway (LAM et al. 1994). Presence of higher amounts of nonessential amino acids (like aspartate and glutamate) might interfere in absorption of other essential amino acids in insect gut and disrupt normal physiology, resulting in death of insects.

Arabidopsis thaliana AS-mutants showed resistance against aphids and whiteflies. While reduction in aphid population in case of ASN-mutants was marginal. AS is responsible for the conversion of aspartate to asparagine and ASN for asparagine to aspartate. AS-mutants either has reduced or hampered the rate of conversion from aspartate to asparagine. This results in decreased concentration of asparagine and increased concentration of aspartic acid. It is reported that the AS-mutants (SALK_028186 and SALK_144656) of Arabidopsis thaliana show decrease in level of asparagine LAM et al (1998), however level of aspartic acid is not measured in same mutants.

Further, mutant of ASN reduced or hampered conversion of asparagine to aspartic acid, which might lead to higher level of asparagine. ASN-mutants of Arabidopsis thaliana did not affect the survival of both the insects, confirming that increase in concentration of asparagine will not affect the insect’s survival.

Present study not only described the importance of certain amino acids in insect survival but also opens a method for insect resistance transgenic plant development by altering the concentration of amino acids. Synthesis of amino acids asparagine, aspartate and glutamate is controlled by the aspartic acid metabolic pathway (figure 1). In plants, glutamate is converted to aspartate by aspartate aminotransferase (EC 2.6.1.1), and asparagine synthetase (EC 6.3.5.4) catalyses conversion of aspartate to asparagine. Down regulation of asparagine synthetase might results in high accumulation of aspartate. This might also accumulate high content of glutamate due to the feedback inhibition of aspartate aminotransferase.Nevertheless, effect of reduced level of asparagine on plant growth and development needs to be examined in such transgenics because asparagine plays major role in nitrogen transport and storage in higher plants (ROGNES et al. 1980; URQUHART and JOY 1981). Enhanced level of aspartate and glutamate content might also be obtained by down regulation of aspartate kinase (AK, EC 2.7.2.4), which catalyzes the synthesis of aspartate-derived amino acids. However, one has to again analyze the impact of aspartate-derived essential amino acids in growth and development of plants. Present report opens a new method for the control of sap sucking insects on crop plants.

ACKNOWLEDGEMENTS

Authors are grateful to the Council of Scientific and Industrial Research, Government of India. CSIR-EMPOWER program for the financial support. SKU and SY are thankful to CSIR and ICMR for research fellowship, respectively.
REFERENCES


APPENDICES

![Figure 1: Aspartic acid metabolic pathway. Glutamate is converted to aspartate by aspartate aminotransferase (AAT, EC 2.6.1.1), and asparagine synthetase (ASN, EC 6.3.5.4) catalyses conversion of aspartate to asparagine. Down regulation of asparagine synthetase might result in high accumulation of aspartate. This might also accumulate high content of glutamate due to the feedback inhibition of aspartate aminotransferase. Enhanced level of aspartate and glutamate content might also be obtained by down regulation of aspartate kinase (AK, EC 2.7.2.4), which catalyzes the synthesis of aspartate-derived amino acids via a help of aspartate semialdehyde dehydrogenase (ASADH, EC 1.2.1.11).](image)
Table 1: Effects of Variation in Selected Amino Acids on Mortality of Aphids and Whiteflies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2nd Day</th>
<th>3rd Day</th>
<th>2nd Day</th>
<th>3rd Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>5.0 ± 1.4^a</td>
<td>12.5 ± 2.5^a</td>
<td>0.0^a</td>
<td>2.5 ± 1.4^a</td>
</tr>
<tr>
<td>Diet with 2x D</td>
<td>27.5 ± 3.5^b</td>
<td>47.5 ± 2.5^c</td>
<td>37.5 ± 2.5^c</td>
<td>50.0 ± 10.0^c</td>
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<tr>
<td>Diet with 4x D</td>
<td>74.0 ± 2.8^b</td>
<td>94.0 ± 6.0^b</td>
<td>80.0 ± 5.0^b</td>
<td>100.0 ± 0.0^b</td>
</tr>
<tr>
<td>Diet with 2x E</td>
<td>27.5 ± 3.5^b</td>
<td>47.5 ± 2.5^c</td>
<td>30.0 ± 5.0^b</td>
<td>65.0 ± 5.0^b</td>
</tr>
<tr>
<td>Diet with 4x E</td>
<td>79.0 ± 15.5^b</td>
<td>94.0 ± 6.0^b</td>
<td>75.0 ± 5.0^b</td>
<td>95.0 ± 5.0^b</td>
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<tr>
<td>Diet with (2x D+2x E)</td>
<td>25.0 ± 0.0^a</td>
<td>40.0 ± 2.5^c</td>
<td>27.5 ± 2.5^c</td>
<td>80.0 ± 10.0^c</td>
</tr>
<tr>
<td>Diet with (4x D+4x E)</td>
<td>86.0 ± 2.8^a</td>
<td>98.0 ± 2.0^a</td>
<td>80.0 ± 10.0^a</td>
<td>100.0 ± 0.0^a</td>
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<tr>
<td>Diet with 2x N</td>
<td>12.5 ± 3.5^a</td>
<td>20.0 ± 5.0^a</td>
<td>0.0^a</td>
<td>5.0 ± 0.0^a</td>
</tr>
<tr>
<td>Diet with 4x N</td>
<td>17.5 ± 3.5^a</td>
<td>17.5 ± 2.5^a</td>
<td>0.0^a</td>
<td>7.5 ± 2.5^a</td>
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<tr>
<td>Normal diet - N</td>
<td>10.0 ±0.0^a</td>
<td>20.0 ± 0.0^a</td>
<td>5.0 ± 1.4^a</td>
<td>12.5 ± 2.5^a</td>
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<tr>
<td>Normal die - D</td>
<td>7.5 ±5.5^b</td>
<td>22.5 ± 2.5^a</td>
<td>2.5 ± 1.4^a</td>
<td>10.0 ± 0.0^a</td>
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<tr>
<td>Normal die - E</td>
<td>12.5±5.5^b</td>
<td>25.0±1.4^a</td>
<td>5.0±1.4^a</td>
<td>10.0±0.0^a</td>
</tr>
</tbody>
</table>

D= aspartic acid; N= Asparagine; E= Glutamic acid.
Means are compared using Duncan’s Multiple Range Test (P <0.05).
Means in column carrying same superscripted later are not different.

Table 2: Percentage Reduction in Insect Pest Population Compared to Control* on Arabidopsis Thaliana Mutants

<table>
<thead>
<tr>
<th>Germplasm Details of Mutant Plants</th>
<th>Reduction (%) in Insect Pest Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Plants</td>
<td>Myzus persicae</td>
</tr>
<tr>
<td>SALK_028186 AT5G10240 AS</td>
<td>72.98± 8.28^a</td>
</tr>
<tr>
<td>SALK_144656 AT5G65010 AS</td>
<td>68.47± 7.68^c</td>
</tr>
<tr>
<td>SALK_064129C AT5G61540 ASN</td>
<td>4.0 ± 2.82^a</td>
</tr>
<tr>
<td>SALK_147134C AT5G61540 ASN</td>
<td>7.5 ± 3.53^b</td>
</tr>
<tr>
<td>SALK_087377C AT5G08100 ASN</td>
<td>6.00 ± 2.82^a</td>
</tr>
</tbody>
</table>

*Col-0 was taken as a control plant
AS=Asparagine synthetase; ASN=Asparaginase
Means are compared using Duncan’s Multiple Range Test (P <0.05).
Means in column carrying same superscripted later are not different.