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EFFECT OF MECHANICAL WOUNDING ON LEVEL OF SECONDARY METABOLITES IN *CAJANUS CAJAN* AND DEFENSE AGAINST *HELICOVERPA ARMIGERA*

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ABSTRACT
Plants defend biotic and abiotic stresses by activating defense system. Hence the present study demonstrates the effect of mechanical wounding on change in secondary metabolite content and its impact on *Helicoverpa armigera* gut enzymes. Total phenolics and flavonoid content was determined by using folin ciocalteau and aluminium chloride reagents respectively. In vitro enzyme inhibitory activity was studied by solution assay. In wound response *C. cajan* leaves accumulated a significant amount of phenolics and flavonoids as compare to control leaves. The gut amylase inhibitory activity of *H. armigera* was greater than proteinase inhibitory activity. This study concluded that metabolites are an effective weapon against insect pests.

KEYWORDS: *Cajanus cajan*, Flavonoids, *Helicoverpa armigera*, gut enzymes, Mechanical wounding, phenolics,

INTRODUCTION

Plants possess morphological, physiological, and biochemical traits which make them possible to reduce the impact of herbivorous arthropods and other biotic stress (Schoonhoven *et al.*, 2005). Wounding is effective to activate the phenylpropanoid metabolism in plant and enhance the accumulation of phenolic compounds (Jacobo-Velázquez and Cisneros-Zevallos, 2012). Plants produce a different group of resistance-mediating metabolites, including a large number of toxic and anti-digestive proteins as well as several anti-nutritive compounds that inhibit an herbivore's digestion after intake of plant material (Kessler and Baldwin, 2002b). These defense components can be expressed as a constitutive or can be induced in response to enemy attacks (Karban and Baldwin, 1997).

Plants develop a variety of metabolic defense responses against biotic and abiotic stresses such as pathogen attack, physical wounding, including MJ and SA exposure (Conrath *et al.*, 2002; *Tan et al.*, 2004). Defense responses to stress induced the production of several secondary metabolites such as phenolics, flavonoids and other low molecular weight substances (Ali *et al*, 2006). The natural defense system in pigeonpea (*C. cajan)* is inducible by wounding, pathogen attack, and insect chewing (Padul *et al.*, 2012a). Funke and Matthias (2005) studied α-amylase inhibitory activity of the different polyphenolic compounds. Some flavonoids such as gentisic acid, chlorogenic acid, protocatechuic acid, caffeic acid, trihydroxyflavone, catechin and lectins are very much toxic to *H. armigera* (War *et al*., 2013). The objectives of this research were to evaluate the effect of wounding stress on the accumulation of total phenolic, flavonoids and their inhibitory effect on *H. armigera* gut proteinases as well as amylase enzymes

MATERIALS AND METHODS

Chemicals and reagents

Chemicals were procured from different reputed companies mentioned in bracket, hexane, chloroform, ethyl acetate, methanol, ethanol, acetone, sodium hydroxide (Rankem), ferric chloride (Sarabhai M. chemicals), Azocasein (Sigma Aldrich), Starch, Trichloro acetic acid (Spectrochem), Glycine (Molychem), Gallic acid, sodium nitrate (Analytical Rasayan), Dinitrosalicylic acid (Merk), Aluminum chloride (Alfa Aesar). All chemicals and reagents used in this study were of analytical grade.

Plant material

The *C. cajan* seeds (Variety BDN 711) were purchased from the local market in Jalna and grown in the field at Dabhadi, Taluka Badnapur, District Jalna, Maharashtra, India in July 2015. Twelve weeks old plants with fully expanded leaves were used for all experiments.

Mechanical wounding

Six healthy plants were selected for induction studies. Wounding was made mechanically (20 holes/leaf by needle) and after 72 h, leaves (damaged and undamaged) were collected and crushed in methanol.

Extraction of Secondary metabolites and HGPs:
The leaves samples were kept for sonication at 20 MHz, 1 h for the extraction of secondary metabolites and centrifuged at 5,000 rpm for 30 min. The supernatent was collected and evaporated the methanol to get dry extract. Furthermore this extract was subjected to solid liquid partition with ethyl acetate and methanol. A methanol extract was used for analysis of secondary metabolites and enzyme inhibitory activity.

For extraction of HGPs, fourth instar larvae of *H. armigera* were collected from the fields. Mid gut tissue was isolated from dissected larvae and stored frozen at 4^0 C. Fresh or thawed mid gut tissue was homogenized with 0.1 M glycine NaOH buffer (1:10 w/v) pH 10 for 15 min at 10^0 C. The suspension was centrifuged at 10,000 rpm for 10 min at 4^0 C and the supernatant was used as a source of *H. armigera* gut proteinases (HGP) and for extraction of HGAs mid gut tissue of *H. armigera* was homogenized with 0.02 M sodium phosphate buffer (1:10 w/v) pH 6.9 for 15 min at 10^0 C. The suspension was centrifuged at 10,000 rpm for 15 min at 4^0 C and the supernatant was used as a source of *H. armigera* gut amylase.

Total Phenolics content:
Amounts of total phenolics were determined by using Folin - Ciocalteau assays with slight modification of the previously reported method (Ainsworth and Gillespie, 2007). Gallic acid was used as a reference standard for plotting of calibration curve. Equal volume (10µl) of each fraction (diluted up to 1.5ml with de-ionized water) was mixed with 0.5ml Folin-Ciocalteau reagent and incubated at room temperature for 3 min. Thereafter, each aliquot neutralized with 1ml sodium carbonate (20% w/v).

Reaction mixtures were incubated at room temperature for few min with intermittent shaking for color development. Absorbance of resulting blue color was measured at 650nm using a colorimeter. Amount of total phenolics was estimated from standard graphs of Gallic acid. Amounts of total phenolics were expressed as GAE (mg/g).

Estimation of Total Flavonoids
Amounts of total flavonoids were determined by the previously reported method with slight modification (Siddhuraju and Becker, 2003). A known volume of extract (100 µl) was placed in a test tube, and distilled water was added to make 2 ml and 150 µl of $NaNO_2$ (5 g 100 mL^{-1}), mixed well, after 5 min added 150 µl of $AlCl_3$ solution (1:100). After 6 min, 2 ml of 1 M NaOH solution was added, and the total volume was made up to 5 ml with distilled water.

The solution was mixed well again, and the absorbance was measured against a blank at 520 nm with a colorimeter. Quercetin was used as the standard for the calibration curve.

Proteinase inhibitor assay
Total HGP inhibitor activity was determined by azocasein assay previously reported method with slight modification (Padul *et al.*, 2012). The enzyme inhibitor mixture was prepared by adding varying amounts of leaf extracts to HGP extract in a 0.1 M glycine NaOH buffer at pH 10. This enzyme inhibitor mixture was incubated at 37^0 C for 20 min and added to pre incubated 0.25% azocasein solution at 37^0 C for 30 min in a total reaction volume of 300 µl. Equal volume of 5% chilled TCA (Trichloro acetic acid) was added to terminate the reaction. After incubation at room temperature for 30 min, tubes were centrifuged at 6,000 rpm for 10 min. 600 µl of chilled 1 N NaOH was added to the supernatant to neutralize TCA and diluted by 3ml distilled water. Activity was estimated by measuring OD at 450 nm.

Amylase inhibitor assay:
The amylase inhibition assay was carried out by a previously described method with slight modification (Kwon *et al.*, 2006). The enzyme inhibitor mixture was prepared by adding varying amounts of leaf extracts to HGA extract in a 0.02 M sodium phosphate buffer (1:10 w/v) pH 6.9. This enzyme inhibitor mixture was incubated at 37^0 C for 20 min, diluted up to 1 ml with distilled water. Then 1ml of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006M sodium chloride) was added to each tube. The reaction mixtures were incubated at 37°C for 15 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid (DNS) color reagent.

The test tubes were then incubated in a boiling water bath for 15min and then cooled to room temperature. It was diluted with 6ml distilled water and absorbance was measured at 540nm.

RESULTS AND DISCUSSION

C. cajan is a major human diet pulse in India and its important metabolites contribute antioxidant and Iron chelating activity, may prove value addition in food products as well as drug industries and hence as a good nutraceuticals which can be exploited for alleviating dreaded health problems like cardiovascular diseases.

Phenolic and flavonoids play an important role in plant defense. A significant difference in phenolic content was observed in wounded and control leaves of *C. cajan*. A slight increase in the level of phenolics (1.2 mg/g) was observed in wounded leaves as compared to control leaves 1.1 mg/g (Table.1). Plant phenols are one of the most widespread and well-known groups of defensive compounds which play a vital role in host plant defense against herbivores (Sharma *et al.*, 2009; Usha Rani and Jyothsna, 2010; Ballhorn *et al.*, 2011). An increased level of flavonoids content was found in wounded leaves than control leaves of *C. cajan*. The level of flavonoids content was 0.927 mg/g leaves in the plants treated with wound while flavonoids content was 0.645mg/g fresh leaves in untreated control *C. cajan* (Table.1). Flavonoids play a central role in various facets of plant life, especially in plant environment interaction (Treutter, 2006)

Table 1. Total Phenolics and flavonoids content of untreated and treated plant of *C. cajan*.

Sr No	Total Phenolic content mg/g fresh leaves	Total flavonoids content mg/g fresh leaves
Untreated plant	1.1	0.645
Treated plant	1.2	0.927

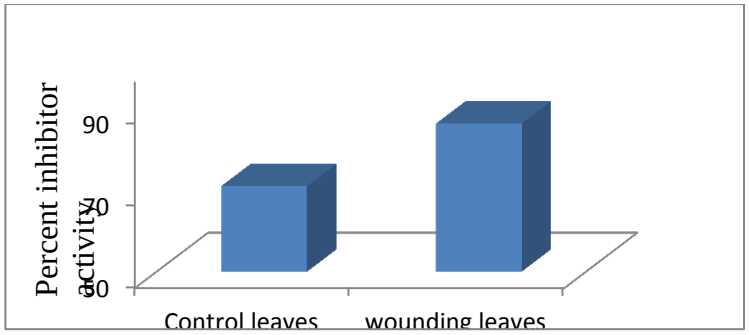

Figure 1. The percent *H. armigera* gut amylase inhibitory activity in the methanol extract of *C. cajan* leaves.

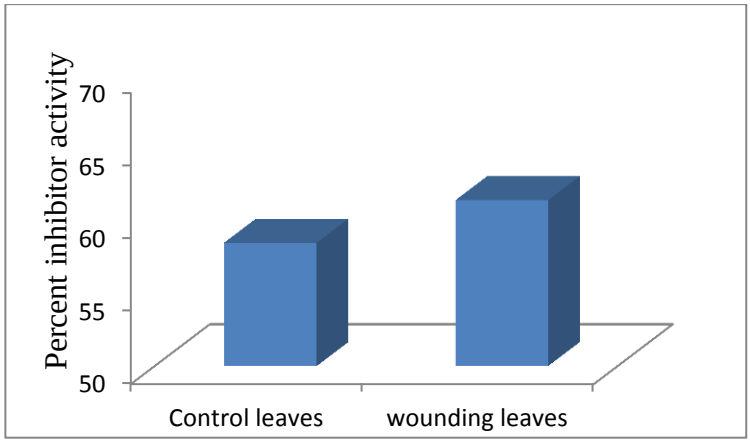

Figure 2. The percent *H. armigera* gut proteinase inhibitory activity in the methanol extract of *C. cajan* leaves.

The percent *H. armigera* gut amylase inhibitory activity of wounded and control leaves were 85.97 and 70.77 respectively (Fig.1). Percent *H. armigera* gut proteinase inhibitory activity of wounded and control leaves were 61.41 and 58.48 respectively (Fig.2). In many plant species leaves, seeds, flowers and tubers contain a high level of serine PIs which play vital role in plant defense against insects (Ryan, 1990; Garcia-Olmedo *et al.*, 1987). The discovery of natural phenols could be an effective approach to develop a "Dietary pesticide" against *H. armigera* infestation. (Joshi *et al.*, 2014). JA and SA induce antioxidative responses in groundnut plants against *H. armigera*, which effected insect growth, behavior and development (War *et al.*, 2015).

It concludes that the wound response of *C. cajan* accumulates phenolic and flavonoids, which are found significant against *H. armigera* gut amylase activity. As there is a correlation between the level of secondary metabolites and enzyme inhibition a search of elicitors which induce accumulation of these defense components becomes the future prospect of this study.

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Abbreviations:
Cajanus cajan (*C. cajan*), *Helicoverpa armigera* (*H. armigera*), *Helicoverpa armigera* gut proteases (HGPs), *Helicoverpa armigera* gut amylase (HGAs), Tri-chloro acetic acid (TCA), Di-nitrosalicylic acid (DNSA), Methyl jasmonate (MJ), Salicylic acid (SA).

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