Mechanism of Entomotoxicity of the Concanavalin A in \textit{Rhopalosiphum padi} (Hemiptera: Aphididae)

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Abstract. The toxicity effect of Concanavalin A (\textit{Canavalia ensiformis} lectin, ConA) to bird cherry-oat aphid, \textit{Rhopalosiphum padi} L. (Hemiptera: Aphididae), was investigated in the laboratory by using artificial diets containing ConA concentrations. Bird cherry-oat aphid performance was affected by the presence of Con A in artificial diets. The lectin added into the liquid diet increased the prereproductive period, mortality, and the average time of generation development (T) and decreased fecundity and the intrinsic rate of natural increase (\(r_m\)). In attempt to unravel the mode of action of ConA, the interaction of the lectin with insect gut and the effect of ConA on feeding behavior were investigated. Extract of gut of treated grain aphid demonstrated DNA fragmentation, and this was accompanied with an increase in caspase 3 activity. Moreover, addition of ConA to the sucrose–agarose gels reduced salivation and passive ingestion of fluids from the gel. The results indicate that the insecticidal activity of ConA on \(R. \ padi\) may involve effects on death of the gut epithelial cells and effects on feeding behavior. This can be employed to create plants that are resistant to aphids.

Key Words: lectin, EPG, antifeeding, apoptosis, caspase 3

Aphids are an extremely successful group that occurs throughout the world, and they are pests of vegetables, grains, tree crops, flowers, and ornamentals. Aphids weaken their host plant diverse ways. First, as phloem feeders, they are diverting for their own profit the nutrients necessary to plant growth and reproduction. Second, during the feeding activity, they inject saliva that could be phytoxic. Third, sooty molds frequently grow on aphids’ honeydew and hinder photosynthetic activity. Finally, aphids are responsible for transmitting ~50% of insect-borne plant viruses, many of which cause diseases of major economic importance in crops (Quiros et al. 2006, Dedryver et al. 2010). Among the 14 most agricultural importance worldwide, aphid pest is the bird cherry-oat aphid, \textit{Rhopalosiphum padi} L. (Hemiptera: Aphididae). \textit{R. padi} is one of the main species from the family Aphididae characterized, in part, by phytophagous phloem feeders with rapid turnover of generations. Moreover, \textit{R. padi} is highly polyphagous with innumerable hosts from the family Poaceae including all the major cereals (Blackman and Eastop 2000, Finlay and Luck 2011). The insect causes the most damage by transmitting a number of viruses, especially barley yellow dwarf virus, for which it is the most important vector (Oswald and Houston 1951, Descamps and Chopa 2011).

Although plant chemicals can be used as biopesticides to control insect pests, aphids are difficult to control because of their unique feeding habits and fast multiplication rates (Majumder et al. 2004). As a consequence, researchers are developing a biotechnological control in which novel genes from plant sources are introduced into plant genomes to enhance the resistance of crop plants to phloem-feeding insects. Several different classes of proteins including lectins, ribosome-inactivating proteins, and protease inhibitors have been tested as alternative resistance factors (Jaber et al. 2010).

Plant lectins are a class of carbohydrate-binding nonimmune origin proteins ubiquitously distributed in a variety of plant species and play different roles and functions in biological processes such as recognition molecules within the immune system in animals (Kilpatrick 2002) and as storage proteins or in defence mechanisms against pest and pathogens in plants. The insecticidal activity of plant lectins against many important insects has been well documented to show their ability to be used as biopesticides (Carlini and Grossi-de-Sá 2002, Jaber et al. 2010). These proteins are particularly important as potential control agents for hemipteran pest/aphids because these insects are not susceptible to any known Bt toxins, and so cannot be controlled by existing plant genetic engineering technologies using Bt toxin genes (Fitches et al. 2008). Previous work showed that mannose and glucose lectins induced the most interesting toxic effects from all classes of lectins tested, snowdrop lectin (GNA) and Concanavalin A (ConA) in particular (Sauvion et al. 2004a). ConA, the first plant lectin with a mannose/glucose-binding specificity, was found in the jack bean (\textit{Canavalia ensiformis}). ConA exists as a homotetramer at physiological pH with molecular mass of 102.5 kDa. Each monomer (non-glycosylated subunits, each of ~27 kDa) consists of Mn2+, Ca2+, and carbohydrate-bind- ing sites (Rüdiger and Gabius 2001, Srirad and Seena 2006). Bioassay has demonstrated that ConA affected survival and delayed development durations, larval weight, and mortality of several aphid species: \textit{Acrithosiphon pismum} (Harris), \textit{Macrosteles albizons} (Essig), \textit{Aphis gossypii} (Glover), \textit{Myzus persicae} (Sulzer), \textit{Macrosiphum euphorbiae} (Thomas), and \textit{Aulacorthum solani} (Kaltenbach) (Hemiptera: Aphididae). What perhaps should be noted here is the fact that the effect of ConA consumption on these species may fluctuate, because some species are highly susceptible to ConA, and the effect on other be less or moderate (Sauvion et al. 2004a, Jaber et al. 2010). Despite current interest in the insecticidal properties of plant lectins, there is no consensus for the mode of action of these proteins toward insects. Three possible modes of action have been suggested. Binding lectins to insect gut structure causes morphological and physiological modifications in the insect intestine (Sauvion et al. 2004b). Another possibility is that the lectins can interfere with digestive enzymes and assimilatory proteins, thereby inhibiting food ingestion (Singh et al. 2006, Sprawka et al. 2012). Moreover, binding of lectins to the carbohydrate moieties associated with membrane proteins of chemosensory sensillae in mouthparts could block the access of chemical signals to receptor proteins, leading to an antifeedant effect (Pyati et al. 2012).

In this article, we have studied the toxicity and the mechanisms of toxicity of ConA in bird cherry-oat aphid. First, we carried out...
experiments to determine the effects of ConA on development, fecundity, and mortality of the bird cherry-oat aphid. Because a recent report that analyzed the action mechanism of lectins at the cellular levels illustrated that effects of binding of lectins to the midgut epithelium may lead to severe anatomical abnormalities with pathological consequences such as apoptosis in insect’s epithelial cells (Hamshou et al. 2010, Shahidi-Noghabi et al. 2010a), the occurrence of apoptosis in the treated insects was investigated. Moreover, to better understand the mechanisms of the interactions between Con A and aphids, we have developed a bioassay to screen for the effect of ConA on R. padi feeding behavior.

Materials and Methods

**Aphid Culture.** The bird cherry-oat aphid (R. padi L.) used in this study were obtained from a stock culture kept at the Siedlce University of Natural Sciences and Humanities. The stock culture was maintained on winter wheat (Triticum aestivum L. cv. Tonacja) in an environmental chamber at 21 ± 1°C, 16:8 (L:D) h photoperiod, and 70% relative humidity (RH). Wingless females were used in all experiments.

**Chemicals.** Lectin Con A were purchased from MP Biomedicals (Santa Ana, California, USA) (CN.150710). Genomic DNA was extracted with the application of Genomic Mini AX Tissue kit (A&A Biotechnology, Gdynia, Poland, www.aabiobiom.com). All dietary components and other chemical reagents were obtained from Sigma (Sigma Chemical Co., Poznań, Poland) and were of analytical or best available grade.

**Aphid Feeding Bioassays.** The liquid diet used for aphid feeding bioassays was prepared as described by Kieckhefer and Derr (1976). The prepared diets (500 mm³) were introduced between two layers of parafilm (Sigma Chemical Co., Poznań, Poland) (sandwich layers), which were placed on plastic rings (h = 1.5 cm, 0 = 3.5 cm³). Con A was incorporated into these mixtures at concentrations 50, 500, 1,000, and 1,500 µg/cm³. Control diets (without Con A) were also included. The diets were sterilized by filtration through 0.45-µm millipore filters. For assays wingless females of R. padi were placed on artificial diet and left to produce nymphs overnight. Adults were then removed, and the nymphs were maintained on the control diet for a further 24 h. After 24 h, nymphs were transferred to new feeding chambers (five insects per dish) containing sachets with test diets. Ten replicates were set up for each treatment for the controls. Ten replicates (five insects per replicate) were used for each treatment for the bioassays. Feeding chambers were kept at 21 ± 1°C, 16:8 (L:D) h photoperiod, and 70% RH, and fresh diet sachets (sandwich layers) were provided every 3 d to avoid fungal and bacterial contamination. Larval development time (prereproductive period), daily fecundity, and mortality of the aphids were monitored daily for 15 d. Population parameters were used to determine the influence of Con A on bird cherry-oat aphid population growth potential. The average time of generation development (T) and the intrinsic rate of natural increase (r_m) were calculated using equations of Wyatt and White (1977):

\[
T = d / 0.74
\]

\[
r_m = [0.74(ln Md)] / d
\]

were d is the length of prereproductive period, Md the number of larvae born during the reproduction period, which equals the d period, and 0.74, the correction factor.

**Lectin-Induced Apoptosis.** In the next experiment, R. padi adult aphids were placed on artificial control diet (without phytohemagglutinin [PHA]) and diet containing 1,500 µg/cm³ of ConA as described above. For feeding chamber, 30 apterae morphs were placed on feeding sachets, and the experiment was repeated three times. After 48 h of diet probing, aphids were collected. Next, the entire guts of adult aphids were dissected under the binocular and analyzed both for DNA fragmentation and caspase-3-like activity.

**DNA Fragmentation Assay.** The dissected aphid guts (60 guts) were collected in sterile deionized water. Genomic DNA was extracted from aphid guts with the application of Genomic Mini AX Tissue kit (A&A Biotechnology, www.aabiobiom.com), following the manufacturer’s protocol. The quantification of DNA samples was conducted using an Epoch Microplate spectrophotometer (BioTek Instruments, Inc.). Additionally, A260/280 and A260/230 ratios were calculated to evaluate the sample integrity and contamination of proteins or other organic substances. DNA preparations of high integrity and purity were accepted for electrophoretic analysis. Separation of DNA samples (8 µg) was performed using a horizontal gel electrophoresis (2% agarose) under standard conditions. DNA fragments were detected using ethidium bromide staining and UV transillumination. The low-molecular weight marker (Genoplast Biochemicals, Poland, 50-bp ladder) was used as standard.

**Caspase Activity Assay.** Dissected gut tissues of R. padi adults were incubated in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, and 5 mM DTT). The caspase 3 activity assay was carried out with the application of Caspase 3 Colorimetric Assay Kit (Sigma-Aldrich, Poznań, Poland, PC CASP-3-C), following the manufacturer’s protocol. This assay is based on the amount of p-nitroaniline released from hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) by caspase 3. The assay can be performed in 1 cm² volume and measured using a spectrophotometer. Activity of caspase 3 was expressed as nanomoles (nmol) of released p-nitroanilin per minute per cm². Three insect sampling were made for each assay.

**Effect of ConA on R. padi Feeding Behavior.** The effect of lectin Con A on R. padi feeding behavior was investigated in vitro, using sucrose–agarose gels. Gels were prepared by incorporating 1.25% agarose (Sigma A-0169) into a 30% sucrose solution. After the mixtures were stirred, they were heated in a water bath (75°C for 15 min). ConA at 50, 500, 1,000, and 1,500 µg/cm³ was added to the mixtures when they had cooled to 37°C; control gels without ConA was also prepared. The cool but molten mixtures were then poured into plastic rings (10 mm in height and 15 mm in diameter) covered with a stretched Parafilm M membrane. Transparent gels formed after 1–2 min and were offered to aphids for probing.

The experiment was investigated using the electrical penetration graph (EPG) technique that is frequently employed in insect probing and feeding behavior studies (Tjallingii 1995, Sauvion et al. 2004a, Pettersson et al. 2007). The EPG technique makes it possible to record different waveform patterns related to aphid activities and stylot locations during penetration, usually within the plant tissue (Sauvion and Rahbe 1999). Apterial adults were collected between 6 and 7 a.m., attached to a golden wire electrode (2 cm in length, 20 µm in diameter) with conductive silver paint (Demetron, L2027, Darmstadt, Germany) and starved for 2 h prior to the experiment. After the aphids were starved probing, the feeding behavior was monitored for 4 h continuously with the two four-channel DC EPG recording equipment (Wageningen, Agricultural University, Entomology Department, The Netherlands). Each aphid was given access to a freshly prepared sucrose–agarose gel. The recordings of all replicates of a same treatment were performed during two consecutive days due to equipment limitations. All experiments were started at 9–10 a.m. Signals were saved on the IBM computer through a DAS 8 SCSI acquisition card (Keithley). During EPG recordings, aphids were in a Faraday cage in the laboratory (21 ± 1°C, 16:8 (L:D) h photoperiod, and 70% RH). EPG recordings were made for 10 aphids on gels without lectin (control) and for 10 aphids for each lectin concentration (50, 500, 1,000, 1,500 µg/cm³).

EPGs were acquired and analyzed with STYLET 2.2 software provided by W.F. Tjallingii. Waveforms were identified by analogy to those waveforms found by others in artificial diets and sucrose–agarose gels (Sauvion and Rahbe 1999, Sauvion et al. 2004a, Golawska and Cid and Fereres 2010, Sprawka and Goławska 2010, Goławska and
Results

**Effects of ConA on Bird Cherry-Oat Aphid Adult in Artificial Diet.**

In this study, the influence of ConA was demonstrated in significant prolongation of prereproductive period and thus in the average time of generation development (T) of this bird cherry-oat aphid. The prereproductive period increased from 6.60 d in control to 11.20 d at 1,500 μg/cm³ i.e. increase of 4.6 d. Thus, the average time of generation development increased by 6.20 d (Table 1). The fecundity and the intrinsic rate of natual increase (rₚ) was also deleteriously and significantly affected (P < 0.01; P < 0.001, t test). As can be seen from table, the mean daily fecundity declined from 1.43 in control diets to 1.16 at 1,500 μg/cm³ of ConA in the diet. The intrinsic rate of natural increase also showed a decline in dose-dependent manner being 0.17 at 1,500 μg/cm³ when compared with control diet. Only for the 50 μg/cm³ concentration of ConA significant differences were not found (P > 0.05, t test). Wingless females of bird cherry-oat aphid on all concentrations of PHA showed a significant (P < 0.001, P < 0.01 t test) mortality in comparison to insects, which fed on control diets (Table 2).

However, there was found significant relationship between studied concentrations of ConA and population parameters: the aphid fecundity (R = −0.92, P < 0.001, Pearson correlations), larval development (R = 0.90, P < 0.001, Pearson correlations), average time of generation development (R = 0.90, P < 0.001, Pearson correlations), intrinsic rates of natural increase (R = −0.95, P < 0.001, Pearson correlations), and mortality (R = 0.95, P < 0.001, Pearson correlations).

Table 2. Effect of ConA on mortality of *R. padi* (values are means ± SD)

| Concentration of lectin (μg/cm³) | Mortality (%) |
|---------------------------------|---------------|
| Control                         | 2.00 ± 4.47   |
| 50                              | 20.00 ± 8.16* |
| 500                             | 25.00 ± 5.77**|
| 1,000                           | 45.11 ± 5.77**|
| 1,500                           | 75.00 ± 5.77**|

Significance of differences from the control values **P < 0.001; *P < 0.01 (Student’s t-test) (n = 10).
The addition of 2 mM Ac-DEVD-CHO blocked caspase-3 activity (ConA/inhibitor). Values are presented as mean (±SD) based on three individual repetitions.

Discussion

Lectins incorporated in artificial diets or expressed in different transgenic plants have been shown to reduce the growth and development of several insect species (Carlini and Grossi-de-Sá 2002, Vanderborre et al. 2009). It is particularly pertinent to the control of homopteran insects pests where no effective Bt strains have as yet been identified for control of insects within this order. In this study, the effects of ConA in artificial diet on survival, development, and fecundity of R. padi were investigated. In general, bird cherry-oat aphid performance was affected by the presence of Con A in artificial diets. When R. padi was fed, an artificial diet containing the lectin ConA, its fecundity was reduced, its prereproductive period and generation time were prolonged, and its morality was increased.

The mannose/glucose-specific lectin ConA has been used a lot in feeding experiments on pest herbivores. When fed to larvae of tomato moth, Lacanobia oleracea L. in artificial diet, or on ConA-expressing potato plants, larval development was retarded, 90% mortality was scored, and also showed a decrease in larval weight (Gatehouse et al. 1999). Toxicity of ConA to Meligethes aeneus F. was also reported by Melander et al. (2003). Similarly, when ConA was added to artificial diets and fed to tara planthopper, Tarophagous Proserpina Kirkaldy, a corrected mortality of 93% was noted (Powell 2001). Moreover, artificial diet studies have shown that this lectin has insecticidal activity, particularly toward Homoptera including the rice brown planthopper, Nilaparvata lugens Stål (Powell et al. 1993), and the pea aphid, A. pisum (Rahbe and Febvay 1993, Rahbe et al. 1995). The effects of ConA on the peach-potato aphid, M. persicae, also show that the lectin has deleterious effects on growth and development in this insect when administrated in artificial diet, resulting in decreased fecundity. In agreement with the diet bioassay results, ConA-expressing potatoes decreased the fecundity of M. persicae by up to 45% (Sauvion et al. 1996, Gatehouse et al. 1999).

Although the biochemical properties and insecticidal activity of lectins are well studied, but our knowledge about the exact mechanism of action of lectins is still limited. The insecticidal activity of plant lectins is to be related to the sugar-binding capacity of these proteins. When plant material is ingested by insect herbivores, the primary site of action for lectins is the insect digestive tract. Because most of the digestive enzymes or transport proteins secreted in the gut of insects or proteins embedded in the epithelial cell membrane contain glycan structures, these glycoproteins are all potential targets for plant lectins (Vandenborre et al. 2011). Ultrastructural studies have shown insecticidal lectins to be bound to suitable glycosylated targets in the insect gut (Habibi et al. 2000, Fitches et al. 2001, Majumder et al. 2004). Immunolocalization studies showed that ConA interacts with glycosylated receptor present at the cell surface or within the midgut epithelium cells. Moreover, immunohistochemical and electron microscopy studies revealed that ConA induced severe cellular swelling of epithelial cells, accompanied by hypersecretion and progressive detachment of apical membrane (Sauvion et al. 2004b). A recent report that analyzed the lectins interaction with specific carbohydrate moieties on the midgut epithelial cells illustrated that it is an important step in lectin-mediated cell killing. Moreover, it was shown that the cytotoxicity of the lectin is mediated via induction of apoptosis (Hamsouh et al. 2010, Shahidi-Noghabi et al. 2010a). The results obtained here confirm this hypothesis. Samples of gut of R. padi showed two main characteristics of cell death or apoptosis. We showed a clear DNA fragmentation in R. padi guts. In addition, we also demonstrated that caspase 3 activity is induced in R. padi tissue, suggesting the involvement of this enzyme in the entomotoxic activity of ConA. Therefore, it can be suggested that caspase 3 is causing DNA fragmentation in the gut epithelium, and this in turn is responsible for the toxicity of ConA in bird cherry-oat aphid. Little research has been done to investigate the induction of apoptosis by plant lectins at insect level. The exposure of insect midgut CF-203 cells to Selerotinia sclerotinum agglutinin resulted in DNA fragmentation, but the effect was caspase-3 independent (Hamsouh et al. 2010). But Shahidi-Noghabi et al. (2010a) reported that the samples of the (mid)gut of A. pisum and Spodoptera exigua Hübner upon feeding on the diet containing Sambucus nigra agglutinins (SNA) showed two main characteristics of apoptosis: a clear DNA fragmentations and the induction of caspase-3-like activity. Similarly, both SNA-I and SNA-II induced caspase-dependent apoptosis, leading to typical symptoms of cell death in midgut CF-203 cells (Shahidi-Noghabi et al. 2010b). However, the induction of apoptosis in mammalian cells under the influence of plant lectin has been studied intensively because lectins elicit apoptosis in different cancer cell lines (Fu et al. 2011). Hitherto, several plant lectins such as wheat germ agglutinin, ricin, abrin, and ConA and PHA have been well studied to possess antiproliferative and apoptosis-inducing activities toward normal or cancer cells. But the mechanisms of apoptotic effects of lectins remain mostly unknown. Apoptosis can be mediated by death receptors initiated by lectins. Fas...
ConA, aphids would spend longer times in penetrating the mesophyll, aphids would be delayed in their initiation of salivation and ingestion, and would spend less time in passive ingestion of phloem sap. Similar tendency was observed by Sauvion et al. (2004a) who also studied the effect of ConA on feeding behavior of the A. pisum. ConA caused some alterations in pea aphid ingestion within the first 4 h. At this stage, ConA did not alter the frequency of behaviors but significantly affected the duration of behaviors: in the presence of ConA, nonprobing and salivation bouts were longer, whereas ingestion phases were significantly shorter. In another study, the mannose-binding snowdrop lectin (GNA) influenced the feeding behavior of the N. lugens; addition of GNA to the diet-reduced diet ingestion (Powell and Gatehouse 1996). Moreover, Sprawka et al. (2013) showed that when the grain aphid, Stitobion avianae, fed on artificial diet with PHA (Phaseolus vulgaris lectin), duration of pathways was increased by higher concentrations of PHA and the number of penetrations was reduced. Detailed understanding of how lectins modulate behavior especially feeding behavior remains unknown. Most studies agree that the effects of plant lectins on insect behavior are a consequence of intoxication rather than a direct sensory-mediated response of the insect (Sauvion et al. 2004a). Alternatively, binding of the lectin to the carbohydrate moieties associated with membrane proteins of chemo-sensory sensilla in mouthparts could block the access of chemical signals to receptor proteins, leading to an antifeedant effect (Pyati et al. 2012).

In conclusion, when ingested in an artificial diet, ConA had dose-dependent detrimental effects on growth, fecundity, and survival of bird cherry-oat aphid. These detrimental effects were associated with the death of the gut epithelial cells and with feeding suppression. Future studies will be necessary to identify and characterize the exact binding target receptor(s) (carbohydrate-specific binding proteins and their glycosylation pattern) for ConA in the cell membrane at the aphid’s epithelial gut cells, taste, and olfactory receptors.

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