

Insecticidal activity of a protein extracted from bulbs of *Phycella australis* Ravenna against the aphids *Acyrtosiphon pisum* Harris and *Myzus persicae* Sulzer

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ABSTRACT

Aphids cause significant losses in many agricultural crops and in many cases cause repeated insecticide sprays, which increase the risk of resistance. Therefore, other alternatives are needed to control them. The toxic, anti-reproductive, and feeding deterrent effects of a mannose-binding lectin isolated from bulbs of *Phycella australis* Ravenna (Amaryllidaceae), named *Phycella australis*-agglutinin (PAA) was assayed on nymphs of the aphids *Acyrtosiphon pisum* Harris and *Myzus persicae* Sulzer fed with an artificial diet. After 72 h of PAA exposure, lethal concentration (LC₅₀) values were 109 and 313 $\mu\text{g mL}^{-1}$ for *A. pisum* and *M. persicae*, respectively, while LC₉₀ values were 248 and 634 $\mu\text{g mL}^{-1}$. Sub-lethal concentrations of PAA significantly reduced the aphid fecundity at a concentration of 80 $\mu\text{g mL}^{-1}$. Only a total of 5.7 descendants per female were recorded for *A. pisum* (32% control progeny) and 12.4 for *M. persicae* (39% control progeny). *Acyrtosiphon pisum* was strongly deterred by PAA under choice conditions, as after 72 h exposed to 80 $\mu\text{g PAA mL}^{-1}$ of diet, the feeding deterrent index was 0.91 for *A. pisum* and only 0.38 for *M. persicae*. In conclusion, the mannose-binding lectin isolated from bulbs of *P. australis* showed acute and chronic insecticidal activity against the pea and green peach aphids.

Key words: Aphid control, botanical insecticides, entomotoxic proteins, lectins.

INTRODUCTION

Aphids are one of the main phytophagous insect groups of economic importance to agriculture worldwide, causing direct damages by sucking the phloem sap and indirectly by producing honeydew on leaves and fruits (Dedryver et al., 2010). In addition, these insects also have great relevance as vectors of many viruses and phytoplasmas affecting crops (Weintraub and Beanland, 2006; Fereres and Moreno, 2009).

The green peach aphid *Myzus persicae* Sulzer is an extremely polyphagous insect, usually settling in very early in the crop development, their colonies stunt plant growth and induce water stress that causes wilting of leaves, flower buds and young fruits (Blackman and Eastop, 2007). On the other hand, the pea aphid *Acyrtosiphon pisum* Harris is a specialist in legumes, including important forage and vegetable crops (Blackman and Eastop, 2007).

Synthetic insecticides have been commonly used to control these pests but the use of some pesticides has been restricted due to the negative impact on the environment. Many synthetic insecticides may harm animals including humans; they have side effects on non-target insects, and become ineffective due to resistance developed by aphids (Foster et al., 2007). All this has stimulated research toward new suitable alternatives for aphid control.

In this context, phytochemicals may play a leading role and become valuable tools for crop protection (Dwijendra, 2014). Plants have developed defensive strategies using different biochemical ways to avoid herbivory (Cornell and Hawkins, 2003), including the production and storing of entomotoxic proteins (Carlini and Grossi-de-Sá, 2002). A group of carbohydrate-binding proteins, called lectins, are among the proteins with the most powerful insecticidal activity (Vandenborre et al., 2011). In plants, most of the lectins can be found in vacuoles of storage tissue cells (e.g. bulbs) (Van Damme et al., 1998). Plant lectins differ in their molecular structure and specificity to carbohydrates and are grouped in families based on their different carbohydrate-binding domains (Van Damme et al., 2008). The *Galanthus nivalis*-agglutinin family (GNA-related lectins), also called monocot mannose-binding lectins, is by far the lectin family that has been studied most intensively for its insecticidal activity (Van Damme, 2008). The mannose-binding lectins have shown strong insecticidal activity against chewing and sap-sucking insects, and particularly in controlling aphids (Sauvion et al., 1996; Sadeghi et al., 2009a; Vandenborre et al., 2011). Plant lectins can have

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detrimental effects on survival, growth, development, and feeding behavior when they are ingested by aphids (Sadeghi et al., 2009a; Sprawka and Golawska, 2010).

Globally, monocot mannose-binding lectins have been typically found in at least six different monocot families, including Amaryllidaceae, in which their occurrence has been better documented (Van Damme et al., 1998). In Chile, this plant family is represented by numerous species, many of which are endemic and have potential as ornamentals. The alkaloid content of some endemic Chilean Amaryllidaceae has been studied (Echeverría and Niemeyer, 2012), but very few knowledge on the lectins that they may contain is available, especially on the potential insecticidal activity. This is the case of *Phycella australis* Ravenna, which can be found in the coastal area of the South-Central Region of Chile (Baeza et al., 2009). Considering the above mentioned, the aim of this work was to study proteins isolated from bulbs of the Chilean Amaryllidaceae *P. australis*, and their defensive potential against *A. pisum* and *M. persicae*.

MATERIALS AND METHODS

Plant material and crude extraction

In October 2013 (spring season in the Southern Hemisphere), 2 kg of *P. australis* bulbs were collected in South-Central Chile (36°04' S, 73°10' W). The species was confirmed in the Herbarium of Department of Botany, University of Concepción, Chile. Fresh plant material was washed with distilled water to remove any debris and stored at 4 °C until the extraction.

For the preliminary extraction, 5 g were homogenized with a mortar in unbuffered 20 mM 1,3-diaminopropane (DAP) (3:1). The extract was transferred into microcentrifuge tubes and centrifuged (13 000 g; 5 min), and the supernatant was collected and stored at 4 °C for 12 h and centrifuged again under the same conditions as described above. The total collected supernatant was analyzed in order to determine protein concentration by Bradford assay (Kruger, 2002), using pure GNA lectin as a standard. Subsequently, the total supernatant was lyophilized and then used in the initials bioassays, previously re-suspended at the desired concentration.

Extraction of *P. australis* mannose-binding lectin (PAA)

Whole bulbs of *P. australis* (1 kg) were homogenized in a blender in 5 L of 20 mM DAP containing thiourea to prevent oxidation reactions in the extract (0.5 g L⁻¹). The extract was passed through glass wool, the pH was set at 7.0, and then CaCl₂ was added (1 g L⁻¹). Subsequently, the extract was stored at 4 °C and after 12 h, the extract was passed through a filter paper and centrifuged at 3000 g for 10 min. The pH of the supernatant was set at 4.0 using 4 M HOAc and the extract was again centrifuged at 3000 g for 10 min, and the supernatant was collected. The purified

lectin, which was named *P. australis* agglutinin (PAA), was purified from the supernatant by affinity chromatography on mannose-Sepharose 4B and further purified by ion exchange chromatography as described previously (Mo et al., 1993). During the purification process, the lectin activity was followed by optical density (OD₂₈₀ > 0.3) and by agglutination of trypsinized rabbit erythrocytes. The purity of the protein was judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Aphids rearing

Acyrtosiphon pisum and *M. persicae* were obtained from stock colonies maintained in the Laboratorio de Fitoquímica, Facultad de Agronomía, Universidad de Concepción, Chillán, Chile. *Acyrtosiphon pisum* was reared on faba bean and *M. persicae* on sweet pepper plants in a bioclimatic chamber at 25 °C, 65 ± 5% RH and 16:8 h photoperiod. The same environmental conditions were used for the experiments. Mature adult aphids were put on plants for 24 h and the resulting offspring (aged 0-24 h) was used in the experiments.

Dose-response assays

The protein crude extract (0.1%-0.8%) or lectin (50-800 µg mL⁻¹) were serially diluted and assayed by ingestion in artificial diet, consisting of water plus aminoacids, vitamins, minerals, and sugar as described above (Febvay et al., 1988). These compounds were added into the diet dissolved previously in DAP (2% as final concentration). The diet was offered to the aphids in a feeding apparatus consisting of a food sachet containing 100 µL of the artificial diet sandwiched between two layers of Parafilm stretched over a plexiglass ring (3.0 cm diameter and 3.0 cm high) as described in Sadeghi et al. (2009b). Twenty nymphs (< 24 h-old) were transferred from the faba bean (*A. pisum*) or sweet pepper (*M. persicae*) plants onto the sachet using a camel-hair brush and then the sachet was confined within a small ventilated Petri dish. Each treatment was replicated four times under the same environmental conditions described for the insect rearing. Mortality percentages were determined after 24, 48, and 72 h of treatment, and LC₅₀ and LC₉₀ values were calculated.

Lectin activity on reproduction of aphids

The activity of sublethal concentrations of PAA on the fecundity of the aphids was assayed by ingestion as described previously. We treated the aphids in two ways: treatment of the aphids only during their nymphal stage (N), or treatment of the aphids only during the adult stage (A). We transferred individually 10 specimens at day 0 into sachets containing artificial diet with different concentrations of PAA (20, 40, 60, and 80 µg mL⁻¹) and control diet (artificial diet and DAP). Each experiment was replicated four times. Every 3 d the aphids were transferred into newly prepared feeding

sachets. The progeny was removed from the rearing chamber, counted, and the number of nymphs produced along the life span of the treated insects was calculated.

Lectin feeding deterrent activity

The PAA deterrent activity for the two aphid species was tested in a binary choice setup. The experiments were carried out using the same feeding units described previously. However, in this case, pairs of rings containing treated and untreated diet were offered. Once the diet was set in each ring, the rings were immediately attached to each other by their open ends and 20 nymphs (< 24 h-old) were located in the middle of this setup, at the same distance of normal and PAA diet. This allowed that the nymphs had the same probability of feeding at treated diet or untreated diet. Four concentrations of lectins were assayed (20, 40, 60, and 80 $\mu\text{g mL}^{-1}$), and four replicates for each one were considered. The feeding units had two side holes (8 mm diameter) covered with fine mesh that provided ventilation. The units were kept into the bioclimatic chamber in a horizontal position so that the aphids could easily walk from one side to the other. The numbers of aphids on treated and non-treated areas were counted after 4, 8, 12, 24, 48, and 72 h. A feeding deterrence index was calculated as follows $(C-T)/(C+T)$, where C is numbers of aphids feeding on the untreated diet and T is numbers of aphids feeding on the treated diet (Powell et al., 1997).

Data analysis

One-way ANOVA was performed to determine significant differences among concentrations ($P < 0.05$). Then, least significant difference (LSD) multiple range tests were used to separate means. Percentages of mortality were transformed into arcsine square root values for analysis. A probit analysis was conducted to estimate the LC_{50} and LC_{90} values with their corresponding 95% confidence intervals (CI) by POLO Plus V 2.0 (LeOra Software, Berkeley, California, USA); LC values are significantly different if their respective 95% CI are not overlapping.

RESULTS

Dose-response assays

According to the Bradford test, total protein content in *P. australis* bulbs was 3.1 mg g^{-1} bulb tissue (fresh weight) and the yield of the mannose-binding lectin PAA reached 1.13 mg g^{-1} bulb tissue, representing 36.4% of the soluble protein.

The insecticidal activity of crude extract and PAA lectin on *A. pisum* and *M. persicae* was time and dose-dependent (Tables 1 and 2). After 72 h exposure, food added with 0.1% to 0.8% of crude extract showed 10% to 100% of *A. pisum* mortality, and 4% to 78% of *M. persicae* mortality. When purified lectin was used in the diet instead of crude extract, 400 $\mu\text{g mL}^{-1}$ PAA lectin killed the 100% of *A. pisum*, and 800 $\mu\text{g mL}^{-1}$ PAA killed 75% of *M. persicae* aphids.

Table 1. Mortality (%) of *Acyrtosiphon pisum* and *Myzus persicae* fed for 24, 48, and 72 h on diet supplemented with increasing concentrations of *Phycella australis* lyophilized crude extract.

Extract concentration (%)	<i>A. pisum</i>			<i>M. persicae</i>		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	0 ± 0a	1 ± 1a	1 ± 1a	0 ± 0a	1 ± 1a	1 ± 1a
0.1	1 ± 1a	7 ± 1b	10 ± 2b	0 ± 0a	3 ± 2b	4 ± 2b
0.2	2 ± 2a	10 ± 2b	24 ± 4c	2 ± 1b	10 ± 3c	12 ± 3c
0.4	6 ± 2b	38 ± 8c	67 ± 6d	8 ± 1c	24 ± 2d	37 ± 2d
0.8	27 ± 8c	92 ± 3d	100 ± 0e	20 ± 0d	57 ± 7e	78 ± 7e

Data are expressed as means ± SE. Means within the same column followed by the same letter are not different, according to ANOVA, least significant difference test ($P > 0.05$).

Table 2. Mortality of *Acyrtosiphon pisum* and *Myzus persicae* fed for 24, 48 and 72 h on diet supplemented with increasing concentrations of *Phycella australis* mannose-binding lectin (PAA).

PAA concentration ($\mu\text{g mL}^{-1}$)	<i>A. pisum</i>			<i>M. persicae</i>		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
50	5 ± 1b	9 ± 2b	28 ± 3b	6 ± 1b	6 ± 1b	6 ± 1b
100	10 ± 1c	31 ± 4c	50 ± 1c	10 ± 1c	31 ± 4c	30 ± 2c
200	13 ± 1d	46 ± 3d	78 ± 5d	23 ± 3d	36 ± 5c	37 ± 4d
400	39 ± 3e	85 ± 5e	100 ± 0e	34 ± 3e	54 ± 2d	56 ± 6e
800	59 ± 4f	92 ± 3e	100 ± 0e	43 ± 3f	64 ± 7d	75 ± 7f

Data are expressed as means ± standard error. Means within the same column followed by the same letter are not different according to ANOVA, least significant difference test ($P > 0.05$).

A comparison of *P. australis* protein crude extract and PAA lethal concentrations (LC_{50} and LC_{90}) against *A. pisum* and *M. persicae* indicated that crude extract and lectin toxicity differs between aphid species, since fiducial intervals do not overlap (Table 3). The crude extract LC_{50} for *A. pisum* and *M. persicae* after 72 h was 0.33% and 0.48%, respectively, while LC_{90} values were 0.54% and $> 0.8\%$. The PAA LC_{50} was 109 and 313 $\mu\text{g mL}^{-1}$ for *A. pisum* and *M. persicae* respectively, whereas the PAA LC_{90} was 248 and 634 $\mu\text{g mL}^{-1}$.

Lectin effect on aphid reproduction

The PAA lectin significantly decreased the fecundity of *A. pisum* and *M. persicae*, when provided at sublethal doses

Table 3. Lethal concentrations (LC) of *Phycella australis* crude extract and purified lectin (PAA) against *Acyrtosiphon pisum* and *Myzus persicae* after 72 h of feeding on diet.

Aphid	Compound	LC_{50}	LC_{90}	Chi square (X^2)	df
		(95% CI)	(95% CI)		
<i>A. pisum</i>	Crude extract (%)	0.33 (0.30-0.36)	0.54 (0.49-0.62)	0.28	2
	Lectin ($\mu\text{g mL}^{-1}$)	109 (91-127)	248 (219-293)	1.17	3
<i>M. persicae</i>	Crude extract (%)	0.48 (0.41-0.55)	> 0.8	0.77	2
	Lectin ($\mu\text{g mL}^{-1}$)	313 (185-538)	634 (453-1366)	6.41	3

LC: Lethal concentrations; df: degrees of freedom. Values in brackets refer to fiducial limits (CI).

through the food (Table 4 and Figure 1a-1d). The number of descendants per female decreased as the concentration of lectin in the diet increased.

Table 4. Total number of progeny per surviving female aphid of *Acyrtosiphon pisum* and *Myzus persicae* reared on sublethal concentrations of mannose-binding lectin isolated from bulbs of *Phycella australis* (PAA).

PAA concentration ($\mu\text{g mL}^{-1}$)	Total fecundity (nymphs per adult)			
	<i>A. pisum</i>		<i>M. persicae</i>	
	N ¹	A ²	N ¹	A ²
Control	17.5 ± 2.4a	15.2 ± 1.5a	31.8 ± 1.1a	33.1 ± 2.7a
20	14.1 ± 1.8ab	13.1 ± 1.1ab	30.9 ± 2.4a	33.7 ± 2.8a
40	12.7 ± 0.8b	11.6 ± 1.3b	25.0 ± 1.8b	29.3 ± 1.4a
60	10.1 ± 1.4c	7.1 ± 0.7c	18.3 ± 2.2c	31.6 ± 2.7a
80	5.7 ± 0.8d	6.9 ± 1.5c	12.4 ± 1.4d	24.9 ± 1.3b

Data are expressed as means ± standard error. Means within the same column followed by the same letter are not significantly different according to ANOVA, least significant difference test ($P > 0.05$).

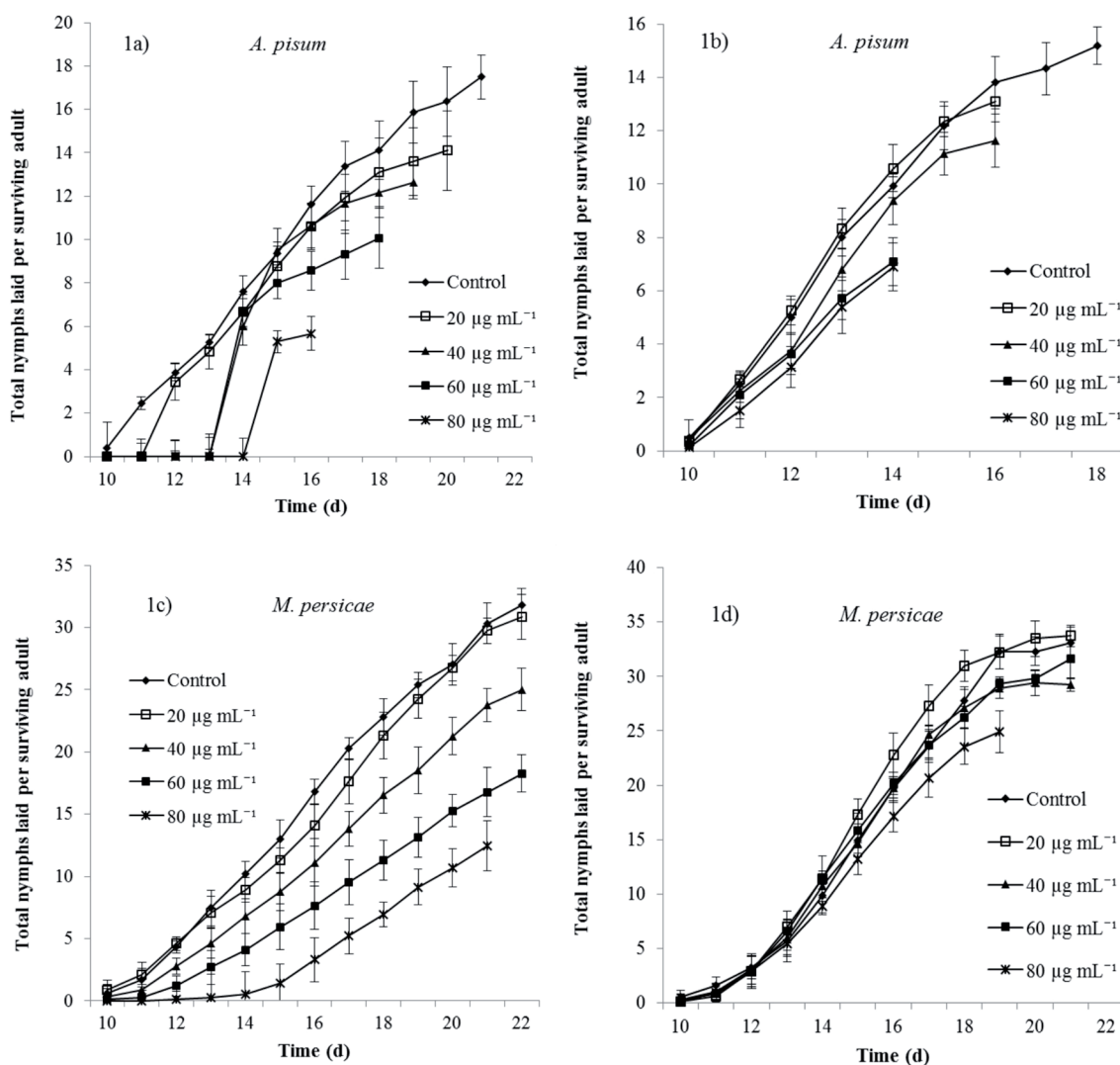
¹N: Aphids were fed on lectin during their entire nymphal stage.

²A: Aphids were fed on lectin only during their adult stage.

For *A. pisum*, there was no difference when the insects consumed the lectin in nymphal or adult stage. The fecundity decreased significantly with concentrations equal or greater than $40 \mu\text{g mL}^{-1}$ of lectin in the diet. With the highest concentration ($80 \mu\text{g mL}^{-1}$) only a total of 5.7-6.9 descendants per female were recorded, which represents a 32%-45% of the control progeny. *Acyrtosiphon pisum* nymphs showed a delay in reaching adulthood when they were fed with PAA lectin (1-4 d), and a shortening of the reproductive period of females compared to the control was also observed (data not shown).

In terms of fecundity, *M. persicae* proved to be more sensitive to PAA when treated in nymphal stage than in adult. It was necessary to incorporate a concentration of $40 \mu\text{g mL}^{-1}$ of lectin in the diet to reduce significantly their fecundity with respect to the control, at the same time only 12.4 nymphs per adult were recorded with the highest concentration tested. In contrast, when *M. persicae* were treated with lectin in the adult stage only the maximum concentration significantly decreased fecundity (24.9 nymphs per female).

Figure 1. Cumulative fecundity of surviving adults. 1a) *Acyrtosiphon pisum* rearing fed on lectin during their entire nymphal stage. 1b) *Acyrtosiphon pisum* rearing fed on lectin only during their adult stage. 1c) *Myzus persicae* rearing fed on lectin during their entire nymphal stage. 1d) *M. persicae* rearing fed on lectin only during their adult stage.



Data are expressed as means ± standard error.

Lectin feeding deterrent activity

Acyrtosiphon pisum was deterred by lectin in the choice experiment (Figure 2). The deterrent activity was time and dose-dependent. The most significant activity was recorded at 72 h exposure. For the highest concentration ($80 \mu\text{g mL}^{-1}$), the feeding deterrent index was 0.91; while for the lower concentrations, the deterrent index reached only 0.12 as maximum. *Myzus persicae* was only slightly deterred by the PAA lectin, because at 72 h the deterrent index reached just 0.38 for the highest concentration tested ($80 \mu\text{g mL}^{-1}$).

DISCUSSION

Considering that more and better options are needed to reduce the incidence of aphids in crops, new alternatives should be studied for this purpose. A search for new lectins with outstanding insecticidal activity could yield interesting new proteins. Focus on endemic plants that grow naturally in restrictive environments can offer many possibilities due to the powerful phytochemical arsenal they have.

The concentration of total protein found in the bulbs of *P. australis* was lower than that found in dormant bulbs of *Galanthus nivalis* L. (Amaryllidaceae) by Van Damme (2008). The low lectin content found in bulbs of *P. australis* could be seasonal, because the plants were blooming when we harvested them, a stage that is not stockpiling (Kamenetsky et al., 2005).

To our knowledge, this is the first report on the insecticidal activity of protein fractions from *P. australis* bulbs. Our results indicated that both the crude protein extract and the pure lectin PAA significantly affected the survival of *A. pisum* and *M. persicae*. The effectiveness of these compounds was dependent on the aphid species, the concentration and time of exposure to the toxic protein.

Based on our findings, the mannose-binding lectin PAA isolated from *P. australis* had a strong effect on survival against first instars of the pea aphid ($\text{LC}_{50} = 109 \mu\text{g mL}^{-1}$) and the green peach aphid ($\text{LC}_{50} = 313 \mu\text{g mL}^{-1}$). Previously,

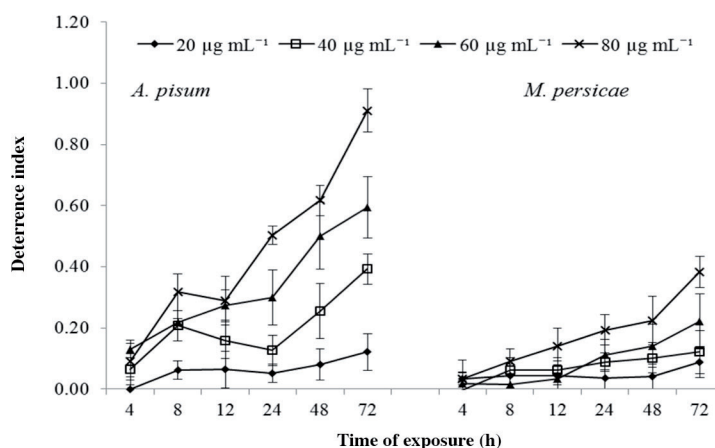
other types of lectins that bind to mannose also adversely affected the survival of aphids. Sadeghi et al. (2009a) reported an LC_{50} value for GNA of $350 \mu\text{g mL}^{-1}$ for pea aphids, which is about three times more than what is required in the current study to kill the same percentage of aphids. Down et al. (2006) reported < 10% mortality of *M. persicae* after 14 d exposure to diet containing 1.4 g L^{-1} of GNA lectin.

The response of each species of aphid to phytochemicals was quite different. So that, *A. pisum* proved to be more sensitive than *M. persicae* to this protein. Previously, Sadeghi et al. (2009a) also concluded that *A. pisum* was more sensitive (76% mortality at $800 \mu\text{g mL}^{-1}$) than *M. persicae* (42% at $1500 \mu\text{g mL}^{-1}$) to GNA lectin when compared their results to the reported by Sauvion et al. (1996). We believe that this difference could be related to a different metabolic activity of the aphids, as a result of their feeding habits (Rahbé et al., 1995; Blackman and Eastop, 2007; Cabrera-Brandt et al., 2010).

Sublethal concentrations of the PAA lectin also adversely affected the reproduction of *A. pisum* and *M. persicae*, resulting in a lower number of progeny (fecundity) for both species. For this parameter, *A. pisum* also proved to be more sensitive than *M. persicae*. These findings are consistent with those reported for *A. pisum* by Sadeghi et al. (2009b), when they fed aphids from nymph and throughout its life with the lectin APA (another GNA-related lectin). In treated aphids, there was delay of adult development and a lower production of nymphs. The mean respective cumulative number of nymphs produced by each surviving adult when fed for 19 d on the diet containing 100, 500, and $750 \mu\text{g mL}^{-1}$ APA was 8.2, 1.9, and 1.7, respectively. It should be noted here that these lectin concentrations of APA were ten times higher than those used in this study.

Our finding also are also consistent with earlier data in which the total reproduction of *M. persicae* was reduced by 29% and 35% when confronted on an artificial diet containing 215 and $450 \mu\text{g mL}^{-1}$ of GNA, respectively (Sauvion et al., 1996). On the other hand, Down et al. (2006) reported that when *M. persicae* was fed for 21 d with an

Figure 2. Feeding deterrent activity of mannose binding lectin obtained from bulbs of *Phycella australis* under choice conditions for *Acyrtosiphon pisum* and *Myzus persicae*.



Data are expressed as means \pm standard error.

artificial diet containing $1.4 \mu\text{g mL}^{-1}$ of GNA, the entry into adulthood was delayed by 2 d, and a reduced reproduction of 14.8 nymphs per female were recorded, contrasting with 73.4 nymphs per female in the control insects.

Our data support the idea that by providing lectin throughout the nymphal stage, it is possible to reduce significantly the fecundity and then the population building-up in both aphids. This could be to the fact that lectins interfere with the normal absorption of nutrients (Rahbé et al., 1995). Indeed, the nymphs exposed to higher concentrations of lectin looked smaller than control, which probably delayed the entry into reproductive status (Down et al., 2006).

Lectins that are both toxic and feeding deterrent may be more effective for pest control in practice. Feeding deterrence prevents settling aphids on the plant and the toxic properties kill the aphids when feeding on the plant/crop that contain the toxic lectin. Our results of the binary choice assay clearly demonstrated a high feeding deterrent activity by PAA lectin to *A. pisum* and lower to *M. persicae*. But apparently, at the tested concentrations, aphids did not detect the lectin present in the food immediately, or at least it was tolerated. In agreement with our results, Sauvion et al. (2004) reported for *A. pisum* that rejection only occurred at 8 h after exposure to the ConA lectin containing diet.

Lectins certainly can disturb the feeding behavior of aphids. Sprawka and Golawska (2010) studied the effects of PHA on the feeding behavior of *Sitobion avenae* F. by the electrical penetration graph (EPG) method. They could conclude that the addition of PHA to an artificial diet affected the aphid probing behavior. Increasing concentrations of PHA significantly reduced the number of aphid probes, reduced ingestion of and phloem sap salivation into phloem sieve elements. Here it should also be noted that the feeding deterrent activity as seen for PAA may have an additional implication, which is to reduce the ability of aphids to transmit viruses to their hosts.

CONCLUSIONS

This study demonstrated that the mannose-binding lectin isolated from bulbs of *Phycella australis* presents a strong insecticidal activity against the pea aphid and green peach aphid. The lectin affects the survival, feeding behavior, and fecundity of aphids, where *Acyrtosiphon pisum* proved to be particularly sensitive.

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