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Effect of the antimicrobial peptide indolicidin on the green peach aphid *Myzus persicae* (Sulzer)

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Abstract: The green-peach aphid, *Myzus persicae* (Sulzer) (Hem., Aphididae), is a major agricultural pest of a wide range of host plants, causing damage by feeding and indirectly by transmitting viruses. In this study we tested the effect of the antimicrobial peptide indolicidin on *M. persicae* survival and on its essential bacterial endosymbionts. Artificial diet bioassays showed a significant dose-dependent lethal response of indolicidin on *M. persicae* survival (LD_{50} of 209 ± 60 μg/ml). Histological analysis of indolicidin-treated aphids revealed a lower number of distorted mycetocytes, whereas control aphids showed abundant number of rounded and filled mycetocytes. These results suggest that aphid survival could be affected via reduction of its endosymbionts. Thus, aphid control based on antimicrobial substances against endosymbionts could be a promising strategy that needs to be further explored.

Key words: *Buchnera*, artificial diet, LD_{50}, pest control

1 Introduction

Aphids (Hem., Aphididae) are phytophagous insects that feed on plant phloem, producing serious damage to crops (Dixon 1998). Among aphid species, the green-peach aphid *Myzus persicae* (Sulzer) is considered one of the most generalists, attacking many host plants (van Emden et al. 1969; Blackman and Eastop 2000). Although pest management has been implemented with relative success against aphids (Parker et al. 2002), the capacity to develop insecticide resistance has become an important problem (Field et al. 1988; Field and Devonshire 1997). Although the use of transgenic crops against this aphid could be overcome by the development of resistance, the use of transgenic plants with more than one type of resistance mechanisms (e.g. *cry* genes, proteinase inhibitors) plus traditional control are more likely to delay the emergence of resistance development. Therefore, new efforts are necessary to improve green-peach aphid control.

Research into transgenic plants expressing compounds that affect negatively pest insects has shown a significant increase (Jouanin et al. 1998). For aphids, the cysteine proteinase inhibitor oryzacystatin I (OC-1) has been used successfully to decrease endogenous protease activity on *M. persicae*, and oilseed rape transgenic plants expressing OC-1 showed a reduced aphid biomass (Rahbé et al. 2003). Another protein tested against aphids is the jackbean concanavalin A lectin, which has been used in artificial diets against *Acyrthosiphon pisum* (Harris) (Rahbé and Febvay 1993), and in transgenic potato plants against *M. persicae* (Gatehouse et al. 1996). Similarly, the snowdrop lectin (GNA) expressed on transgenic tobacco plants reduced *M. persicae* populations by up to 60% (Zhou et al. 1998).

It has been recently found that a group of small peptides (< 10 kDa), which are part of the immune system of both animals and plants, play an important role in protecting them from pathogens (Hancock and Lehrer 1998; Banzet et al. 2002; Thomma et al. 2002). Although most of the studies have reported antibacterial and antifungal properties of these peptides, few studies have assessed the effect on pest insects (Jennings et al. 2001; Chen et al. 2002).

Aphids are known to harbour intracellular bacteria (endosymbionts) of the genus *Buchnera*, which provide aphids with essential amino acids, nutrients in short supply in phloem sap. Such aphids are partially or completely independent of a dietary supply of these nutrients (Douglas 1998; Douglas et al. 2001). *Buchnera* are located in specialized insect cells, known as mycetocytes (or bacteriocytes), in the aphid body cavity and are transmitted to aphid offspring via the ovary (Douglas 1998). The experimental elimination of *Buchnera* from aphids by the antibiotic rifampicin avoids aphid reproduction (Douglas 1996) and can kill aphids at small doses (Rahbé and Febvay 1993). In this case, the antibiotic has no direct deleterious effects on aphid physiology, and the general malaise observed on treated aphids is only attributed to the impact on the

endosymbionts (Wilkinson 1998) compared with the effect of traditional insecticides on the physiology of insects. Thus, aphid control based on antimicrobial substances against endosymbionts could be a promising strategy that needs to be further explored. In this study artificial diet assays were used to study the effect of the small antimicrobial peptide indolicidin on the survival of the aphid *M. persicae* and to evaluate the possible effect on mycetocytes and its intracellular bacteria by performing a histological comparison between treated and non-treated aphids. Indolicidin is known to reduce population growth of *Escherichia coli* (Selsted et al. 1992), which is a closely related species of *Buchnera* (Baumann et al. 2000). However, this peptide has not been tested on insect-endosymbiont bacteria.

2 Materials and Methods

2.1 Insects

Two parthenogenetic cultures of *M. persicae* were reared for several generations on potted sweet pepper plants [*Capsicum annuum* var. *grossum* (Sendt)], at 20 ± 0.5°C (L16 : D8 photoperiod). Each had been established as a clone from a single aphid collected from the field on separate sweet pepper orchards in Talca, and used as aphid stock for all bioassays. To ensure that clones studied were genotypically different, we performed microsatellite genotyping of individual DNAs using four microsatellite loci (Myz2, Myz3, Myz9 and Myz25) previously described for *M. persicae* (Sloane et al. 2001). DNA extraction, amplification and electrophoresis were performed following the protocol described by Fuentes-Contreras et al. (2004). Nymphs aged 0–16 h were used for all artificial diet assays.

2.2 Artificial diets

The antimicrobial peptide indolicidin, I-0144 (from bovine neutrophils) was purchased from Sigma-Aldrich Co (Sigma-Aldrich, St. Louis, MO, USA). This peptide was offered to aphids following the artificial diet procedure of Rahbé and Febvay (1993) used to test protein toxicity to the pea aphid. Standard artificial diet described as suitable for *M. persicae* (Dadd and Mittler 1966) was used as a control and a basis for peptide dilutions. Peptides were incorporated to the standard diet to obtain diets with 10, 50 and 250 μg/ml. These concentrations are in the range of those used in other studies testing toxicity of different proteins (Rahbé and Febvay 1993; Cherqui et al. 2003; Saguez et al. 2005). An aliquot of 10 μl of the diet, previously filter sterilized with a 0.45 μm Millipore filter (Millipore Corp., Bethford, MA, USA), were included in a Parafilm® (American National Can, Menasha, WI, USA) sachet encompassing an uncapped Eppendorf microtube (1.5 ml capacity) and maintained at −20°C until use. For toxicity assays, aphids were placed on sachets that were covered with another uncapped microtube to avoid aphid escape.

2.3 Toxicity test

In order to synchronize the age of test aphids, ca. 100–200 wingless adult parthenogenic females were transferred from the sweet pepper plants to Parafilm sachets of artificial diet mounted on a Petri dish of 7.5 cm diameter. After 12–16 h, four new-born aphid nymphs were placed on each uncapped Eppendorf microtube for toxicity test. Five of these uncapped Eppendorf microtubes ($n = 5$) were used for each compound concentration. All treatments and replications were randomly arranged in a culture chamber where they were maintained under controlled conditions (Conviron chamber model E7 (Winnipeg, USA) at 20°C, 50–60% RH). Daily observation consisted in counting the number of surviving aphids and the total number of new nymphs that were produced. Both parameters were recorded until mortality in controls reached 20%, thus assays lasted about 10–12 days. To avoid diet contamination, all sachets were replaced at day 4 and day 8. Tests were repeated three times for each concentration.

2.4 Histological analysis

For light microscopy, 12-day-old treated (250 μg/ml indolicidin) and non-treated aphids were fixed with Duboscq-Brasil solution, whereas for electron microscopy aphids were fixed for 4 h in 3–4% glutaraldehyde in 0.1 M sodium phosphate (pH 7.2) and post-fixed in 1% osmium tetroxide. To improve the penetration some aphids were beheaded. In both fixation methods, infiltrations in EPON 812 resin were performed under vacuum at room temperature. For light microscopy, 1 μm sections of the embedded samples were stained with 1% toluidine blue in 1% sodium borate, whereas for electron microscopy thin sections were double stained with alcoholic uranyl and lead citrate. Sections were viewed under a Zeiss EM 109 electron microscope (Oberkochen, Germany). To assess the effect on mycetocytes, the number of mycetocytes was scored for treated (250 μg/ml, $n = 4$) and untreated ($n = 4$) aphids. The mycetocytes inside embryos were excluded from this record. Serial sections (ranging from 182 to 320 sections by aphid) were analysed under light microscopy following the procedure of Douglas and Dixon (1987).

2.5 Statistical analysis

Survival data were analysed with a two-way ANOVA with repeated measures using doses (0, 10, 50 and 250 μg/ml) and clone (two *M. persicae* clones) as factors and days (five last days of the assay) as the within-subject factor. New nymphs produced were compared with day 12 by one-way ANOVA. Univariate analyses of variance were performed to identify significant differences among doses in a given time followed by a multiple comparison test. Both analyses were performed using SPSS software (SPSS Inc. 1997). To ensure normal distribution of data and homocedasticity of variances, data were transformed with the arcsin square root method. Mortality data were corrected using Abbott's formula (Abbott 1925). The lethal concentration needed to achieve 50% aphid mortality (LC_{50} value) was determined by probit analysis with the SPSS software (SPSS Inc. 1997). Fit to probit model was assessed with chi-square for goodness-of-fit test, considering $P > 0.05$ as fitting to the model. Comparisons of mycetocyte number and aphid survival between two peptides at a given dosage and time were performed with a Mann–Whitney *U*-test.

3 Results

3.1 Toxicity effect of indolicidin

The effect of indolicidin was not significantly different among both clones ($F_{1,16} = 3.26$; $P = 0.09$). A significant effect of dosage and time ($F_{3,16} = 4{,}28$; $P = 0.02$; $F_{4,64} = 21.6$;

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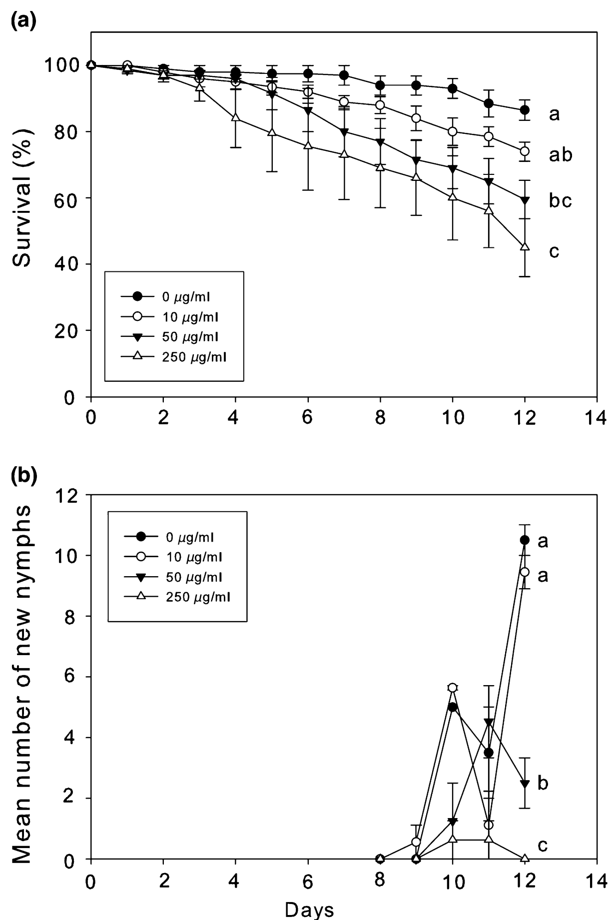

Fig. 1. *Survivorship curves and number of new nymphs (mean ± SE) produced by* Myzus persicae *neonates on artificial diets containing different concentrations of indolicidin. Data of both clones studied were pooled. Different letters among bars at day 12th indicates that differences among mean values are significant at P < 0.05 (multiple comparison Tukey HSD test)*

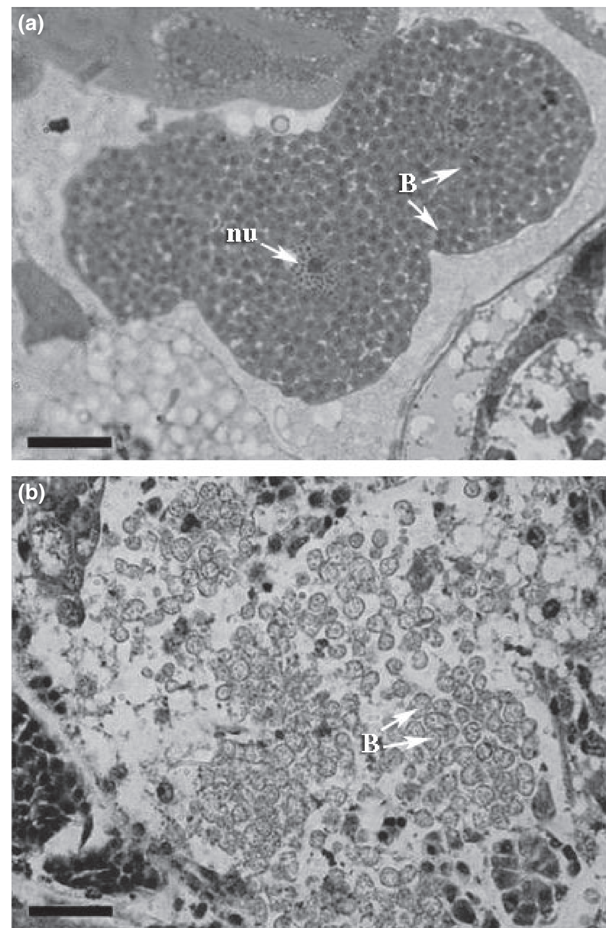

Fig. 2. *Light microscopy micrographs of transverse sections of non-treated (a) and indolicidin-treated (b) aphids. B,* Buchnera*; nu, mycetocyte nucleus. Scale bar: 10 μm*

P < 0.001 respectively) on percentage of surviving aphids was found. Interactions were not significant. Differences among dosages over time were apparent at day 12, with a high lethal effect at the highest concentration tested (250 μg/ml; fig. 1a). Mean number of new nymphs produced was significantly lower compared with the control at the dosage of 50 and 250 μg/ml (P = 0.002 and P = 0.001, multiple comparison Tukey HSD test respectively; fig. 1b), while dosage 10 μg/ml was not different to the control (P = 0.592, multiple comparison Tukey HSD test; fig. 1b). Lower concentrations, namely 10 and 50 μg/ml, showed lower lethal effects (fig. 1). Probit analysis gives an LD_{50} of 209 ± 60 μg/ml ($\chi^2_1 = 0.010$; P = 0.940).

3.2 Histological analysis

Light microscopy of transverse sections of treated aphids (250 μg/ml indolicidin) showed a significantly lower number of mycetocytes compared with untreated aphids (an average of 23 and 32, respectively, t = 4.68; P = 0.003). Mycetocytes appeared less rounded and the bacteria present within scattered, as opposed to the compact structure observed in control insects (fig. 2-a,b). Indolicidin-treated aphids revealed few and distorted mycetocytes, whereas control aphids showed abundant number of rounded and filled mycetocytes. Electron micrographs confirmed differences found with light microscopy sections between treated and non-treated aphids. As expected, every *Buchnera* cell is enclosed by a vacuole and surrounded by the mycetocyte's cytoplasm, consisting of a granular matrix with mitochondria, smooth endoplasmic reticulum and vesicles (fig. 3a,b). However, this matrix was less dense in mycetocytes of treated aphids and the bacteria within them were slightly larger (fig. 3b).

4 Discussion

Indolicidin showed a statistically significant negative effect on *M. persicae* performance. For instance, survival and reproduction were reduced to 45% and 80%, compared with the control at the highest dosage respectively. When aphids treated with the highest indolicidin concentration were histologically analysed, a negative effect on mycetocyte organization was apparent. In addition, the number of mycetocytes was significantly lower in indolicidin-treated aphids. Indolicidin (Selsted et al. 1992) belongs to a large and diverse group of cationic antimicrobial peptides with defensive function against bacteria and fungi in

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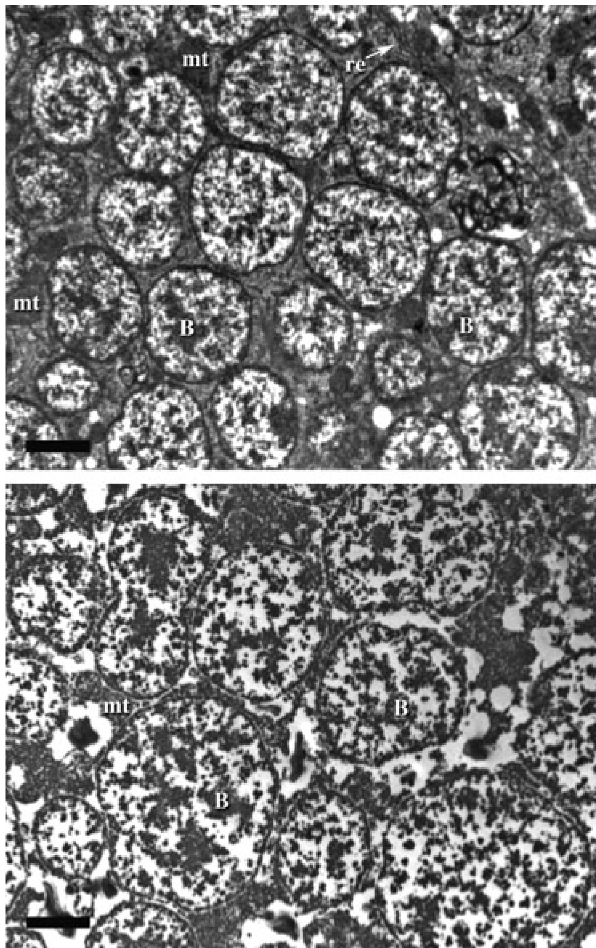

Fig. 3. *Electron microscopy micrographs of transverse sections of non-treated (a) and indolicidin-treated (b) aphids. B,* Buchnera*; mt, mitochondria; re, smooth endoplasmic reticulum. Scale bar: 1 μm*

animals and plants (Zasloff 1987; Matsuzaki 1999). This compound is cytotoxic to lymphocytes and protozoa (Schluesener et al. 1993; Aley et al. 1994) and is also capable of disrupting the erythrocyte membrane (Ahmad et al. 1995). As aphids do not have a peritrophic membrane, it is likely that indolicidin can pass through the gut epithelium, as observed in the case of both the proteins oryzacistatin (Rahbé et al. 2003) and β-glucoronidase (Cherqui et al. 2003), and thus the mortality caused on *M. persicae* could be the result of a direct damage of this peptide either on gut cells or in other internal tissues of the aphid. It is also known that proteins and peptides can pass through the gut of *M. persicae*, reaching the mycetocytes (Cherqui et al. 2003; Rahbé et al. 2003). Inside these cells are located bacterial endosymbionts of the genus *Buchnera* (Munson et al. 1991). As our histological analysis suggests, indolicidin may have also affected mycetocytes and endosymbionts, which could have subsequently reduced aphid survival. It is known that aphids deprived of endosymbionts, for example treated with the antibiotic rifampicin, show general malaise associated with poor larval growth and reproduction (Douglas 1998; Wilkinson 1998). A combined effect including the direct effect of indolicidin on aphid cell damage and the indirect effect via reducing endosymbiont survival cannot be neglected.

The effective use of antimicrobial peptides in transgenic plants, particularly in the case of indolicidin, will be subjected to reach concentrations similar to the ones tested here, in the phloem of transformed plants. In relation to this, protein concentrations used in this study are in the range of gene products expressed in transgenic plants (Cherqui et al. 2003). Thus, the effect of indolicidin on aphid survival and its mycetocytes are likely to be found in transformed plants.

In summary, the peptide indolicidin tested in the present work could be potentially used on transgenic plants to protect them from the aphid *M. persicae*. Further studies on the effects of this antimicrobial peptide expressed in transgenic plants against aphid endosymbionts need to be accomplished.

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