In vitro pathogenicity of northern Peru native bacteria on *Phyllocnistis citrella* Stainton (Gracillariidae: Phyllocnistinae), on predator insects (*Hippodamia convergens* and *Chrisoperna externa*), on *Citrus aurantifolia* Swingle and white rats

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Abstract

In Peru, the leaf miner *Phyllocnistis citrella* attacks citrus crops, including the economically important species *Citrus aurantifolia*, adversely affecting production. The objective of this work was to determine the in vitro pathogenic ability of enterobacteria isolated from within *P. citrella*. In addition, the pathogenic effects of these enterobacteria were tested on the predator insects *Hippodamia convergens* and *Chrisoperna externa*, on the host plant *C. aurantifolia* and on rats. The insects were captured in plantations of *C. aurantifolia* in the Piura Region. *Phyllocnistys citrella* was the most frequently occurring pest (98%), among other identified pests. From diseased larvae of *P. citrella*, the bacteria *Serratia* sp., *Pseudomonas* sp., and *Enterobacter aerogenes* were isolated. The three bacterial species had a similar pathogenic effect on *P. citrella* after 48 h (74.1% average mortality). *Serratia* sp. caused the highest mortality after 24 h in *H. convergens* (40%) and *C. externa* (30%), whereas the Lowest mortality rates were induced at 72 h by *E. aerogenes* on *C. externa* (3%) and by *Pseudomonas* sp. on *H. convergens* (10%). The bacteria did not affect neither *C. aurantifolia* or the rats, which gained the same weight as control animals.

Additional key words: Enterobacter, entomopathogenic bacteria, Piura, plant health and crop protection, *Pseudomonas, Serratia*.

Resumen

Patogenicidad in vitro de bacterias nativas del norte del Perú sobre *Phyllocnistis citrella* Stainton (Gracillariidae: Phyllocnistinae), sobre insectos predadores (*Hippodamia convergens* y *Chrisoperna externa*), *Citrus aurantifolia* y ratas blancas

En Perú el minador de los cítricos *Phyllocnistis citrella* ataca a cultivos de *Citrus aurantifolia*, afectando negativamente a su producción. El objetivo de este trabajo fue determinar la capacidad patogénica in vitro de enterobacterias, aisladas de *P. citrella*, sobre esta plaga, comparándola con el efecto de estas bacterias sobre los insectos predadores *Hippodamia convergens* y *Chrisoperna externa*, sobre la planta hospedera *C. aurantifolia* y sobre ratas blancas. Los insectos fueron capturados en plantaciones de la Región Piura. *Phyllocnistys citrella* fue la especie mas frecuente (98%) entre otras plagas identificadas. A partir de larvas enfermas de *P. citrella* se aislaron las bacterias *Serratia* sp., *Pseudomonas* sp. y *Enterobacter aerogenes*. Se determinó su actividad patogénica contra *P. citrella*, los insectos controladores *Chrysoperla externa e Hippodamia convergens*, sobre el hospedero *C. aurantifolia* y ratas blancas. Las tres bacterias tuvieron un efecto bacteriano similar (74.1% mortalidad promedio), desde las 48 h de inoculación, contra *P. citrella*. *Serratia* sp. indujo la mortalidad mas alta, desde las 24 h, sobre *H. convergens* (40%) y *C. externa* (30%). La mortalidad más baja fue inducida a las 72 h por *E. aerogenes* sobre *Ch. externa* (3%) y por *Pseudomonas* sp. sobre *H. convergens* (10%). Las bacterias no afectaron a *C. aurantifolia* ni a las ratas, las cuales aumentaron de peso igual que el control.


Abbreviations used: CFU (colony-forming units), GA3 (gibberellic acid), NA (nutritive agar), rpm (revolutions per minute), UdeP (Universidad de Piura, University of Piura), UNP (Universidad Nacional de Piura, National University of Piura).
Introduction

Key lime, *Citrus aurantifolia* (Rutaceae), is an important agricultural crop in Peru, both for export and domestic consumption. *Citrus aurantifolia* plantations cover 10,528 ha or 38.6% of the area dedicated to orchards in the Piura Region. Citrus leaf miners (*Phyllocnistis citrella*), can limit or reduce citrus fruit production (Ginocchio, 1993). This pest attacks citrus trees by infesting the buds and decreasing the leaf area. They reduce the leaf photosynthetic rate and cause them to roll up and drop off (Schaffer *et al.*., 1997). Larvae of *P. citrella* can eat 1 to 7 cm² of leaf per day.

The pathogenic bacteria, *Pseudomonas* sp., isolated from diseased larva of *P. citrella* induced 80% mortality on this pest in micro-plot field bioassays (Sepúlveda *et al.*, 2001). *Pseudomonas aeruginosa* was pathogenic against the orthopterans *Melanoplus bivittatus* and *Camnula pellucida*; this bacterium does not normally multiply in the digestive tract of insects, but it can in the haemocoel (Angus, 1965). In a similar study, (Goptal; this bacterium does not normally multiply in the digestive tract of insects, but it can in the haemocoel (Angus, 1965). In a similar study, (Goptal and Gupta, 2002), detected high concentrations (10⁹-10¹⁰ cells mL⁻¹), of *Pseudomonas alcaligenes* in the haemolymph of dead *Oryctes rhinoceros* grubs, a tropical coconut pest. Approximately 52% of the grubs succumbed to septicemia. Although *P. alcaligenes* is a normal bacterial component in the gut of healthy grubs, under other conditions, it can be an opportunistic pathogen. The main problem with this pathogen has generally been its adaptation and survival when used in new environments (Ohba and Aizawa, 1986). Therefore, the origin of a control agent is important.

The bacterial genus *Serratia* consists of ten recognized species; one group is an important nosocomial pathogen and the other species cause less frequent infections (Carrero *et al.*, 1995). However, *S. marcescens* has been isolated from the haemolymph of boll weevils (*Anthonomus grandis*) (Schmitz and Braun, 1985) and strains of *Serratia* have been isolated from different soils and the gut of invertebrates (Ashelford *et al.*, 2002). *S. marcescens* isolated from boll weevils can cause disease in guinea pigs (*Cavia* sp.), mice (*Mus musculus*) (Lyrley and Kreger, 1983), and insects by causing evolution of exoproteases during pathogenesis (Stock *et al.*, 2003). *Serratia proteamaculans*, isolated from the spider *Nephilila clavata*, could have mutualistic or synergistic relationships with exoprotease production by the spider in order to digest its victims. However, this does not exclude the possibility that the bacterium could also be pathogenic to the spider as well as the insects (Dece-
All of the insects were in non-adult states (eggs, larvae or pupae). Insect species were determined in the Entomology Laboratory, Faculty of Science, Universidad Nacional de Piura. To calculate relative abundance, the number of individuals per species was determined at each sampling. The cumulative relative abundance (%) was calculated and plotted against time. Field and laboratory work was performed in 2004 and 2005.

**Isolation of bacteria**

*Phyllocnistis citrella* larvae were collected in Sullana and in Chulucanas. Dead, diseased, and healthy *P. citrella* larvae were collected directly, by cutting live twigs with their attached foliage from *C. aurantifolia* trees. Individual larvae were collected without removing them from the tunnels that they had excavated in the leaves. Twigs were kept alive by placing them in a solution of kinetin and gibberellic acid (GA₃, 200 ppm each) for at least 2 d. Healthy larvae were reserved for biological tests. They were fed on a diet of *C. aurantifolia* leaves. Collection of insect larvae was constant throughout the project.

Conspicuously diseased larvae were used for bacterial extraction because they were most likely to contain pathogenic bacteria. Bacterial isolation was performed in the laboratories of the University of Piura and the National University of Piura, Peru. Diseased larvae were sterilized externally by immersion in sodium hypochlorite (0.1%, 1 min) and were then rinsed in sterilized distilled water. Twenty larvae were liquefied and homogenized with a mortar and pestle in 1 mL of distilled water. From the homogenate, 0.1 mL of supernatant was inoculated into nutritive agar (NA) and agar 5% peptone and incubated at 26ºC. After 24 h, the number of colonies found represented the number of bacteria in 1 mL or CFU. Based on growth curve analysis, bacteria were harvested at the mid-log phase of growth.

By using the culture method and counting CFUs, the bacterial concentration in the different inocula was determined. In the biological test on *P. citrella* larvae the average concentration of the inocula were 2.2 x 10⁶ (Serratia sp.), 4.5 x 10⁶ (Pseudomonas sp.), and 2.4 x 10⁶ (E. aerogenes) bacteria mL⁻¹. In the test of acute toxicity in rats, the final bacterial concentration in inoculated wheat was determined; 1 g of inoculated wheat grain was stirred for 5 min in 10 mL of distilled water and 1 mL was used to determine the CFU. The inoculated wheat had an average concentration of 16 x 10⁶ CFU of the three pathogenic species. This was similar to the concentration of used in the pathogenicity test on *P. citrella*.

**Entomopathogenic bacterial activity**

The ability of each bacterial species to cause disease was determined. The experimental units were groups of 20 healthy larvae in their leaf mines. Each leaf could have one or more larvae. Each bacterial treatment was replicated three times.
A waxy cuticle covers the photosynthetic tissue or mesophyll of each leaf. Leaf miners carve tunnels as they eat into the mesophyll. They do not eat the cuticle. Cuticles covering the mines were perforated in front of each larva with a sterilized needle to ensure contact between the bacteria and the larvae. A drop of 0.05 mL of liquid inoculum plus a dispersant agent Agridex (1 mL L⁻¹) was placed in the leaf tunnel in front of the larvae. When the drop did not totally enter the tunnel in 10 s, excess was absorbed with filter paper. Leaves used as the control were inoculated with sterilized culture medium without bacterial inoculum. Afterwards, larvae were observed in their leaves in a specially designed chamber. Every 24 h, mortality (%) was calculated and the cumulative mortality (%) was plotted against elapsed time since the start of the test. Only bacterium cultures that sickened and killed *P. citrella* larvae in prior tests were used in the experiment.

The pathogenic effects of the bacterial cultures were tested on individuals of *Chrisoperla externa* Hagen (Neuroptera) and the convergent lady bird, *Hippodamia convergens* Guérin-Méneville (Coleoptera). Both prey on insect pests of *C. aurantifolia*. *Chrisoperla externa* individuals were obtained from egg samples donated by the National Service of Agrarian Health, SENASA, Peru, and individuals of *H. convergens* were obtained from field collections. For each bacterial treatment, three groups of 30 individuals of the predator species were maintained in plastic vials (250 mL) covered with a fine cloth. On the first day only, the predators were fed with green bugs (*Toxoptera aurantis* B. de F.) that had been externally infected by submersion in a liquid culture of each bacterium. Control insects were fed untreated green bugs. Mortality (%) was evaluated as in the previous biological test.

To determine the pathogenic effect of the bacteria upon *C. aurantifolia*, liquid inoculums of each bacterium were sprayed (from 20 cm away, for 2 s) on leaves of nursery stock trees (N = 20, three repetitions). Fruit from adult trees were sprayed with the same bacterial suspensions. Groups of 20 limes were used, with three replicates for each treatment. Appearance of symptoms was monitored daily to determine probable phytopathogenic effects. Symptoms on fruits and leaves were expressed as affected area (%) relative to the total surface.

**Test of acute mammalian toxicity**

In this experiment, the objective was to determine whether the isolated bacteria affected the health of albino rats (*Mus musculus*). Inferences of the bacteria’s effect could be made by extension. Three-month-old rats, weighing 18 g on average, were fed wheat grain inoculated with the three bacterial species. To obtain inoculum of each bacterium, 100 mL of culture medium (peptone 5%) was inoculated with a colony of *Serratia* sp., *Enterobacter cloacae*, or *Pseudomonas* sp. and incubated at 26°C with constant stirring (100 rpm) to obtain an absorbance of 1.5 (550 nm). The cultures were used in the acute toxicity assay. One kg of wheat grain was mixed with 300 mL of each bacterial inocula; the mix was incubated for 24 h at 26°C and the concentration of each bacterium (CFU) on the wheat was determined. There were 10 rats per group, with three replicates, three bacterial treatments and a control. Rats were fed 2 g of wheat daily. Wheat inoculated with bacteria was used only on the first day. Control rats were fed wheat inoculated with pure liquid culture medium. Afterwards, all rats were fed non-inoculated wheat. The test was conducted for 40 d, during which rat behaviour and mortality were recorded daily.

**Bacterial re-isolation**

At the end of each test dead insects were sterilized externally and the bacteria were re-isolated using the same procedure as for general bacterial isolation. The re-isolated bacterium species were matched with the bacteria with which the dead insects had been inoculated.

**Evaluation**

Insect mortality (%) in the biological assays was calculated by subtracting control mortality (without bacteria). Accumulated mortality (%) was plotted against time (h). The mortality dynamic was determined with using a paired *t*-test for dependent samples with a 95% (*P* ≤ 0.05) confidence interval.

**Results**

**Population dynamics**

The pest control or predator insects *C. externa*, *H. convergens*, and the pollinating insect *Apis* sp., and the pests *Scirtothrips citri* Moulton (Thysanoptera), *Tox-
optera aurantii B. de F. (Homoptera), Aleurothrixus floccosus Maskell (Homoptera), and P. citrella were identified in C. aurantifolia plantations.

From October to January, the relative abundance (Fig. 1) of P. citrella was similar to that of the other pests (p = 0.305) and higher than that of the pest control insects (p = 0.009). From February through the evaluation period, the pest-control insects maintained their populations (p = 0.07); while populations of other pests decreased (p = 0.009), and the P. citrella population (p = 0.0006) increased over that of other pests and pest-control insects (p = 0.0004).

Bacterial isolation

Bacteria were isolated from larvae infected by the pathogens. Bodies of dead larvae were opaque, dark yellow in colour, and very soft. Diseased larvae were sluggish and generally with amber in colour. The bodies of healthy larvae were slightly yellowish and transparent. They were capable of active movement.

Five bacteria (Table 1) from P. citrella larvae were isolated and identified. The Serratia sp. which was isolated produced gas from glucose and was negative for lysine and urease activity metabolism. Due to differential characteristics, this species was 86% similar to S. pymuthica, 83% and 50% similar to two biotypes of S. marcescens and less similar to other species. Serratia sp. was isolated from insects and was more than 95% similar to S. entomophila, a species isolated from soil and chitinolytic microorganism. The strain obtained here was named only Serratia sp. It is extracted from insects and is very different from Serratia sp associated with human diseases.

Pseudomonas sp. was slightly similar to P. aerugi-nosa. It grew well under anaerobic conditions but did not reduce nitrate, and did not produce H₂S gas. On the other hand, it was only 58% similar to P. mendocina and did not induce death in rats. The Enterobacter isolation had 100% identification (all nine tests) with the response patterns of E. aerogenes. Finally, two isolates were identified as Streptococcus sp. and Staphylococcus sp., but were not important because they were not pathogenic on P. citrella larvae.

Pathogenic activity of bacteria

After 24 h the entomopathogenic effect of Serratia sp., Pseudomonas sp., and E. aerogenes under in vitro conditions (Fig. 2A) was similar (p = 0.295). By 48 h, these bacteria had induced average mortalities of 80.2% (Serratia sp.), 70.2% (Pseudomonas sp.), and 71.9% (E.

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<tr>
<th>Test</th>
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any damage or symptoms on leaves or fruit of *Citrus aurantifolia*.

If one compares net maximum mortality among insect species; *Serratia* sp. induced the highest mortality (between 48 and 72 h). This bacterium was more efficient on *P. citrella* (80.4% mortality) than on *C. externa* (43%) and *H. convergens* (53%). *Serratia* was 46.5% more efficient on the pest insect than on the biological control insects.

*Pseudomonas* sp. was more efficient at 72 h on the pest *P. citrella* (70% mortality) than on *C. externa* (20%) and *H. convergens* (10%). The mortality of the most affected controller insect (*C. externa*) was 71.4% lower than on the pest insect. *Enterobacter aerogenes* had a similar effect to *Pseudomonas*, killing 73% of *P. citrella* and inducing the lowest mortality on *C. externa* (3%) and *H. convergens* (30%). The maximum mortality induced by *H. convergens* was 58.9% lower than on the pest insect. The mortality of *P. citrella* was statistically similar to that induced by the other two bacterial species.

**Acute toxicity of the bacteria to rats**

Mortality with *Serratia* sp (3.3 ± 4%, one rat) was no different from the control at 40 d. The rat died due to natural causes, not from the infection by *Serratia* sp. With the other bacterial species there were no deaths. Therefore, bacteria pathogenic to *P. citrella* did not have a pathological effect on rats. The rats, in all treatments, gained weight from 21.5 ± 0.3 g to 26.6 ± 0.2 g. The rats did not show significant difference (p = 0.64) in weight due to the bacteria.

**Discussion**

On *C. aurantifolia*, the biocontrol insects *C. externa* and *Hippodamia* were present. The *P. citrella* population was 98% higher than that of other pests, associated with seasonal high relative humidity. The *P. citrella* population density was highest from February to June; the population of other pests and pest control insects decreased or was constant during the rest of the year. In other work from Peru (Granda et al., 2001; Arce, 2003), the *P. citrella* population increased exponentially from April to June and reached a maximum in the second week of June and again in the first week of December. This difference may be correlated with the high relative humid-
ity of Piura’s subtropical summer induced by the effect of the “El Niño” phenomenon. The insects adapt their population dynamics to annual climatic changes.

*Serratia* sp. was the most virulent bacterium. It induced high mortality in *H. convergens* and in *C. externa*. It also killed a significant number of *P. citrella* larvae.

On the other hand, *S. marcescens* is reported to be a nosocomial pathogen (Carrero et al., 1995) and a facultative anaerobe that multiplies quickly in the gut of many insect species, causing septicaemia and death. It is often isolated from diseased and dead insects (Benoit et al., 1990; Rodríguez, 1995; Escobar et al., 2001; Prabakaran et al., 2002; Green et al., 2005). Other species, such as *S. entomophila*, induce pathologies in pest insects when the bacterium is ingested (Hurst and Jackson, 2002).

The species of *Serratia* isolated, in this study, had a low matching with *S. marcescens*, but a high matching with the group *S. entomophila*; this isolate was innocuous to laboratory rats (Their et al., 1993, Weidenmaier et al., 2004). Hence, the *Serratia* sp. of this study was used in pest-control experiments on plantations, using traps designed to keep the insects inside (Sepúlveda, unpublished data). Mortality was very high at a low bacterial concentration. As for symptoms of the diseases in insects, sick larvae or those that are killed by bacteriosis become dark-brown or black in colour and appear to be dried and mummified (Bach, 1985; Leucona, 1996) as observed here. *Serratia* sp. and *Pseudomonas* sp. have been reported as pathogens of *Anastrepha fraterculus*, *Ceratitis capitata*, and *Rhynchoporus palmarum* (Briceño, 2004); *Serratia* and other bacteria isolated from fruit flies (*A. fraterculus*, *C. capitata*), and *R. palmarum* induced a crossed effect of a 66.7% of mortality in *P. citrella* larvae (Campos et al., 2007).

Species of *Enterobacter* are reported to be normal, or eventual, inhabitants of the gut of healthy insects (Bach, 1985). Natural concentrations of *Enterobacter* sp. did not induce mortality in *C. capitata* or *A. fraterculus* but were pathogenic at high concentrations. The principal symptoms of infection of insects by gram negative bacteria are septicaemia, inhibition of feeding a lack of motility, and death at 24 to 72 h (Briceño, 2004).

Due to their low virulence against *C. externa* and *H. convergens*, but high virulence against larvae of *P. citrella*, it seems that the *Pseudomonas* sp. and *E. aerogenes* used in this study would have potential for use in experimental pest control, under controlled conditions, such as the above-mentioned traps.

Enterobacteriaceae are not easy to use for biological control because they are sensitive to dehydration and sunlight, both of which tend to cause variations in bacterial virulence. Further, *Pseudomonas* and *Serratia* species include strains with different levels of mammalian pathogenicity (Angus, 1965). However, these species are responsible for natural mortality in insects. This can be taken advantage of, if suitable studies are made and/or the right methods were used (i.e., special traps). For example, *S. entomophila* and *S. proteamaculans* are used as effective biological pesticides; they cause amber disease which inhibits insect growth and induces death of *C. zealandica* (New Zealand grass grub) (Hurst et al., 2000; Hurst and Jackson, 2002).
From diseased larvae, it was possible to isolate *Serratia* sp. *Pseudomonas* sp. and *Enterobacter aerogenes*; all of them enterobacteria pathogenic to *P. citrella* larvae. The isolated bacteria did not have any pathological effect on rats. This could be important in deciding to use these bacteria in programs or systems for pest control of *P. citrella*. *Serratia* sp. was the most virulent against the *P. citrella* predator insects, *C. externa* and *H. convergens*. The other bacteria were almost harmless to the predator insects, but caused death of the pest. This is an important consideration because the bacterial concentration can be determined to achieve maximum pest death and minimum death of the biocontrol species in pest control programs. This technology needs further development to be implemented at all levels of production.

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