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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 enterobacterias 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.

Palabras clave adicionales: bacterias entomopatógenas, *Enterobacter*, Piura, *Pseudomonas*, sanidad y protección de cultivos, *Serratia*.

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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 cm^2 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 *Cammula 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 and Gupta, 2002), detected high concentrations (10^9-10^{10} 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 septicaemia. 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*) (Lyerly and Kreger, 1983), and insects by causing evolution of exoproteases during pathogenesis (Stock *et al*., 2003). *Serratia proteamaculans*, isolated from the spider *Nephilia clavata*, could have mutualistic or synergic 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 (Decedue *et al.*, 1979). *Serratia entomophila* and *S. proteamaculans* cause amber disease in grass grub *Costelytra zealandica* (Coleoptera); *S. entomophila* has been isolated from insects and the environment, but not from animals other than insects. A product based on *S. entomophila* has been successfully developed to control the pest *C. zealandrica* (O'Callaghan and Gerard, 2005).

The aim of this work was to determine the *in vitro* pathogenic ability of enterobacteria isolated from *P. citrella* on this pest; this was compared with the pathogenic effects of the bacteria on the predator insects, the convergent ladybird, *Hippodamia convergens,* and *Chrisoperna externa*, on the host plant *Citrus aurantifolia,* and with rats.

Material and methods

Location of the study

The Piura Region is located on the northwest coast of Peru between 3º and 7º S. It is divided into eight provinces, including Sullana (4°52´49"S, 80°41´07"W, elevation 70 m) and Chulucanas (05°05´57"S, 80°41´07"W, elevation 115 m). Ecologically, the Piura region is divided between tropical dry forest in the north and the Sechura desert in the south. The northern part of the Sechura desert has an annual temperature of 26ºC and an average annual rainfall of 50 mm concentrated in the months of January, February, and March. In the study area, winters are hot and humid with temperatures of 28ºC and the maximum average temperature exceeds 35ºC. The average temperature during the region's dry summers ranges between 24º and 20ºC.

Estimation of the *Phyllocnistis citrella* population

The relative abundance of *P. citrella* larvae was determined over 9 months on a 1 ha plantation of *C. aurantifolia*, plantation at the Fundo Tungazuca farm in Cieneguillo Village, Sullana Province. *Phyllocnitis citrella* and the other insect pests of *C. aurantifolia* deposit their eggs on *C. auranitfolia* leaves or are found as pupa and larvae in leaf galleries. These leaves can be collected with the insects inside or on them. Special insect catching traps were not necessary. Other common insects such as bees (*Apis* sp.) and predators were easy to catch with nets. Insects from 10 branches from each cardinal point of different citrus trees were collected.

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 (GA_3, 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 to for bacteria 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 culture media, and incubated at 26ºC (room temperature of the bioassay chambers).

After 24 h incubation, bacterial colonies were isolated and identified by morphological and biochemical tests in the laboratory of the Department of Biology, UNP and the Referential Health Laboratory, LARESA, Piura, Peru. Seventeen different tests were performed on the colonies. The tests were: gram staining, motility, fluorescence, nitrate, oxidase, methyl red, H_2S, indole, lysine, coagulase, catalase, mannitol, citrate, urea, lactose, production of gas from glucose, and gel at 22ºC. These tests have different specific culture media, indicated in the international protocols. Only in the test for H_2S the laboratory reported the use of special triple sugar iron agar (Table 1). The results were matched with the responses to these tests of enterobacterial species from the genera studies as reported by Ramírez (1968), Edinger *et al.* (1985), Aragone *et al.* (1992), Vivas *et al.* (2000), Bindu *et al.* (2003), Traub *et al.* (1970), and Euzéby (2003). To determine the degree of similarity among bacterial isolates with bacterial species described by the above authors, the number of similar responses between isolated and reference bacteria was divided by the total number of biochemical tests and expressed as a percentage. Pure cultures of each bacterium species were obtained and maintained under standard conditions. Matching was done with reported species of *Pseudomonas* and *Enterobacter*, and with the species *Serratia marcescens* (principal and variants), *S. liquefaciens, S. rubida, S. odorifera* (two biotypes or variants), *S. plymuthica, S. ficaria, S. entomophila* and *S. fonticola*.

To prepare a bacterial inoculum, one colony of each isolated bacterium species was incubated (26ºC, 100 rpm in 5% peptone liquid culture). Absorbance was determined (550 nm) hourly to obtain growth curves of each isolated microorganism. Every hour, 1 mL of pure culture or the suitable dilution was inoculated into solid 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 10^6 (*Serratia* sp.), 4.5 x 10^6 (*Pseudomonas* sp.), and 2.4 x 10^6 (*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 determinate the CFU. The inoculated wheat had an average concentration of 16 x 10^6 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 determinate 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 26ºC 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 26ºC 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% ($P \leq 0.05$) 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. aeruginosa*. It grew well under anaerobic conditions but did not reduce nitrate, and did not produce H_2S 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.*

Table 1. Characteristics of bacteria isolated from *Phyllocnistis citrella* larvae; +: positive reaction, -: negative reaction, (): low reaction, [a:] using triple sugar iron agar

Test	*Serratia* sp.	*Pseudomonas* sp.	*Enterobacter aerogenes*	*Staphylococcus* sp.	*Streptococcus* sp.
Gram	-	-	-	+	+
Fluorescence	+	+	+	-	-
Motility	+	+	+	-	-
Nitrate		-			
Oxidase		+			
Methyl red	-	-	-		
H_2S	-[a]		-[a]		
Indole	-	-	-		
Lysine	-		+		
Lactose	-	-	+		
Gas-glucose	-	-	+		
Urea	-	(+)	-		
Citrate	+	+	+		
Mannitol	+		+	+	+
Catalase		+		+	
Coagulase				+	-
Gel 22ºC	+		+		-

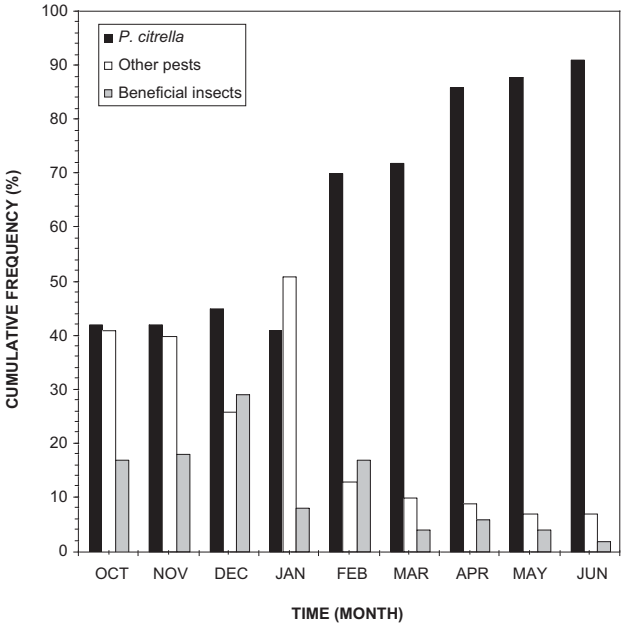

Figure 1. Relative abundance of *Phyllocnistis citrella* correlated with the other pests [*Scirtothrips citri* (Thysanoptera), *Toxoptera aurantii*, and *Aleurothrixus floccosus* (Homoptera)], and with the pest control insects *Chrysoperla externa* and *Hippodamia convergens*. Insects captured in plantations of *Citrus aurantifolia* (Piura, Peru) from October 2004 to June 2005.

aerogenes), which had statistically similar effects. Average mortality, at 48 h, of *P. citrella* associated with the three pathogenic bacteria was 74.1%. This was 95% higher ($p = 0.0001$) than the mortality caused by *Staphylococcus* sp. and *Streptococcus* sp. These latter two species were not pathogenic and their effect was similar to that of the control. At the end of the tests, the pathogenic bacteria that were re-isolated from larvae corresponded to the bacteria that were inoculated with.

Serratia sp. induced the highest ($p = 0.019$) accumulated mortality on *C. externa* (Fig. 2B): 30% at 24 h and 43% at 72 h. *Pseudomonas* sp. induced mortality of 14% at 48 h, and a maximum of 20% at 72 h; *E. aerogenes* induced the lowest mortality (3% at 72 h), which was similar to the control. On *H. convergens*, *Serratia* sp. induced the highest mortality rate (Fig. 2C): 40% at 24 h and 53% at 72 h ($p = 0.007$). *Enterobacter aerogenes* had an intermediate effect, with maximum mortality of 30% at 72 h; *Pseudomonas* sp. induced the lowest mortality, 10% at 72 h, similar to the control results. At the end of the tests, the pathogenic bacteria were re-isolated from the larvae corresponding to those that had been inoculated. The assayed bacteria did not induce 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 ($3.3 \pm 4\%$, 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-

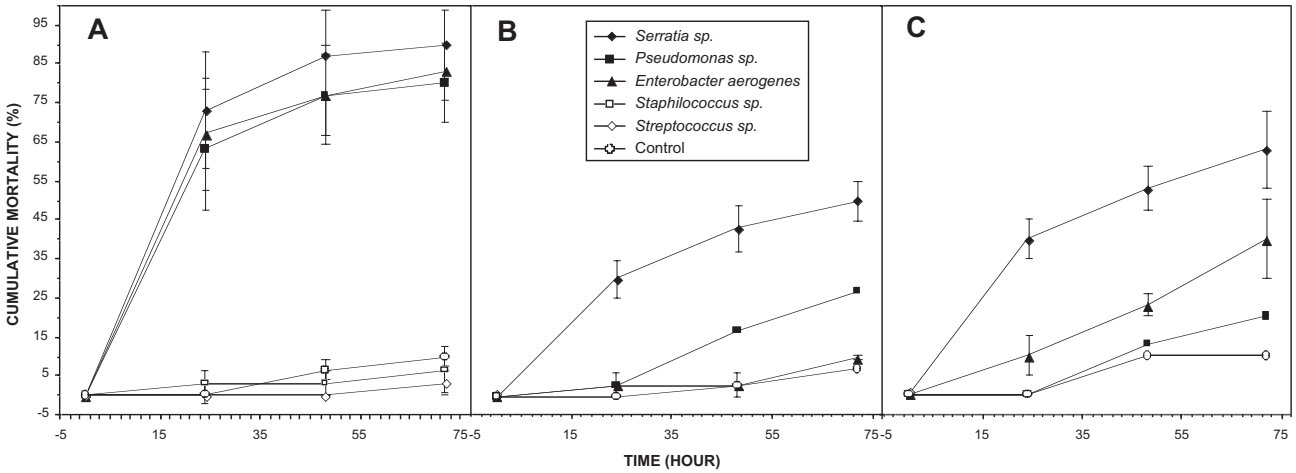

Figure 2. Entomopathogenic effect of isolated bacterial species on: (A) *Phyllocnistis citrella* larvae. (B) *Chrisoperla externa.* (C) *Hippodamia convergens*. The bacterial concentration was in the order of 10^6 bacteria mL^{-1} for all bacteria. Leaves used as controls were inoculated with sterilized culture medium, without bacterial inoculum.

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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