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Cytotoxin production in phytopathogenic and entomopathogenic *Serratia marcescens*

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ABSTRACT. In this work, culture filtrates of entomopathogenic and phytopathogenic *Serratia marcescens* strains induced cytotoxic effects on CHO, Vero and HEp-2 cell lines. Morphological changes on sensitive cells were characterized by cell rounding and detachment as soon as 30 min of incubation, culminating in cell death after 24 h. The cytotoxic effect was completely neutralized by specific antiserum indicating that occur antigenic similarity among cytotoxins produced by these strains. The toxicity assays on plants showed that the culture supernatants did not provoke any visible morphological change and did not affect their growth. By contrast, the plants treated with bacterial suspension showed disease symptom, such as shriveling and decay of stores bulbus in onion and lettuce plantlets. In conclusion, this study show that phytopathogenic and entomopathogenic *S. marcescens* may produce a cytototoxin similar to that produced by clinical isolates and it is toxic to different mammalian cell lines. These results are especially important for studies involving this bacterium as biological control agent.

Key words: *Serratia marcescens*, cell culture, cytotoxins, virulence factors.

RESUMEN. En este trabajo, filtrados del cultivo de cepas de *Serratia marcescens* entomopatógenas y fitopatógenas indujeron efectos citotóxicos sobre las líneas celulares CHO, Vero y Hep-2. Los cambios morfológicos sobre las células sensibles se caracterizaron por el redondeamiento de las células y el despegado de las placas, a partir de los 30 min de incubación y terminando a las 24 h, con la muerte celular. El efecto citotóxico fue neutralizado completamente por un antisuero específico, lo que sugiere que existe similitud antigénica entre las citotoxinas producidas por estas cepas. Los ensayos de toxicidad sobre plantas, mostraron que los sobrenadantes del cultivo no provocaron ningún cambio morfológico visible y no afectaron su crecimiento. Por el contrario, las plantas tratadas con la suspensión bacteriana mostraron síntomas de la enfermedad, tales como en plantas de cebolla y lechuga. En conclusión, este estudio muestra que *S. marcescens* entomopatógena y fitopatógena puede producir una citotoxina similar a la producida por aislados clínicos y que es tóxica a diferentes líneas celulares de mamíferos. Estos resultados son especialmente importantes para estudios que involucran esta bacteria como un agente de control biológico.

Palabras clave: *Serratia marcescens*, citotoxinas, factores de virulencia.

INTRODUCTION

Serratia marcescens is a Gram-negative *bacillus* classified as a member of the family Enterobacteriaceae commonly found in soil, water, plants and animals.[7] This bacterium has been recognized as an important nosocomial pathogen, causing urinary and respiratory tract infections,[11,14] bacteremias,[17] meningitis,[2] and septic arthritis.[13]

In plants, *S. marcescens* can be found on healthy plants or as a phytopathogen, such as in onion (*Allium cepa* L.), causing decay of stores bulbs.[1] The disease caused on vegetables like soft rot of potato, tomato, etc., is very similar to those caused by *Erwinia* strains.[8]

S. marcescens produces several exoenzymes such as gelatinase, lecithinase, proteinase and chitinase,[9] that may be harmful to insects, and the pathogenicity depends on stress conditions affecting the host insect.[10] A detailed study on the species and biotypes of *S. marcescens* associated with insects showed that the major group was the pigmented *S. marcescens* biotype A2a.[7] Pigmented biotypes of *S. marcescens* are more frequently recovered from natural environments and are rarely responsible for an outbreak of nosocomial infection.[2]

Recently, it was demonstrated that clinical isolates of *S. marcescens* can produce a toxin active on epitelial cells grown *in vitro*.[3] This cytotoxin is extracellular, heat-labile and culture conditions identified as optimal were incubation at temperatures ranging from 30 to 37ºC for 24h, under shaking, in medium adjusted to pH 8.5.[5]

In the present study we investigated the ability of *S. marcescens* isolated from plants and insects to produce cytotoxins active on mammalian cell lines and to compare it with the cytotoxin produced by clinical isolates of *S. marcescens*. The effect of the toxin in plants and the serological relationship with cytotoxin produced by clinical *S. marcescens* were also evaluated.

MATERIAL AND METHODS

Bacterial strains. The phytopathogenic *Serratia marcescens* strains isolated from lettuce (numbers 5/6 and 6/5) and onion (*Allium cepa* L.) (number 980) and the entomopathogenic strain isolated from *Heliotis virescens* worm (number 981) were obtained from the culture collection of the Laboratório de Bacteriologia Vegetal do Centro Expe-

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rimental do Instituto Biológico de São Paulo, Campinas, Brasil. The previously characterized cytotoxin-producing *S. marcescens* (number 458) isolated from urinary infection[3] was used as a reference strain.

Preparation of bacterial culture filtrates. *S. marcescens* strains were cultured in 10 ml of Trypticase Soy Broth (TSB, Difco Lab., Detroit, MI) at 37ºC for 18 h, with shaking at 110 rpm. Bacterial cultures were centrifuged (10,000 x *g* for 10 min at 4ºC) and the culture supernatants were filtrated through 0.2 µm filters. Alternatively, after centrifugation of bacterial culture, the supernatant was 10-fold fractionated with ammonium sulfate at 80% saturation and the pellet was extensively dialyzed with Tris-HCl buffer (pH 7.0).

Cytotoxicity assays. The cell lines HeLa (human cervix), Vero (African Green Monkey kidney), HEp-2 (human larynx) and CHO (Chinese hamster ovary) cells, obtained from the American Type Culture Collection (Rockville, MD) were tested for the cytotoxicity as previously described.[3] Briefly, the cells were grown in tissue culture flasks in Eagle medium supplemented with 10% fetal calf serum. Cells were detached from the flasks with trypsin-EDTA, resuspended to approximately 104 cells/ml in Eagle medium and 0.1 ml samples were pippeted into each well of a 96-well microtiter plate. Confluent monolayers were inoculated with bacterial culture filtrates and the plates incubated at 37oC in 5% CO_2 for 24 h. As negative controls some wells received only TSB medium or *E. coli* K12/711 supernatant filtrates, and as positive control, filtrates of *E. coli* H30 (producing verocytotoxin) and *S. marcescens* 458 were used. Cell monolayer morphology was observed under an inverted microscope and checked for cytotoxic effect daily for 3 days.

Determination of cell viability. Cellular viability was determined for assays with mammalian cells, as described previously.[5] After cytotoxic assay (incubation with filtrates for 24 h), the media containing the bacterial filtrates were removed and cultures were washed with sterile phosphate-buffered saline (PBS), 0.025 M, pH 7.4. To each well, 0.2 ml Eagle medium containing 50 µg/ml neutral red was added and the plate was incubated for 3 h at 37ºC. The media containing the dye were removed and each well was washed for 2-3 min with formol-calcium solution (40% formaldehyde, 10% anhydrous calcium chloride) to remove unincorporated neutral red. Finally, 0.2 ml of an acetic acid-ethanol mixture (1.0 ml glacial acetic acid in 100 ml 50% ethanol) was added to each well and the plate was kept for 15 min at room temperature in order to remove the dye from the viable cells. Plates were transferred to a spectrophotometer (Titertek Multiskan model 340) and read with a 540 nm filter. Control cell cultures received medium without bacterial filtrates. Cell viability was determined by comparison to the absorbance values obtained for control wells (without toxin), which were taken as 100% cell viability.

Toxicity assays on lettuce tissue culture. Seeds of lettuce were surface-sterilized by immersion in 70% ethanol for 1 min, followed by 2.5% sodium hypochloride for 60 min and thoroughly rinsed in sterile distilled water. They were transferred to Petri dishes containing medium for germination (water-agar) and maintained in culture room at 25 ± 3ºC. After 7 days, cotyledons of plantlets growth were removed, cut and inoculated on plates containing Murashige & Skoog (MS) medium[12] in the presence of culture filtrate of phytopathogenic, entomopathogenic and clinical *S. marcescens* two-fold serially diluted. Ten explants for each plate were inoculated and plates were incubated as described above, for observation of the callus formation. Evaluation was carried out after 15 days of growth, using as parameters: callus absence; small callus on some points of explants; small callus on all periphery of explants; callus more developed and involving all explants.

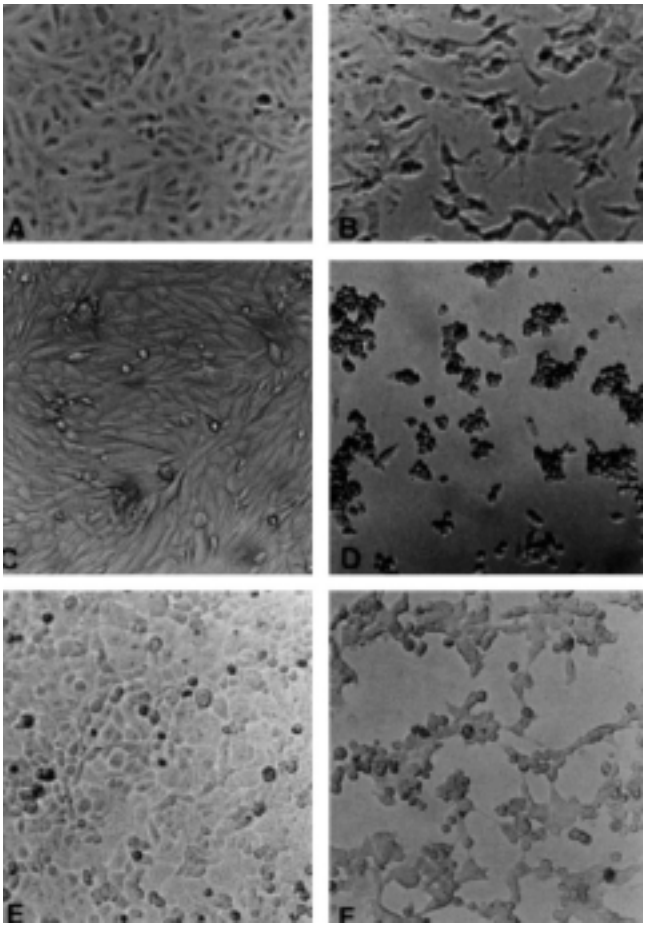

Figure 1. *Effect of environmental* S. marcescens *supernatants number (980) on morphology of mammalian cells. (A) Untreated Vero cells; (B) Vero cells 24 h after treatment; (C) CHO negative control; (D) CHO cells 24 h after treatment; (E) Untreated HEp-2 cells; (F) HEp-2 cells 24 h after treatment.*

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Phytotoxicity on onion bulbs. The assay was carried out for all *S. marcescens* strains by injecting 1 ml of culture filtrates or 1 ml of the same filtrates concentrated 10-fold with ammonium sulfate (80%) in onion bulbs. The bulbs were maintained at room temperature during 5 days with the root immersed in water. As negative control were used bulbs treated with sterile distilled water and as positive control suspension of *S. marcescens* (1 x 10^8 CFU/ml). After 5 days, the bulbs were sliced by half and observed for decay.

Phytotoxicity on lettuce plantlets. Seeds of lettuce were sterilized and put into 24 wells microplate, containing water-agar medium and placed in the culture room at 25 ± 3°C until development of plantlets. After this period, to each well was applied 0.5 ml of culture filtrates of *S. marcescens* stra-

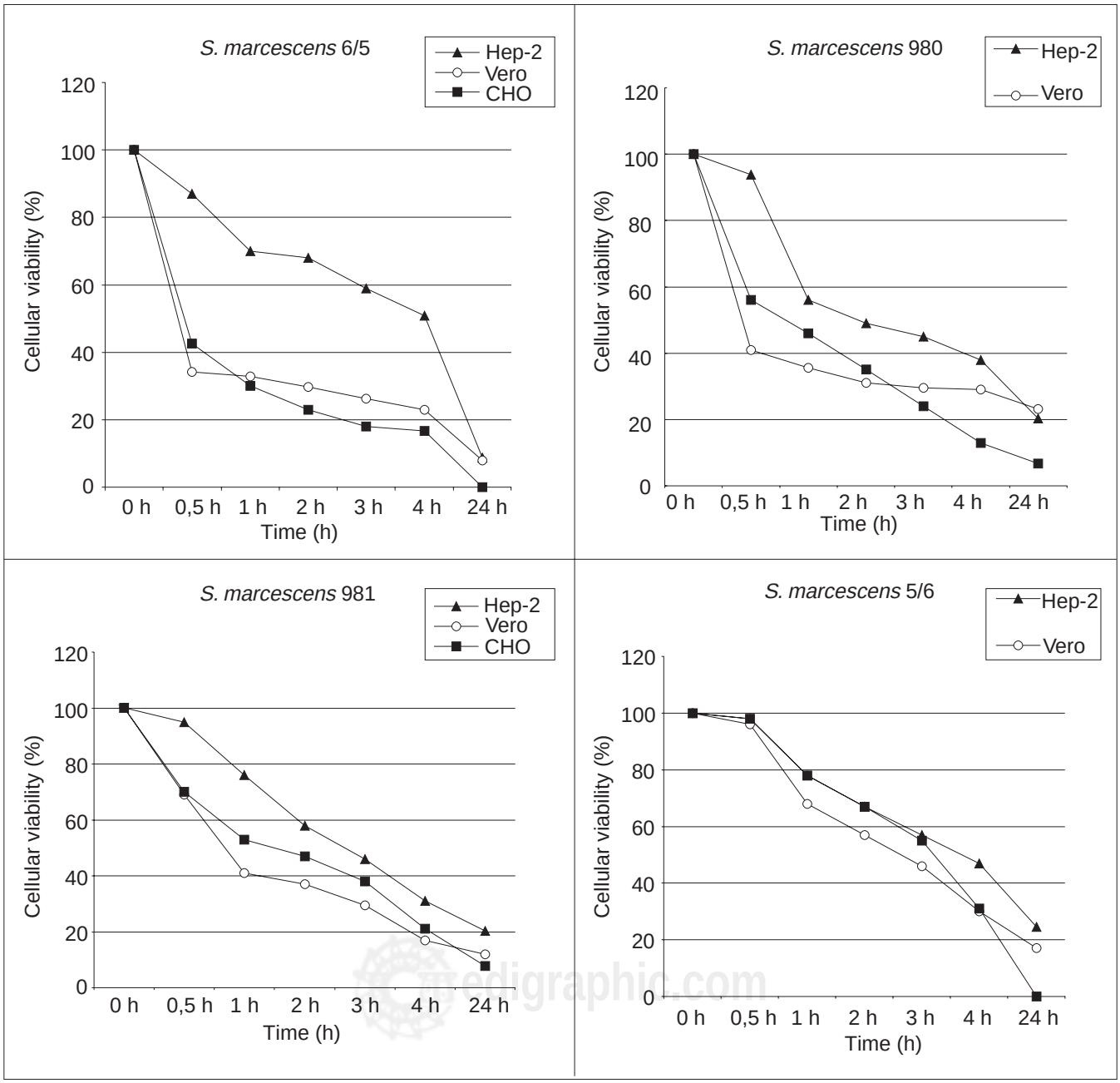

Figure 2.

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ins 10-fold concentrated with ammonium sulfate (80%). Negative controls included seeds treated with TSB medium and as positive control, the seeds that received suspension of phytopathogenic, entomopathogenic and clinical *S. marcescens.* After 7 days of incubation in culture room, the plantlets were analyzed with a magnifying glass lens.

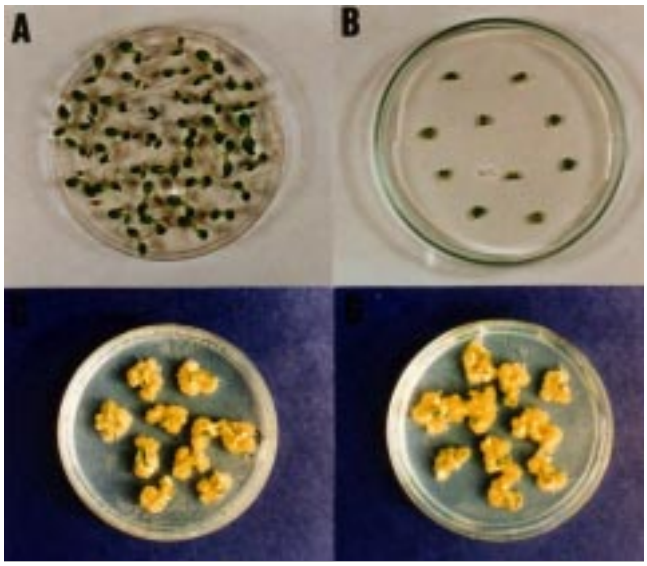

Figure 3.

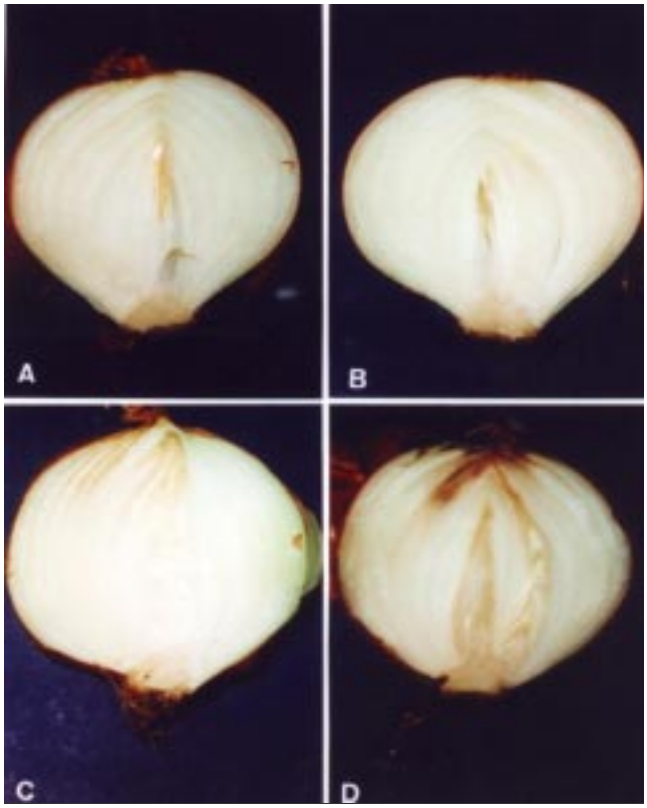

Figure 4.

Preparation of antiserum. Rabbits were injected intramuscularly with cytotoxin produced by the clinical strain. Samples of 50 µl of purified cytotoxin (75µg/ml) were emulsified in an equal volume of complete Freund adjuvant, with subsequent injections in incomplete adjuvant. Animals were bled at 2-week intervals and checked for neutralizing titres. Antiserum obtained was inactivated at 56°C for 30 min.

Seroneutralization assay. The seroneutralization assay was carried out as described previously,[15] with antiserum produced in rabbits against purified cytotoxin produced by the clinical *S. marcescens* strain 458. Anti-cytotoxin sera were serially diluted in Eagle medium and mixed with filtrates of phytopathogenic and entomopathogenic *S. marcescens*. Negative controls with preimmune serum were also included. The mixtures were incubated for 1 h at 37°C prior to inoculation in CHO cells.

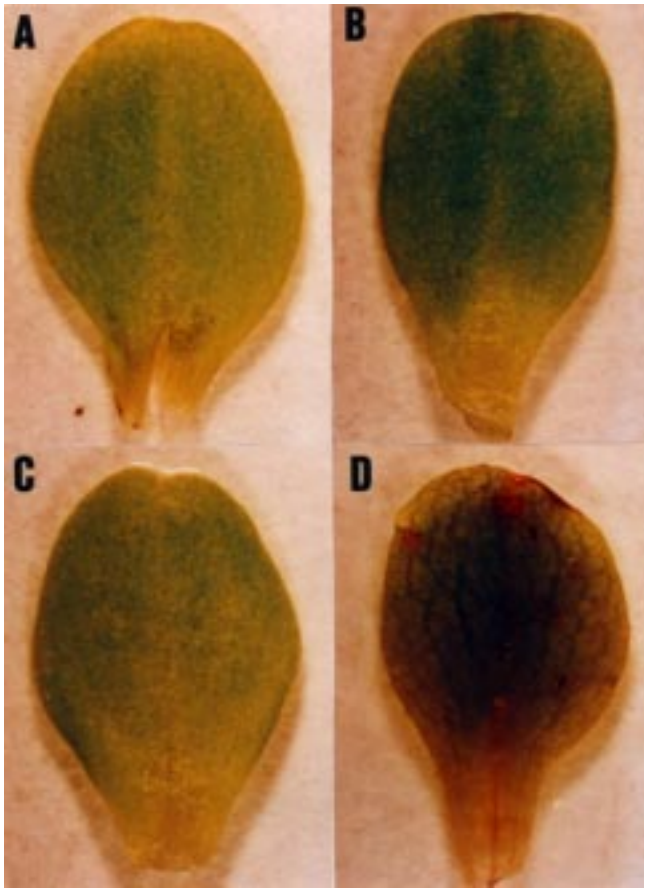

Figure 5.

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RESULTS

Cytotoxicity assays on mammalian cells. Cytopathic effects were observed in CHO, Vero and HEp-2 cells treated with culture supernatant from phytopathogenic and entomopathogenic *S. marcescens*. After 24 h of incubation, there was a change from spindle-shaped cells characteristic of normal cells to round and shrivelled cells, and these changes were followed by gradual destruction of the monolayer (Fig. 1). The HeLa cells were unresponsive and did not exhibit cytopathic effects under conditions in which other cell lines did.

Determination of cellular viability. Cellular viability of the Vero, CHO and HEp-2 cells was determined for all *S. marcescens* strains and the results were closely similar. Figure 2 shows that cell death occured rapidly, as early as 0.5 h after incubation with supernatant of 6/5, 5/6, 981 and 980 environmental strains. Toxicity assays on plant tissue culture. As shown in Figure 3, the culture filtrates of phytopathogenic, entomopathogenic and clinical *S. marcescens* were not toxic to lettuce seedling *in vitro*. All the explants inoculated in MS medium supplemented with several dilutions of supernatants filtrates showed callus developmented, involving all explants.

Phytotoxicity on onion bulbs assay. Figure 4 shows the results obtained with culture filtrates of phytopathogenic, entomopathogenic and clinical *S. marcescens* inoculated in onion bulbs. It can be observed that apparently did not cause any tecidual alteration, in contrast with bulbs inoculated with bacterial suspension, that shows decay .

Phytotoxicity on lettuce plantlets assay. Culture filtrates of phytopathogenic, entomopathogenic and clinical *S. marcescens* did not cause morphological changes on analyzed lettuce plantlets, even when concentrated 10 times with 80% ammonium sulfate. With the inoculation of bacterial suspension, it can be observed the appearance of symptoms such as shriveling and necrotic stains on plantlets cotyledons (Fig. 5).

Seroneutralization assays. The cytotoxic activity from phytopathogenic and entomopathogenic *S. marcescens* on CHO cells was completely neutralized by antiserum obtained against cytotoxin of the clinical *S. marcescens*, with titre of 1/128.

DISCUSSION

Cytotoxins have been considered as important virulence factors in several species of bacteria[6] and are detected on the basis of the degree of the damage imposed to several mammalian cell lines, evidenced by morphological changes.[6]

Serratia marcescens isolated from clinical specimens can produce an extracellular cytotoxin active on epithelial cell lines in culture.[5] However, as far as we know there are no data on cytotoxin production by environmental *S. marcescens*.

In this work, supernatants of the *S. marcescens* isolated from plants and insects were capable of damaging mammalian cell lines, in vitro. Morphological changes on the sensitive cell lines were characterized by cell rounding and detachment (Fig. 1), culminating in cell death. The cytopathic effect observed resemble those described in cells inoculated with cytotoxin produced by clinical isolates.[3]

The sensitivity of several cell lines to cytotoxin produced by entomopathogenic and phytopathogenic was compared in this study. Figure 2 shown that CHO, Vero and HEp-2 lines were similarly sensitive for all strains tested, with cytopathic effects observed as soon as 30 min of incubation with culture filtrates and it was confirmed by measured of the cellular viability ranging 40%. Moreover, the HeLa cells were resistant to all culture filtrates, as shown in cellular viability assays.

The cytotoxic activity of the supernatants obtained from culture of phytopathogenic and entomopathogenic *S. marcescens* on CHO cells was completely neutralized by specific polyclonal antiserum anti-cytotoxin produced by clinical *S. marcescens*. It indicates that these strains produces toxins antigenically related. Moreover, the cytotoxin shows similar biological activity on CHO cells with the cytotoxin produced by clinical *S. marcescens*.

The toxicity assays on plants shows that the culture supernatants not provoked any visible morphological changes and did not affect their growth. By contrast, the plants treated with bacterial suspension shown disease symptom, such as shriveling and decay of stores bulbus in onion (Fig. 4) and lettuce plantlets (Fig. 5). On the basis of these results, it appears that the extracellular factor capable of damaging cultured cells was not responsible by disease in plants.

In conclusion, this study shows that phytopathogenic and entomopathogenic *S. marcescens* may produces a cytotoxin similar to produced by clinical isolates and it is toxic to different mammalian cell lines. These results are especially important for studies involving this bacterium as biological control agent. At present, more detailed study of biological activity of this cytotoxin is in progress in our laboratory.

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