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Ecotoxicity studies of antifungal metabolites of B*acillus* sp. IBA 33.

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Abstract: The increments of plant diseases caused by the proliferation of fungicide-resistant pathogens intensified the research of new active metabolites against them. The study of biological methods as an alternative to chemical control has reach relevance in recent years. It has been suggested that the use of metabolites from *Bacillus*, is an alternative or supplementary method to chemical plant protection, if they are not toxic for the consumers and environmentally friendly. The toxicity of different concentrations of antifungal metabolites produced by *Bacillus* sp. IBA 33 was evaluated. Their ability to induce cytotoxic effects was investigated by different tests as hemolysis, and *Artemia salina* and *Allium cepa* L immobilization. With 2.56 mg/ml of antifungal metabolites hemolysis reached 24.07%, to 1.28 mg/ml was 14%. HC_{50} was 10.41 mg/ml. *A. salina* nauplii exposed to 3.2, 1.6 and 0.8 mg/ml of antifungal metabolites showed 80, 20 and 10% of mortality respectively; LD_{50} was 2.24 mg/ml. In *Allium cepa* L test after 72 h of treatment, the roots lengths were 8.75, 10.35 and 23.75 mm with 3.2, 1.6 and 0.8 mg/ml of antifungal metabolites respectively. The EC_{50} was 0.078 mg/ml. After 144 h the EC_{50} was 4.11 mg/ml. Only with 3.2 mg/ml of metabolites, chromosomal aberrations, cytoplasm vacuolization and decentralization of nuclei were observed in the cells of *Allium cepa* L. No micronuclei were detected. Under the experimental conditions, the antifungal metabolites concentrations assayed are considered non toxic.

Keywords: lipopeptides, hemolysis, *Artemia salina*, *Allium cepa* L., citotoxicity, ecotoxicity.

Resumen: Estudios de la ecotoxicidad de metabolitos antifúngicos de Bacillus sp. IBA 33.

El incremento de enfermedades de plantas causado por la proliferación de patógenos resistentes a fungicidas intensificó la investigación de nuevos metabolitos activos contra ellos. El estudio de métodos biológicos como una alternativa al control químico ha alcanzado relevancia en años recientes. Se ha sugerido que el uso de metabolitos de *Bacillus*, es una alternativa o un método suplementario a la protección química de las plantas, siempre y cuando ellos no sean tóxicos para los consumidores y sean amigables para el medio ambiente. Se ha evaluado la toxicidad de diferentes concentraciones de metabolitos antifúngicos producidos por *Bacillus* sp. IBA33. Se investigó su capacidad para inducir efectos citotóxicos mediante diferentes tests como hemólisis e inmovilización de *Artemia salina* y *Allium cepa* L. Con 2.56 mg/ml de metabolitos antifúngicos la hemólisis alcanzó 24.07%, para 1.28 mg/ml fue 14%. La HC_{50} fue 10.41 mg/ml. Nauplios de *Artemia salina* expuestos a 3.2; 1.6 y 0.8 mg/ml de metabolitos antifúngicos mostraron 80; 20 y 10% de mortalidad respectivamente después de 24 h de tratamiento; la LD_{50} fue 2.24 mg/ml. En el test de *Allium cepa* L después de 72 h de tratamiento, la longitud de las raíces fueron 8.75; 10.35 y 23.75 mm con 3.2; 1.6 y 0.8 mg/ml de metabolitos antifúngicos respectivamente, con una EC_{50} de 0.078 mg/ml. Después de 144 h la EC_{50} fue 4.11 mg/ml. Solamente con 3.2 mg/ml de metabolitos fueron observadas aberraciones cromosómicas, vacualización y descentralización de los núcleos en las células de *Allium cepa* L. No se observaron micronúcleos. Bajo las condiciones experimentales, las concentraciones de metabolitos antifúngicos ensayadas son consideradas no tóxicas.

Palabras claves: lipopéptidos, hemólisis, *Artemia salina*, *Allium cepa* L., citotoxicidad, ecotoxicidad.

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Introduction

The greatest causes of loss in fruit industry are postharvest diseases by the proliferation of strains resistant to fungicides, resulted in the need to investigate new bio-fungicides with potential industrial application. Factors that contribute to the fungicide-resistant plant pathogens included the prolonged and occasional excessive use of synthetic agrochemicals. Overuse of chemicals fungicides causes several environmental problems.

Therefore, the search for alternatives to chemical control has reach importance in recent years. It has been suggested that the use of strains of the genus *Bacillus*, or their metabolites, may be an alternative or supplementary method to chemical plant protection [1].

Several species of bacteria are known for antifungal activity, *Bacillus* are a great choice because produce endospores (tolerant to heat and desiccation) [2]. Production of antifungal protein of *Bacillus* spp in the suppression of various diseases is a common phenomenon. These antifungal metabolites are usually polypeptides composed entirely of amino acids, but some may contain other residues like a fatty acid chain. These kinds of metabolites are named lipopeptides and there are three families of *Bacillus* lipopeptides; surfactins, iturins and fengycins [3]. Many microorganisms, particularly bacteria, release biosurfactants to their growth medium. The chemical nature of these compounds is rather heterogeneous, including glycolipides, lipoproteins and lipopeptides [4]. Lipopeptides as compared with synthetic fungicides are in general more selective, effective, stable and environmentally friendly with a number of very interesting andpromising technical applications [5]. Previous work demonstrated that *Bacillus* sp IBA 33 produces lipopeptides from iturins family with antifungal action against pathogens of postharvest disease of lemon fruits [1]. Lipopeptides application in products for human consumption, for instance, requires an accurate characterization of possible toxic effects.

The preliminaries tests applied to determine cytotoxicity are: hemolysis of red blood cells and mortality of *Artemia salina*, also the test of *Allium cepa* L. detects potentially genotoxic agents.

The aim of this paper was applied the cited methods to study the toxicity of the antifungal metabolites produced by *Bacillus* sp. IBA 33, according to the effective concentration (1.6 mg/ml), to control *G. citri-aurantii* in lemon fruits. These studies contribute to increase the knowledge to the safety of antifungal metabolites for the future use as a biocontroller.

Materials and Methods

Microorganism:

Bacillus sp. IBA 33 strain, producer of antifungal metabolites (AFM), was isolated from the soil of lemon plantations [6] maintained in Luria Bertani medium, transferred every 30 days and kept at 4ºC.

Antifungal metabolites:

AFM from *Bacillus* sp. IBA 33 were produced in Landy medium [1]. Three stock solutions of AFM were prepared in distilled water or saline water: 3.2, 1.6 and 0.8 mg/ml.

Cytotoxicity assay:

Hemolysis:

Human erythrocytes were freshly prepared before the experiments from red blood cell concentrates. Cells were washed with buffer Tris-saline (150 mM NaCl, 50 mM Tris-HCl pH 7.6) and finally suspended at a hematocrit of 26%. AFM was added from a 3.2 mg/ml stock solution in buffer Tris-saline pH 7.6 and then were diluted until 0.64 mg/ml. After 30 min at room temperature, the samples were centrifuged and the hemolysis was determined by measuring the absorbance of release hemoglobin at 540 nm. The total amount of hemoglobin was established by lysis of the erythrocytes with water plus NaCl 50% (positive control) [7]. As negative control of hemolysis, the erythrocytes were suspended in buffer Tris-saline pH 7.6. The results were expressed as hemolysis percentage.

Toxicity test on Artemia salina:

Artemia salina is a brine shrimp, an organism living in saline water. A little amount of eggs from *Artemia salina* (VitaFish) was cultured into saline water (2.5% NaCl). The culture was incubated at 30ºC, 200 rpm during 24 h. The freshly hatched individuals, called nauplii, are used for testing. 10 nauplii of *Artemia salina* were placing into Petri dishes with 6 mL of different concentrations of AFM suspended in saline water (3.2, 1.6 and 0.8 mg/ml) at 30ºC without aeration. $K_2Cr_2O_7$ (20 mg/l) was used as positive control and saline water as negative control of toxicity. For each treatment three groups of 10 individuals were assayed. Mortality of nauplii was checked at 4, 8, 20 and 24 h.

Toxicity test on Allium cepa L.

Onion bulbs (*Allium cepa* L.) were scraped at the root and were place in 50 ml flasks, containing tap water during three days to promote the emergence of new roots until reached 15-20 mm in length. These bulbs were placed onto flasks filled with different concentrations of AFM (3.2; 1.6 and 0.8 mg/ml) at 25ºC. At 72 and 144 h the length of roots (mm) were measured. As positive and negative control were used $K_2Cr_2O_7$ (20 mg/ml) and tap water respectively. All solutions were renewed every 24 h. A series of three bulbs for each concentration and control groups were assayed.

Macroscopic parameters: Thereafter, the length of the whole root bundle was measured as described by Fiskesjo [8]. Were plotted the root length expressed as a percentage of the control against concentrations of AFM to estimate EC_{50} values. Other signs of toxicity such as changes in root consistency and color, the presence of tumors, hook and twisted roots were also, recorded as indicative of general toxicity

Microscopic parameters: Of each *Allium cepa* L. bulbs treated with the AFM during 48 h under the usual laboratory conditions described above, three root tips were used for the microscopic analysis to determine genotoxicity (mitotic index and the scoring of micronuclei and chromosome aberrations). At the end of the exposure period, the roots were fixed in ethanol-glacial acetic acid (3:1) and kept at 4ºC overnight. After hydrolysis during 15 min in 1N HCl were rinsed in distilled water. Then, only the meristematic region of the rootlets were fragmented, under microscope using histological needles, stained with 2% orcein-50% acetic acid. Microscope slides were prepared by squashing. The mitotic index was determined by scoring approximately 900–1000 cells per slide and was calculated as the ratio between the number of cells in mitosis and the total number of cells. The frequencies of micronuclei were analyzed by observing 1000 interphase cells for each bulb. Chromosome aberrations were characterized in 100 mitotic cells per slide and classified as stickiness and abnormal ana/telophases, which include bridges, vagrant chromosomes, chromosome missegration.

Data analysis: The data of roots lengths after 144 h of treatment were analyzed using Kruskal-Wallis test and ANOVA two ways, with repeat observations. Dunnett test was used for comparing several treatments groups with the negative control. The level of statistical significance was in all cases $p \leq 0.05$. IC_{50}, EC_{50} and HC_{50} value were calculated using the Nonlinear Curve Fit (DoseResp) with Origin 8.0 Pro software [$y = A1 + (A2-A1)/(1 + 10^{(Log\ x0-x)*p})*$]. Each data point represents the arithmetic mean ± standard deviation (S.D.) of three independent experiments.

Results

Hemolysis

The AFM has been tested at different concentrations for its hemolytic activity. The results are given in Table 1. Indicating that at 2.56 mg/ml of AFM, the hemolysis reached 24.07%, to 1.28 mg/ml was 14.8%; with lower concentrations no hemolysis was observed. With positive control (NaCl 50%) and buffer Tris-saline pH 7.6 (negative control), the hemolysis was 100% and 0% respectively. Based on the results, we determined HC_{50} values, which is defined as the concentration of AFM that burst 50% of the red blood cells was equal to 10.41 mg/ml with R^2=0.93001 (Fig. 1).

Table 1. *Effect of different concentrations of AFM from Bacillus sp. IBA 33 on hemolysis.* ***Negative control:*** *Buffer Tris-saline pH 7.6.* ***Positive control:*** *NaCl 50%. The data are* expressed ± S.D.

AFM (mg/ml)	Hemolysis (%)
0.64	0*
0.96	0*
1.28	14.8 ± 0.65*
1.92	18.51 ± 2.66
2.24	20.37 ± 2.62
2.56	24.07 ± 2.60*
Negative control	0
Positive control	100

*Significative difference p>0.05

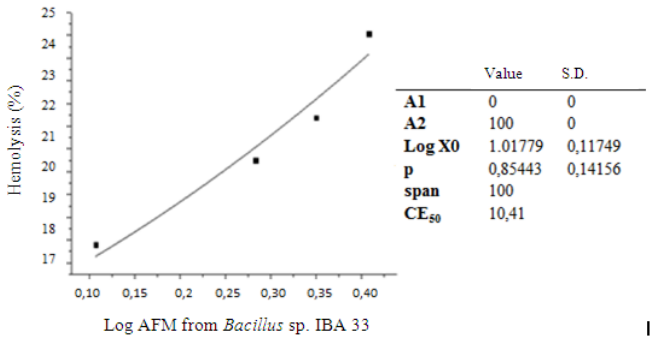

Fig. 1 HC_{50} obtained through the Dose-Resp analysis at 30 min of exposure of erythrocytes to different concentrations of AFM from *Bacillus* sp. IBA 33.

Toxicity on brine shrimp

The negative control (saline water) of *A. salina* nauplii registered 0% mortality after 24 h of treatment. *A. salina* nauplii exposed to 3.2, 1.6

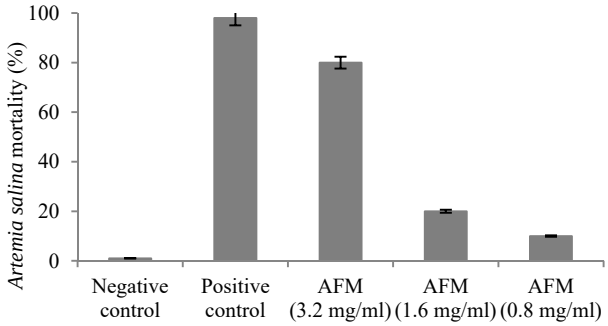

Fig. 2 Effect of different concentrations of AFM from *Bacillus* sp. IBA 33 on *Artemia salina* mortality (%). Negative control: Tap water. Positive control: $K_2Cr_2O_7$ 20 mg/l. Standard deviation is represented by the error bar on each point.

and 0.8 mg/ml of AFM showed 80, 20 and 10% of mortality respectively. The positive control registered 100% of mortality after the assay (Fig. 2). These results suggest that AFM showed a significant dose-dependent effect.

The data of nauplii mortality at 4 h of incubation have not significant differences with the three doses of AFM assayed (p= 0.1497). However, at 8, 20 and 24 h of AFM exposure show statistically significant differences (p<0.05) (Data not shown). When the survival of *Artemia* nauplii (3.2 mg/ml AFM) was compared with positive control no significant difference was observed (p=1).

With 1.6 and 0.8 mg/ml of AFM showed significant differences respect to positive control (p<0.05). When these concentrations were compared with negative control, the Kriskall Wallis analysis showed significant differences (p<0.05).

The level of AFM that produce a response in 50% of the population under study is known as LD_{50}, (lethal dose). LD_{50} was obtained from Fig. 3. The LD_{50} was 2.24 mg/ml with R^2=0.9596. The LD_{50} for $K_2Cr_2O_7$ was 12.5 mg/l [8].

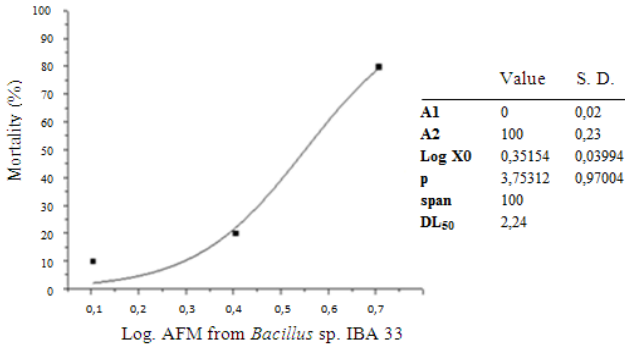

Fig. 3 LD_{50} obtained through the Dose-Resp analysis at 24 h of exposure of *Artemia salina* to different concentrations of AFM from *Bacillus* sp. IBA 33.

Toxicity on Allium cepa L.

No macroscopic changes were evident in growing roots under the three concentrations of AFM examined, the dichromate solution induced hook formation. The effect of AFM on root elongation of *A. cepa* L. bulbs is summarized in Table 2. After 72 h of treatment, the roots lengths were 8.75, 10.35 and 23.75 mm with 3.2, 1.6 and 0.8 mg/ml of AFM respectively, and the control was 16.16 mm. After 144 h of treatment, the roots in the control had an average length of 44.41 mm. With 3.2, 1.6 and 0.8 mg/ml of AFM the roots length reached 25.9; 36.73 and 49.79 mm (Table 2).

Table 2. Roots length (mm) after treatment with AFM from *Bacillus* sp. IBA 33. ***Negative control:*** tap water and ***Positive control:*** $K_2Cr_2O_7$ (20 mg/l). The data are expressed mean ± SD.

	Length (mm)	
	72 h	144 h
Negative control	16.16 ± 0.707	44.41 ± 4.563
Positive control	7.73 ± 0.251	7.9 ± 0.100
AFM (3.2 mg/ml)	8.75 ± 1.322*	25.9 ± 0.115
AFM (1,6 mg/ml)	10.35 ± 0.351*	36.73 ± 0.472
AFM (0.8 mg/ml)	23.75 ± 1.258*	49.79 ± 3.664

*Significative difference $p>0.05$

The measurements of roots length after 72 and 144 h of treatment were analyzed by Kruskal-Wallis test, showing length significantly difference (p<0.05).

The ANOVA analyses of variance between the negative control and the AFM treatments (144 h) shows that there are no significant difference in the roots length (p>0,05).

At the concentrations assayed, significant and dose-dependent reductions in length were observed after treatment (144 h). The EC_{50} for treatment of 72 h and 144 h were 0.078 mg/ml and 4.11 mg/ml respectively. The EC_{50} for $K_2Cr_2O_7$ was 30 mg/l [10].

Microscopic parameters

Mitotic Index: The mitotic index of the cells of *Allium cepa* L. treated with 3.2, 1.6 and 0.8 mg/ml of AFM were 97.8, 99 and 100% respectively. The mitotic index for the negative control was 100%.

Chromosomal aberrations: Normal mitotic phases in meristematic cells were observed (Fig. 4a, b, c and d). The chromosomal aberrations in cells exposed to 3.2 mg/ml of AFM were observed in telophase as losses (Fig. 4e) and sticky (Fig. 4f) chromosomes. Also c- mitosis cells were examined (Fig. 4g). In cells treated with lower concentrations of AFM no aberrations were observed.

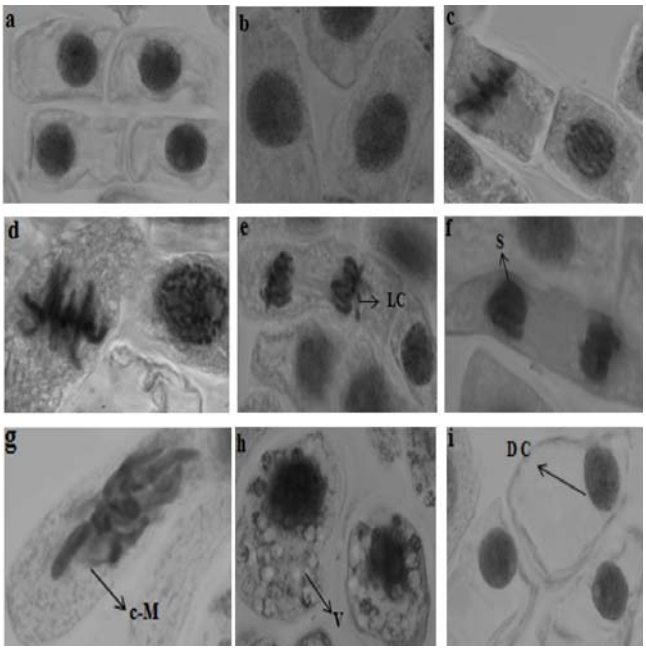

Fig. 4 Chromosomal aberrations of meristematic cells of *Allium cepa* L. induced by AFM from *Bacillus* sp. IBA 33 (100X magnification) a) Normal interphase, b) Normal prophase, c and d) normal metaphase, e) anaphase with losses chromosomes (LC), f) anaphase with sticky chromosome (S), g) c-Mitosis (c-M), h) cytoplasm vacuolization (V), i) decentralization nulcei (DC).

Nuclear and Cytoplasm Abnormalities: The cytoplasms of meristimatic cells treated with 3.2 mg/ml of AFM had morphological alterations. These alterations included vacuolization of the cytoplasm (Fig. 4h) and decentralization nuclei (Fig. 4i). With the all concentrations of AFM assayed, no micronuclei were observed.

Discussion

In previous works realized in our laboratory, the AFM obtained from *Bacillus* sp. IBA 33 were partially identified as lipopeptides like iturins family [1]. The AFM concentrations assayed were chosen according to the effective concentration to control *G. citri-aurantii* in lemon fruits (1.6 mg/ml) [6].

The AFM isolated from *Bacillus* sp. IBA 33 produces disruption of plasmatic membrane of *G. citri-aurantii* (data not shown). The ability of lipopeptides to disrupt a membrane is related to two parameters. The first one is its capacity to penetrate into the membrane at low concentrations; the second one is the molar ratio between lipopeptides and membrane lipids [11]. The capacity of iturin A to cause hemolysis of human erythrocytes has been reported before [12]. Aranda *et al.*

[13] suggest that iturin A induced hemolysis of red blood cells and also induced the leakages of K^+ ions in a concentration dependent way. The hemolysis occurred gradually, inferring that the lipopeptides produced membrane lesion [14].

The behavior of AFM with human erythrocytes was similar to obtained with lipopeptides in a dose–dependent manner. The HC_{50} of AFM from *Bacillus* sp. IBA 33 (10.41 mg/ml) was higher than the lipopeptides obtained from *Bacillus subtilis* ATCC 6633 (0.06 mg/ml) [15]. According to these results, AFM have less toxicity than that other lipopeptides.

A. salina is a target organism for the study of organophosphate pesticides [16] and was chosen as testing organism, considering the feasibility of culturing large populations, facility to manage, short term results given in short life cycle and low cost [17].

Siqueira *et al.* [18] defined a substance as highly toxic when the LD_{50} is less than 0.08 mg/ml, moderately toxic, when values are between 0.08 and 0.25 mg/ml and with values over 0.25 mg/ml a substance is considered non-toxic. According to our research, *A. salina* proves to be an excellent organism for toxicity studies of AFM with LD_{50}=2.24 mg/ml. Elkahoui *et al.* [19] studied the toxicity of antifungal extract produced by *B. subtilis* and *B. cereus,* and their results depended of culture medium used for antifungal extract production. The results of the *A. salina* assays showed no evidence of cytotoxicity with surfactin produced by *Bacillus* strain, the LD_{50} was 0.6122 mg/ml [20]. The difference observed between LD_{50} of the lipopeptides of AFM and surfactin should be to the grade of purity of them, by the way, both are non-toxic compounds.

The *Allium cepa* L. is frequently used as a bioindicator of environmental pollution [21]. It is used to determine the toxicity of crude extracts of cyanobacteria [22], and to evaluate the genotoxic potential of medicinal plants [23]. Is an excellent *in vivo* model, where the roots are growing in direct contact with the AFM causing possible chromosomal alterations. The damage of the DNA eukaryotic cells could be predicted.

The results for macroscopic parameters used in the test revealed a dose-dependent inhibition of root growth. 3.2 mg/ml of AFM produce the major inhibition of the root length. Also was observed that the time of treatment was important due to after 144 h the root length was recuperated. It was demonstrated that the toxicity effect was reversible, because the EC_{50} at 72 h was lower than the EC_{50} at 144 h.

The cytotoxicity levels of an agent can be determinate by the mitotic index [24]. According to Hoshina [25] mitotic index significantly lower than the negative control can indicate alterations deriving from the chemical action in the growth and development of exposed organisms. The three concentrations of AFM tested have mitotic index similar to the negative control demonstrating that the AFM have not cytotoxic effect.

The analysis of the mitotic phases in meristematic cells allows determined chromosome aberrations. There were a few cells with chromosome aberrations when they were treated with highest AFM concentration. Micronuclei have been considered by many authors as the most effective and simplest endpoint to analyze the mutagenic effect promoted by chemicals [24]. The absence of micronuclei in meristematic mitotic cells revealed that the AFM were non-mutagenic.

Under our experimental conditions, the three different AFM concentrations assayed may not be considered toxic. However, the results of these studies are promising and can be fundamental for more complex toxicity studies.

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