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Full Length Research Paper

Secondary metabolites induction in *Mammillaria huitzilopochtli* (Cactaceae) and evaluation of the fungicidal activity

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Bioactive compounds isolated from *Mammillaria huitzilopochtli* were evaluated in a biological activity test (% inhibition) against eight phytopathogen fungi. The fungi evaluated were: *Cladosporium* sp., *Phoma* sp., *Alternaria* sp., *Rhizoctonia* sp., *Fusarium* sp., *Fusarium moniliforme, Helmintosporium* sp. and *Phaecilomyces* sp. A suspension culture of *M. huitzilopochtli* was established using MS medium with 0.1 mg/l of 2,4-D and elicited with fungic homogenate. The defensive compounds produced were segregated to culture medium. Maximum production was obtained four days after the induction. The total extract showed inhibition for all fungi to 100 µg/ml, but at 400 µg/ml the *Phoma* fungus was inhibited 100%. This extract could be used eventually against the *Phoma* fungus, a pathogen of potatoes.

Key words: Cactus, suspension culture, secondary metabolites, phytoalexins, phytopathogens, fungus, elicitors.

INTRODUCTION

Mammillaria huitzilopochtli is an endemic cactus of the Cuicatlan Valley, in the State of Oaxaca, Mexico. Its population covers an approximated area of 80 Km^2, growing on scarped soils (Bravo and Sánchez-Mejorada, 1991). With a view of countering obvious problems in the environment and avoiding the toxic effects of synthetic chemicals on non-target organisms, investigations on exploiting pesticides of plant origin are becoming increasingly important in the field of plant pathology. Studies carried out through *in vitro* investigation confirm the fungicidal potential of the compounds present in some plants. Various approaches for redirecting transport and biosynthesis of secondary metabolites in plant-cell suspension culture have been attempted in an effort to increase productivity of secondary metabolites (Brodelius and Pedersen, 1993). Increasing the activity of metabolic pathways by elicitation, in conjunction with end-product removal and accumulation in an extractive phase, has proven to be the most successful strategy. Higher plants respond to several environmental stimulants, activating their secondary metabolism. Some derived compounds of this process are vital for plant life. Most have proven to be defense mechanisms against bacteria, virus and fungus attack in an immune system analogue of animals (Vickery and Vickery, 1981). Others can function in a process of attraction or as a repellent of insects (Bell, 1981). The chemical composition of cacti has been studied. This family produces pigments (betalains), alkaloids, and triterpenes (Gibson and Nobel,

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **BAP,** benzyl amino purine; **BA,** benzyladenine; **MS,** Murashige and Skoog (1962) medium.

1990). Not many studies have been carried out on chemical response to microbial infections in dessert plants. In *Cephalocereus senilis*, a species from south-western Mexico, an aurone was accumulated in suspension culture after elicitation with quitin and this compound inhibited the growth of gram negative bacteria (Paré et al., 1991). It was also observed that this aurone, named cephalocerone, inhibited the growth of *Erwinia cacticida* (Paré and Mabry, 1993), a cactus pathogen, which is responsible of the disease-cacti known as "soft rooting".

In this work the establishment of the suspension culture of *M. huitzilopochtli*, the production of secondary metabolites induced by biological elicitors and the fungicidal activity of these compounds on different phytopathogen fungus are reported.

MATERIALS AND METHODS

Suspension cultures

The inoculum (callus) of *M. huitzilopochtli* used in this study was obtained according to Arriaga (1994). The suspension culture was obtained using as basal medium (100 ml), of the MS mineral salts and vitamins (Murashige and Skoog, 1962) plus 30 g/l of sucrose with 0.1 mg/l of 2,4-dichlorophenoxyacetic (2,4-D). The pH was adjusted to 5.8 prior to adding agar (8 g/l) and autoclaving (121°C, 15 min). The inoculum (callus) was 2 g/100 ml culture medium. Cultures were kept in a growth chamber at 27 ± 2°C, photoperiod was of 16 h light/ 8 h dark and illumination (0.01 watt/m^2).

Biological elicitors and induction

Fusarium moniliforme homogenates were used as biological elicitors (Saad, 1996). All fungi were grown in agar potato-dextrose medium. 200 g of potato were peeled, washed and cooked in 500 ml of distillated water. It was filtered in a stainless steel strainer (200 mesh) and 14 g of dextrose and 16 g of agar were added and this was autoclaved (121°C, 15 min). After a week, the fungus was filtered through a stainless steel strainer (200 mesh), washed with distillated water, acetone was added to eliminate the residual water and this was macerated in a mortar until a homogenate was obtained. 100 mg of homogenate was put in test tubes and autoclaved (121°C, 20 min). For elicitation, 100 mg of homogenate was added to each vessel of suspension culture. In each vessel (100 ml MS medium) 2 g of *M. huitzilopochtli* callus was used. The effect of the elicitor was evaluated each 24 h. Some compounds were segregated to culture medium.

Fungicide extraction

The culture medium was filtered in a stainless steel strainer (200 mesh) and later the cells were centrifuged for 15 min at 3,000 rpm. The culture medium was extracted with ethyl acetate (30 ml/100 ml of culture medium). The cells were homogenized for 5 min (Ultra-turrax) and extracted with dichloromethane:methanol (9:1). Both extracts were concentrated in a rotavapor. An analysis was made of them separately with UV-spectrophotometry and kept at 4°C for their later use.

Biological activity test

The total extract was evaluated in a test of biological activity against the phytopathogenic fungi: *Cladosporium* sp., *Phoma* sp., *Alternaria* sp., *Rhizoctonia* sp., *Phaecilomyces* sp., *Helmintosporium* sp., *Fusarium* sp. and *Fusarium moniliforme*. A potato-dextrose agar medium was prepared and 100 µg/ml of the total extract obtained from the culture medium of elicited cells of *M. huitzilopochtli* was added. The addition was made under sterile conditions. The extract was sterilized by filtering through a millipore membrane (0.65 µm). Later it was dissolved in 95% ethanol and the ethanol was found to be non-toxic to the fungi (Saad, 1996). The test was repeated three times. Additionally, a greater concentration (100-400 µg/ml) for the *Phoma* sp. was tested. The determination of funguicide activity was carried out in the following way: an inoculate of each fungus was placed in the center of a petri dish and the radial growth of the fungus was determined. The results are expressed as a percentage of inhibition compared to the control and are the average of three repetitions.

RESULTS

Suspension cultures

To establish the suspension culture, MS medium was added with different concentrations of BA (0, 1, 5 and 10 mg/ l) and of 2,4-D (0, 0.01, 0.1 and 1 mg/l). The treatment without BAP and 0.1 mg/l 2,4-D showed greater production of biomass in the suspension culture. These were the conditions that were used for the production of biomass to carry out different experiments with elicitors. Figure 1 shows the curve of growth of cells in suspension of *M. huitzilopochtli* in the MS medium with 0.1 mg/l of 2, 4-D added.

Fungicide induction

There was 100% induction of defense compounds by the homogenate (1 mg/ml) in cultures in suspension of *M. huitzilopochtli* (intense red color in medium and cells in suspension). The presence of a red color in the cells and yellow in the culture medium was observed between 24 - 48 h after the induction with the elicitors. The yellow color of the medium stayed stable indefinitely. The maximum absorbance was observed to 444 nm. The curve of production of phytoalexins, both, released in the culture medium (Figure 2) and accumulated in the cells of the suspension cultures of *M. huitzilopochtli* (Figure 3) are shown. It was observed that the segregation of defense compounds to culture medium reached the maximum four days after inoculation (96 h, 0.692 absorbance to 444 nm) and the greatest production of metabolites in the elicitated cells was also reached four days after the inoculation, (96 h, 2.24 absorbance to 444 nm).

Fungicidal activity of elicited compounds

The colors in the cells and medium of elicitated cacti indicate a phytoalexin type effect (Paré and Mabry, 1993). The elicitation of the suspension culture of *M. huitzilopochtli*, presented the same effect. The tests of biological activity were carried out using the total extract obtained

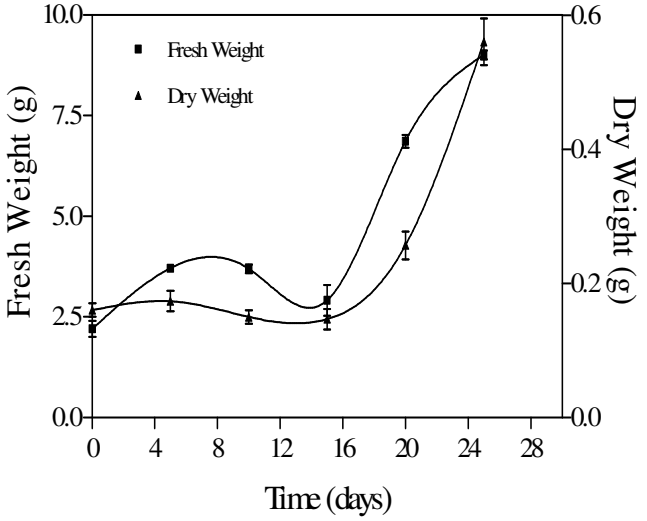

Figure 1. Growth of suspension cultures of *Mammillaria huitzilopochtli* (MS medium plus 0.1 mg/l of 2,4-D). Fresh weight and dry weight in grams was measured. The average is the result of three repetitions.

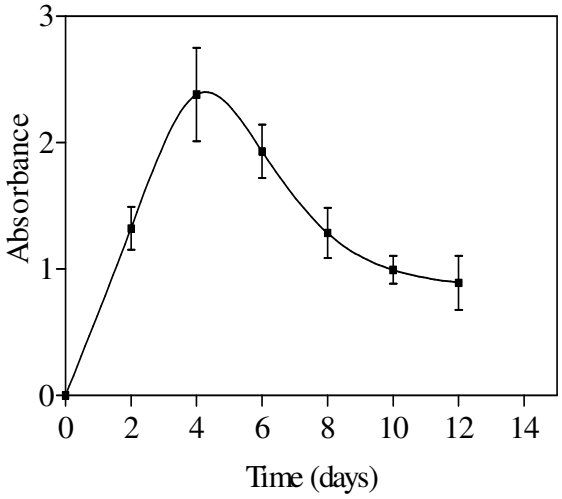

Figura 3. Production of phytoalexins. Extracts of filtered cells of the suspension culture medium of *Mammillaria huitzilopochtli* (MS medium plus 0.1 mg/l of 2,4-D) elicited with homogenates fungi. Absorbance, λ of 444 nm. The results are an average of three repetitions.

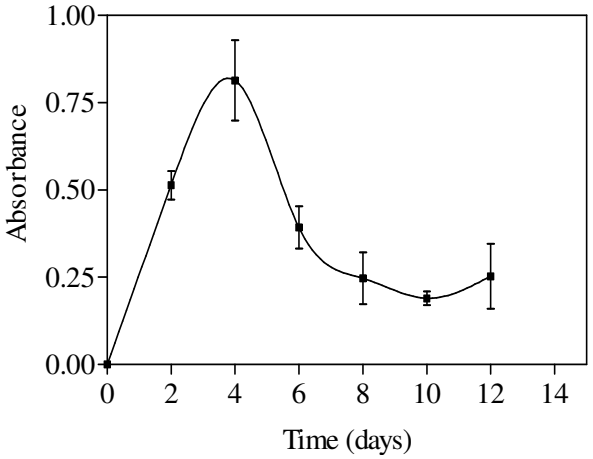

Figure 2. Production of phytoalexins. Extracts of the suspension culture medium of *Mammillaria huitzilopochtli* (MS medium plus 0.1 mg/l of 2,4-D) elicited with homogenate fungi. Absorbance, λ of 444 nm. The results are an average of three repetitions.

from the cells and culture medium.

The effect of the total extract of culture medium and cells on the radial growth of eight phytopathogens fungi was determined (Table 1). All the evaluated fungi showed some degree of inhibition by the extracts at 100 µg/ml. However, the *Phoma* sp. was most affected by the extract with a percentage of inhibition of 50%. The *Phaecilomyces* sp. fungus had 45.11% inhibition. The least affected was the *Alternaria* sp. fungus with 14.61% inhibition. Despite the differences in the percentage of inhibition, the statistical analysis demonstrates that there were no significant statistical differences between the fungi: *Cladosporium* sp., *Phoma* sp., *Rhizoctonia* sp., *Fusarium* sp., *Helmintosporium* sp. and *Phaecilomyces* sp.

In *Phoma* sp. it was observed that increasing the concentration of the extract also increased the percentage of inhibition and that 400-450 µg/ml concentrations inhibited 100% of its radial growth. The statistical analysis showed that the treatments with 400-450 µg/ml of total extract were statistically different from the others (Table 2).

DISCUSSION

Diverse reports indicate that material such as, homogenate fresh fungus, homogenate dry fungus, chitin and chitosan have an effect on the production of phytoalexins in plants (Paré et al., 1992; Bonness et al., 1993; Liu et al., 1993; Toivonen, 1993; Marinelli et al., 1994; Saad, 1996).

In the Cactaceae family it has been demonstrated that the pigments produced in the flowers and under certain conditions of stress are of the betalain type, specifically betacyanins (red) and betaxhantins (yellow). These pigments absorb UV-VIS to a wavelength of 480 and 540 nm respectively (Gibson and Nobel, 1990). However, the pigments produced by elicitation with fungic homogenates do not correspond to these types of natural pigments. The greatest defensive activity in plant cells of different species appears in the first hours after inoculation. Our results agree with results reported by other authors (Chappell and Nable, 1987; Ren and West, 1992; Afek et al., 1995; Bestwick et al., 1995; Doares et al., 1995). A similar effect was observed in *M. huitzilopochtli*.

A common effect was observed in the cultures of *M. huitzilopochtli* and other cultures induced with elicitors

Table 1. Effect of the total extract (100 µg/ml) against the radial growth of eight phytophatogen fungi.

Fungus	Radial growth inhibition (%)*
Cladosporium sp.	18.60[ab]
Phoma sp.	50.00[b]
Alternaria sp.	14.60[a]
Rhizoctonia sp.	46.06[b]
Fusarium sp.	36.04[b]
Fusarium moniliforme	17.06[a]
Helmintosporium sp.	34.08[ab]
Phaecilomyces sp.	45.08[b]

*Compared against a control without extract. The average is the result of three repetitions.
Different letters correspond to significant differences at a 0.05 level.

Table 2. Effect of different concentrations of the total extract obtained by elicitation of suspension cultures of *Mammillaria huitzilopochtli* in the radial growth of *Phoma* sp.

Concentration	Radial growth inhibition (%)*
50	32.95[a]
75	42.96[ab]
100	47.24[bc]
125	38.90[ab]
150	55.83[bc]
200	30.17[a]
250	56.99[bc]
300	69.83[bc]
350	84.35[bc]
400	100[d]
450	100[d]

*Compared against a control without extract. The average is the result of four repetitions.
Different letters corresponding to significant differences at a 0.05 level.

(Chappell and Nable, 1987; Ren and West, 1992); it has been observed that the elicitation process induces the formation of secondary metabolites and even favors their liberation in the culture medium, presumably as a defense mechanism. In *M. huitzilopochtli* suspension cultures, the compounds released in the medium are yellow in color, while the cells themselves are red and there seems to exist an accumulation of different types of compounds, but both extracts absorb to 444 nm. The fact that the cells liberate metabolites to the culture medium is a factor valued in all biotechnological processes because this phenomenon diminishes the production costs at an Industrial level (Dicosmos and Misawa, 1995).

There was reported for *Rhizoctonia solanis* that an extract of elicitated compounds in *Verticillium biguttatum* at a concentration of 138 µg/ml inhibited its growth completely (Morris et al., 1995). In the present report it was observed that at a concentration of 100 µg/ml, the fungus of this same genus is inhibited 46.06% with respect to the control without extract. As far as we know there are few reports of phytoalexins induced by elicitation in cacti. In *Cephalocereus senilis*, compounds produced by elicitation were evaluated against gram negative bacteria, in addition to *E. cacticida*, a cactus pathogen that degrades the cellular wall (Paré et al., 1991). With these considerations, our results present a perspective worth studying.

To date, there is no existing report of induced phytoalexins from cacti that has been proven against fungi; the well-known reports have been focused only towards degradative bacteria of the cell wall in cactus like *E. cacticida* and some other gram positive bacteria.

Of the fungi evaluated here, pathogenicity in some species is well-known. For example, it is known that *Fusarium* sp. is the cause of diverse damages in plants such as *Lilium* sp. and *Ocimum basilicum* that can result in death (Davis and Marshall, 1993; Straathof et al., 1993). To find a future an application for these compounds, it is important to know the concentration at which these phytoalexins are able to eliminate the fungus.

Mexico has a great diversity of cacti and most are endemic. Many of them are threatened to some degree by extinction. Biotechnology offers the possibility of studying their potential for sustainable development. At the present time, the most interesting and important insecticides, bactericides and fungicides are those of biological origin because they do less damage to the environment. It is important to continue these studies and evaluate future applications of these compounds.

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