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Evaluation of Sunlight and Humidity Protection of a Bioherbicide for *Eichornia crassipes* Biocontrol

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Abstract: *C. piaropi* (*Cercospora piaropi*) and *A. zonatum* (*Acremonium zonatum*) have proved to be effective in reducing water hyacinth growth. However, efficacy of these fungi in field is limited by the effect of solar UV (ultraviolet) light and desiccation. In this study, three compounds used as sunscreens and one seed plant that produce mucilage were tested for their effects on the infection produced by *C. piaropi* and *A. zonatum* inoculum under laboratory and field conditions. In laboratory conditions, TiO_2 (titanium dioxide) and metamucil did not inhibit *C. piaropi* and *A. zonatum* viability. Moreover, the addition of TiO_2 and metamucil to the inoculum suspension increased fungi infection. The protective effect of TiO_2 and metamucil was more evident when the inoculum suspension was applied 4 h before sunset. These results suggest that addition of TiO_2 and metamucil provides necessary humidity and solar protection for increasing *C. piaropi* and *A. zonatum* infection on water hyacinth plants.

Key words: *C. piaropi*, *A. zonatum*, solar radiation, TiO_2, water hyacinth.

Nomenclature

Eichornia crassipes (Martius, Solms)
Cercospora piaropi (Tharp)
Acremonium zonatum (Saw) W. Gams

1. Introduction

Water hyacinth (*Eichornia crassipes* (Martius, Solms)), with geographical origin in South America, is one of the most important invasive and exotic species in the world. The Global Invasive Species Database Lists include this plant among the "100 of the world's worst invasive species". The beauty of water hyacinth flowers led to the plant's introduction into many countries as a decorative plant and finally its conversion into a weed in response to high level of nutrients in water bodies due to urban, industrial and municipal wastewater discharges [1]. In México, water hyacinth is the most important exotic aquatic plant—more than 40,000 ha of reservoirs, lakes, canals and drains are infested with water hyacinth [2]. This weed was probably introduced in México in the early 1900s [3]. Chemical and mechanical control methods have been used to manage water hyacinth, but these methods have expensive and unsatisfactory results, because repeated applications have been needed [2]. This lack of control is due to the weed's rapid growth rate and its ability to re-infest via the seed bank or by flood-borne plants. For these reasons, the only long-term and sustainable solution is applying an integrated approach to water hyacinth management in which biological control agents should play a key role. The biocontrol of this weed has been attempted by using insects, such as weevils of the genera *Neochetina* [4]. However, the use of a single level of biotic stress has not been completely effective in reducing plant growth and reproduction [5, 6]. Because of the reproductive capacity and fast growth of water hyacinth, it is necessary to use a set of biocontrol agents to increase the biotic stress in order to reduce population resurgence [7, 8]. Among the natural enemies of water hyacinth, plant pathogens can be useful because they are often host-specific and easy to propagate and disseminate. In a survey performed in México [9], the fungus *C. piaropi* (*Cercospora piaropi*) (Tharp) and *A. zonatum*

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(*Acremonium zonatum*) (Saw) W. Gams were identified as potential biocontrol agents for water hyacinth. The results indicated that damage produced by fungus is enhanced when used in combination with insects [8, 10-12]. Nevertheless, performance of plant pathogens as biocontrol agents might be limited by solar radiation, temperature and leaf wetness [13-17]. In many cases, pathogens applied in field can lose at least 70% of their original activity within the first day due to UV (ultraviolet) inactivation and/or partial fungal dehydration [18]. The SUV (sunlight-UV) inactivation of viral and other microbial insecticides applied in field has been attributed to a direct damage on DNA or by generation of highly reactive radicals or both [19, 20].

Several attempts have been reported to increase biocontrol agents stability, e.g., by adding UV protectors such as Congo Red [21], vitamin B [22], uric acid [23], dyes [24] and brighteners [25]. Although others have evaluated these synthetic organic chemicals as sunlight protectors in viruses [18, 25, 26], bacteria [15], protozoa [23] and nematodes [27], no reports were found about their use in plant pathogens. In order to increase the efficiency of *C. piaropi* and *A. zonatum* as biocontrol agents, the purpose of this study was to evaluate the feasibility of using some ingredients of several sunscreens, which were developed by cosmetic industry as sunlight protectors, such as TiO_2 (titanium dioxide), and some seed plants that can give a source of humidity for these two plant pathogens.

2. Material and Methods

2.1 Selection of Testing Compounds

The selection of the three testing compounds to be used as solar protectants in this study was based on the reported active ingredients of 10 commercial UV sunscreens intended for human use: oxybenzone, TiO_2 and octyl methoxynnamate (Merk Index Nos. 7088, 9612 and 6864). The compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The seed powder of a plant that produces mucilage and soluble in cool water was evaluated as desiccation protection: *Plantago psyllium* (Metamucil ® Procter & Gamble).

2.2 Laboratory Evaluation

The effects of three solar compounds and one powder of seeds on the viability of *C. piaropi* and *A. zonatum* were evaluated for a period of 25 days using spore germination and mycelium development as endpoints. Ten roux flasks containing 150 mL of potato dextrose broth (Difco, Detroit, MI, USA) were inoculated with 5 mL of a solution prepared with 30 g of *C. piaropi* or 30 g of *A. zonatum* (previously cultivated on potato dextrose agar during 25 days) and were bio-homogenized in 100 mL of sterile and distilled water. Based on concentration of the active ingredient used in commercial sunscreens for human use, the flasks were amended with 5 ppm of oxybenzone, TiO_2 or octyl methoxynnamate. The same concentration of metamucil was used as protective humidity cover since it creates a clear, colorless and gelling agent that is hydrophilic. Three repetitions per treatment were incubated at 25 ± 0.5 °C for 25 days (Lab-Line, model 302, IL, USA). As previous observations had shown that mycelium and conidia were developed in *C. piaropi* and *A. zonatum* after 8 days, monitoring of these two points was made after 10, 15, 20 and 25 days of incubation using a compound microscope (Zeiss, SV6, Germany). TiO_2 and metamucil did not inhibit viability of *C. piaropi* and *A. zonatum*.

2.3 Plant Material

Healthy water hyacinth plants were surface disinfected in 0.26% sodium hypochlorite solution and rinsed 3 times with tap water to remove traces of the disinfectant. The plants were maintained for 2 weeks in 50% Hoagland's solution [28]. One day before the experiment began, plastic pots (0.75 m × 0.45 m × 0.21 m) were filled with 50% Hoagland's solution and 10 water hyacinth plants of approximately 25 cm of height were transferred to each pot. Each triplicated treatment was applied on each pot (10 replicates).

2.4 Fungal Culture

The Mexican indigenous strains Mx-WH-15.1 of *C. piaropi* and Mx-WH-26 of *A. zonatum* were taken from a fungal collection made in a previous work [9]. These two strains have a deposit in the American Type Culture Collection (PTA-983 and PTA-984). The mycelia suspension was prepared by growing *C. piaropi* in Roux flasks containing 150 mL of potato-dextrose broth plus 5% yeast and PD (potato dextrose) medium in Petri dishes for *A. zonatum*. Cultures were maintained in the dark in an incubator at 25 ± 2 °C. 21 days after incubation, the culture matrix was weighed, diluted in sterile distilled water to a concentration of 20% w/v (weight/volume) and comminuted in a food blender for 10 s at the high-speed setting. The final inoculum consisted of mycelia suspensions of *C. piaropi* and *A. zonatum* added of 5 ppm of TiO_2 and 5 ppm of metamucil .

2.5 Bioassays

Treatments involved the addition of TiO_2 and metamucil to inoculum of *C. piaropi* and *A. zonatum*. By using manual sprayers, plants were completely wet with *C. piaropi* and *A. zonatum* mycelia suspension added of 5 ppm of TiO_2 and 5 ppm of metamucil. Plants were sprayed at 0 (at night), 2 and 4 h before sunset. Each triplicated treatment was applied on 10 replicates. Plants were arranged in a completely random design and maintained outdoors. Incident solar radiation, relative humidity and temperature were recorded during the 15 days of the experiment. The test control was prepared by wetting the plant with mycelia suspension of *C. piaropi* and *A. zonatum* in water without TiO_2 and metamucil. In México, spring time is when water hyacinth infestations increase in water bodies, but at the same time it is when the climatic conditions (high temperatures and low relative humidity) are slightly favorable for the development of plant pathogens. These optimal conditions are present in summer during the rainy season. For this reason, the protective effects of TiO_2 and metamucil were evaluated in spring and summer.

As previous observations have shown that first symptoms of infection appear 8 days after inoculation, water hyacinth plants were allowed to grow for 15 days after inoculation. After this period, plants were evaluated for disease. The plants analysis was based on the observation of characteristics symptoms caused by *C. piaropi* (dark-brown, ovate leaf spot with a whitish center) and *A. zonatum* (concentric pale-brown leaf spots with dark-brown rings) on the leaves of water hyacinth, and by using a pictorial disease scale developed by Freeman and Charudattan [11] and Rintz [29]. The percentage of infection was calculated by using the formula of Townsend and Heuberger (Eq. (1)) [30]:

$$P = \frac{\sum(n \times V)}{N \times V} \quad (1)$$

where, P: degree of infection on leaves (%); n: total number of leaves per category; V: value of each category; N: total number of leaves from the sample. Values of n and V were obtained according to pictorial disease scale prepared by Freeman and Charudattan [11].

2.6 Data Analysis

Student t-test was used to determine significant differences between treatments. Means were considered significantly different at $P < 0.05$.

3. Results and Discussion

Diverse compounds have been studied to reduce the impact of the loss of humidity and the germicidal effect of UV light on survival of microorganism used as biocontrol agents. No reports were found about TiO_2 and metamucil as protectors of plant pathogens. TiO_2 is the most widely used white pigment in the world. It has the ability to absorb solar UV light while scatters visible light [31, 32]. Unlike other two organic solar protectants tested in this study, TiO_2 was the only one which did not inhibit viability of *C. piaropi* and *A. zonatum* under laboratory conditions.

Therefore, in this study, the feasibility of using TiO_2 and metamucil was evaluated against the germicidal effects of UV light and desiccation of *C. piaropi* and *A. zonatum* inoculums.

C. piaropi and *A. zonatum* enter the host through wounds or natural openings most commonly, and such stomata and disease development is dependent on extended periods of high humidity [33]. In particular, *Cercospora* conidia can germinate with wet weather and temperatures between 20-30 °C [34]. If these pathogens do not find these conditions when they are applied in field, its efficiency as biocontrol agents will be limited.

Preliminary evaluation made in spring time showed no infection in plants infected and exposed during all day long. For this reason, the following stage of this study consisted of testing how long the pathogen protection effect last against solar exposure. A period of less solar and dryness intensity during the day was selected to inoculate plants. This time was 4 h before sunset.

Evaluation made in spring time has shown that plants inoculated with a mixture of both fungi showed a bit more infection persistence on water hyacinth (Fig. 1, C (*C. piaropi*) + A (*A. zonatum*)). Nonetheless, very little percentage of infection is achieved under unfavorable conditions for plant pathogens. Probably, this low percentage of infection was produced by the germicidal effect of sunlight plus high temperature during daytime that may cause fungus dehydration. Therefore, if adequate protection was provided to *C. piaropi* and *A. zonatum* against the germicidal effects of UV light and desiccation produced by solar radiation and wind, its infection performance might be increased with the consequent reduction in water hyacinth growth.

In this regard, when a solar protection (TiO_2) or humidity protection (metamucil) was added to the combined inoculum, an increase of the percentage of infection was observed (Fig. 1). This increase seems to be improved when both protectors were added to *C. piaropi* and *A. zonatum* inoculum (Fig. 1, C + A + TiO_2 + metamucil).

When plant controls were inoculated 4 h before sunset (in spring), very low infection (0.60%) was observed (Fig. 2). These plants had been exposed to sunlight (952.3 $W \cdot m^{-2}$) during daytime which could have enhanced plant dryness and therefore, inoculum was destroyed. These results might be either attributed to the low humidity (14.5%) and high temperature

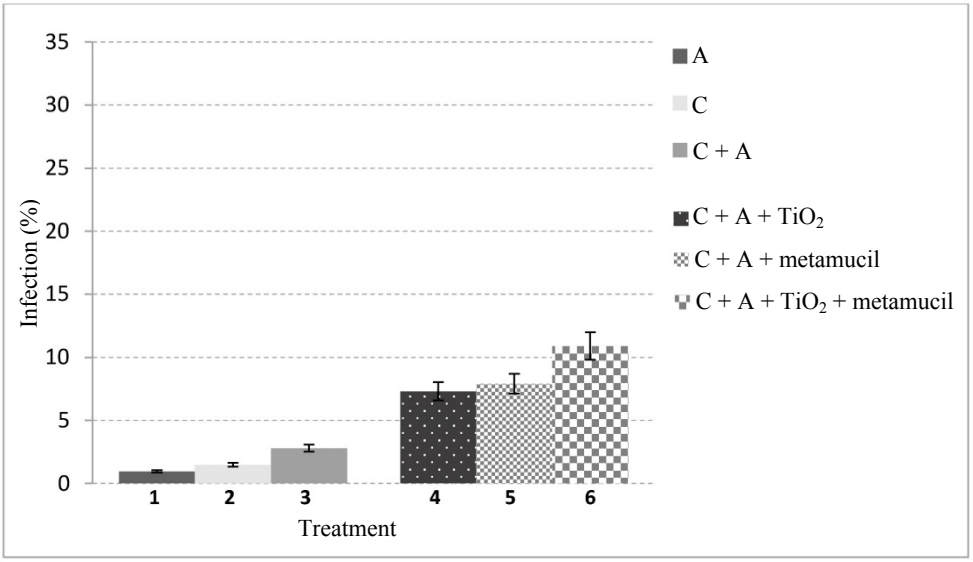

Fig. 1 Percentage of infection of water hyacinth produced by *A. zonatum* (bar 1), *C. piaropi* (bar 2), *C. piaropi* + *A. zonatum* (bar 3) inoculum with addition of TiO_2 (bar 4), metamucil (bar 5) and the addition of two fungi and two protectants (bar 6). Each series bar represents the mean of 10 replicates and three repetitions. The evaluation was made in spring time.

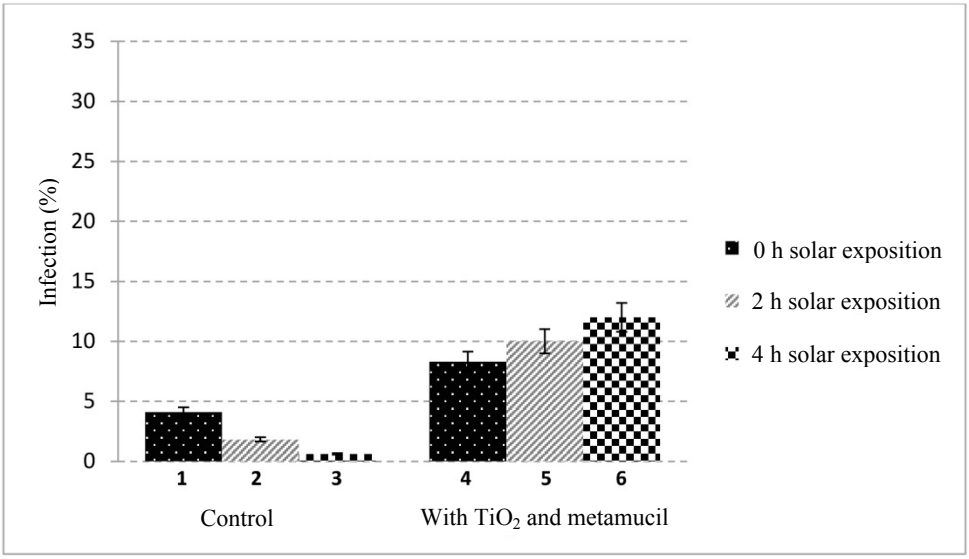

Fig. 2 Percentage of infection of water hyacinth produced by *C. piaropi* and *A. zonatum* inoculum with and without TiO_2 and metamucil. Each series bar represents the mean of 10 replicates and three repetitions. The evaluation was made in spring time.

(34 °C) recorded during the experimental period. During spring time, other uncontrolled factors contributed to low percentage of infection, such as differential dryness at the surface of the plant leaves at the time of inoculation and wind. Strong winds just before sunset are not uncommon during this season. Moreover, because plants were randomly positioned during the experimental period, it is possible that some plants were more exposed than others to the effects of wind. However, comparing with the control, when TiO_2 and metamucil was added to the inoculum, an increase of infection was observed (Fig. 2), reaching a significant difference ($P < 0.001$) in the percentage of infection in plants inoculated for 4 h of sun light exposure (12.10%). These results suggest that TiO_2 and metamucil, even under these climatic conditions, increase the infection produced by *C. piaropi* and *A. zonatum*.

Evaluation made in summer shows that the addition of metamucil and TiO_2 increases the percentage of water hyacinth infection produced by *C. piaropi* and *A. zonatum* (Fig. 3). When TiO_2 and metamucil were evaluated at different hours of sunlight exposure (Fig. 3), the protective effect was significant ($P < 0.001$) after 4 h (27.78%), since these compounds create favorable conditions for pathogenic infection (Fig. 3, bar 6). These conditions probably were provided by TiO_2 and the mucilage was produced by metamucil. Thus, wetting periods lasted longer to infect the plant by the pathogen. Pedersen and Morrall [35] observed that temperature and moisture period are important factors for disease development. On the other hand, TiO_2 provides a UV protection without which the inoculum will be destroyed. This is the case of *Cercospora kikuchi* where approximately 8 min of UV light exposure (500 $W \cdot cm^{-2}$) was required to kill 95% to 99% of conidia [36].

On the opposite, control exposed for 4 h of sunlight (Fig. 3, bar 3) shows a low percentage of plant infection (7.37%), which is probably due to the combined effect of sunlight (722.5 $W \cdot m^{-2}$), temperature (30 °C) and wind that might have contributed to fungal partial dehydration. In this respect, Rotem et al. [16] observed that solar radiation directly reduces survival of *Alternaria* spores and Caesar and Pearson [37] found that UV sunlight reduced survival of *Sclerotinia sclerotiorum* ascospores.

In spring, very low percentage of infection was observed, probably due to the negative effect of environmental factors, such as sunlight, humidity and

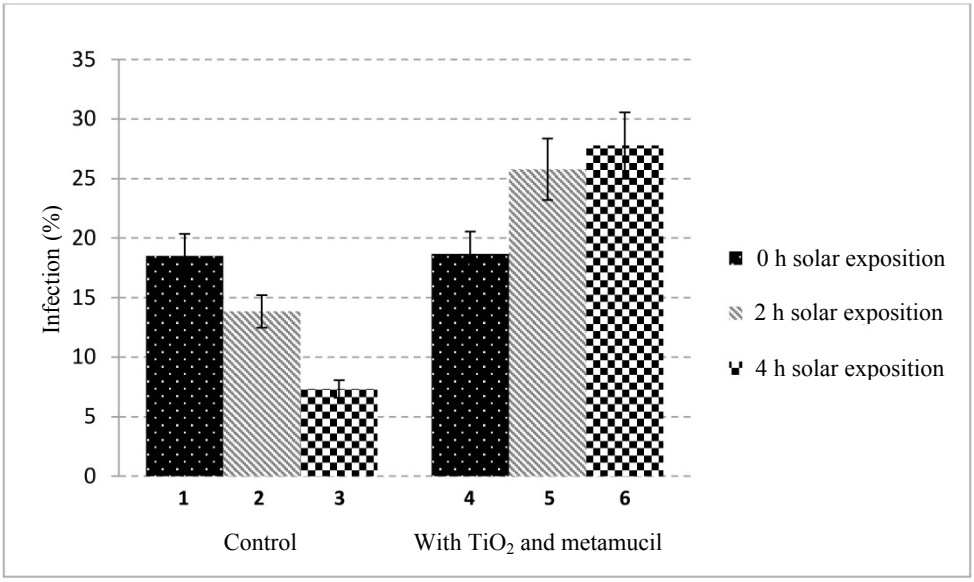

Fig. 3 Percentage of infection of water hyacinth produced by *C. piaropi* and *A. zonatum* inoculum with and without TiO_2 and metamucil. Each series bar represents the mean of 10 replicates and three repetitions. The evaluation was made in summer time.

wind on *C. piaropi* and *A. zonatum* infection (Fig. 2). On the opposite, in summer, during rainy season, large period of clouds, high relative humidity (78%), and optimal temperatures (28-30 ºC) contributed to fungal development (Fig. 3). The results suggest that TiO_2 and metamucil have a protective effect against solar radiation and desiccation of fungal inoculum of *C. piaropi* and *A. zonatum* in summer, when better weather conditions for fungal development are presented. This protective effect will allow to make the application of this bioherbicide up to 4 h before sunset and to apply it in wide infected areas.

4. Conclusions

In order to improve the efficacy of water hyacinth biocontrol agents, this study evaluated and showed results of the feasibility of using TiO_2 and metamucil as potential protectors of sunlight and humidity of inoculum of two plant pathogens (fungus) of water hyacinth. This is the first study that uses TiO_2 as solar protectant and metamucil as desiccation protection for plant pathogens to improve its infection persistence. These results are relevant in setting up the conditions for the development and production of efficient bio-herbicides and by this way to improve management practices for water hyacinth biocontrol.

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