Anais da Academia Brasileira de Ciências (2017) 89(3 Suppl.): 2101-2111
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201720160040
www.scielo.br/aabc | www.fb.com/aabcjournal

Potential effects of fungicide and algaecide extracts of *Annona glabra* L. (Annonaceae) on the microalgae *Raphidocelis subcapitata* and on the oomycete *Pythium*

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Manuscript received on January 20, 2016; accepted for publication on June 1, 2016

ABSTRACT

Annona glabra L. is a semi-deciduous tree that contains several active substances, including secondary metabolites, with antifungal activity. Phytopathogenic strains of the genus *Pythium* cause billion dollar losses all over the world on natural and crop species. Searching for eco-friendly algaecides and fungicides, we analyzed the effects of acetone extracts of *A. glabra* leaves on the algae *Rhaphidocelis subcapitata* (Korshikov) and on the oomycete *Pythium aphanidermatum* (Edson). We evaluated ten extract concentrations for each organism - 0 to 400 mg L^{-1} for algae and 0-1000 µg $disc^{1}$ for oomycete. The results showed no effect on algae up to 75 mg L^{-1}, but a significant inhibitory effect at 125 mg L^{-1} and above, which reduced the growth rate and the final biomass of the algae. Extract concentrations above 200 mg L^{-1} were completely inhibitory. The half maximal inhibitory concentration for 72 and 96 h of exposure to our crude extracts are comparable to those obtained with commercial fungicides and herbicides used in aquatic ecosystems. The *P. aphanidermatum* inhibition concentrations have effects comparable to fungicides as Cycloheximide and Bifonazole. Some substances isolated from the extracts are described as antifungals, which could explain part of anti-oomycete activity. Our results highlight the importance of searching bioactive compounds from plants.

Key words: algal growth, allelopathy, leaf extract, secondary metabolites.

INTRODUCTION

Annona glabra L. (Fig. 1) is a semi-deciduous plant adapted to flooded environments, including brackish- and saltwater, native from tropical America and West Africa (Pinto et al. 2005), present in tropical and subtropical swampy environments, such as Florida (USA), Caribbean, Central and South America (Allen et al. 2002) and being considered invasive in Asia and Oceania (Mielke et al. 2005, Sugars et al. 2006). This species forms clusters along coastal wetlands and its fruits produce several seeds that germinate better in sunny conditions (Mata and Moreno-Casasola 2005, Setter et al. 2008). *A. glabra* has been reported to have parasiticide and insecticide activity, which

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supports its use in traditional medicine (Liu et al. 1999, Mendonça et al. 2005). Some of its isolated compounds have antimicrobial, antifungal, and anticancer properties (Padmaja et al. 1995, Zhang et al. 2004). Previous studies in our laboratory showed an allelopathic potential of the leaf extracts of *A. glabra* to the germination of weed species and etiolated wheat coleoptile (Matsumoto et al. 2010, 2014).

A. glabra has glabrous leaves, which may favor the leaking and leaching of intracellular compounds (Gross 2003). Several studies on allelopathy in aquatic ecosystems pointed to an influence of macrophytes on algae through *in situ* and laboratory experiments (Macías et al. 2007, Wu et al. 2009, Zhang et al. 2011).

An excess of nutrients in water bodies stimulates plant growth, accelerates eutrophication, and may lead to explosive phytoplankton growth with negative environmental impacts. As immediate consequences, we observe a decrease in water quality and a reduction of light penetration. In addition, depending on the dominant algae species, toxins may be released, affecting the biota (Hu and Hong 2008). Thus, the search for specific, natural products from plants that reduce microalgae growth is important for the control of phytoplankton blooms in the environment. Plants and their secondary metabolites are good candidates (Zhu et al. 2010).

Oomycetes has been recently separated from Fungi kingdom, and contain several plants pathogens, such as *Phytophthora* and *Pythium* (Van West et al. 2013). Most of phytopathogenic *Pythium* species often live in wet or flood soil conditions and causes fruit, root or collar rot and seedlings damping-off (Nechwatal and Mendgen 2007). *Pythium aphanidermatum* is a filamentous organism that causes severe diseases in many plant species and responsible for huge economic losses in agriculture worldwide - fruit trees, sugarcane (Hendrix and Campbell 1973), rice

Figure 1 - *Annona glabra* tree (**a**) and fruit (**b**) in the Massaguaçu River Estuary, Brazil (23º37'20''S and 54º21'25''W).

(Eberle et al. 2007), soybean and corn (Broders et al. 2007), carrots (Barr and Kemp 1975) and some hydroponic crop culture, as lettuce and tomato (Patekoski et al. 2010). The control of these pathogens is traditionally based on fungicides and chemical substances, however, biological control using endophytic actinomycetes (El-Tarabily et al. 2009) or bacteria (Paulitz et al. 1992) showed promising results under controlled conditions (i.e. *in vitro* experiments).

Long term application of synthetic chemicals for pest control cause food and environmental contamination due to their residues and slow degradation and the search for effective natural substances to control plant fungal diseases is very important (Salamci et al. 2007, Pawar 2011). According to Lu et al. (2008) and Duke et al. (2010) natural toxins are environmentally and

toxicologically safer molecules than synthetic toxins, because they do not have potentially dangerous substances in their composition while chemical algaecides have broad toxicity to aquatic organisms and can persist in the environment, whereas natural products may be more specific and less toxic to higher trophic levels. Some of the advantages of using natural compounds to control phytoplankton populations are that they do not have toxic metals, pro-oxidants, and organic amines in their composition, in contrast to the chemical algaecides available in the market.

After extraction, 8 compounds were isolated and identified from *A. glabra* leaves extract: (1) *ent*-kaur-16-en-19-oic acid; (2) β-sistosterol; (3) stigmasterol; (4) *ent*-19-methoxy-19-oxokauran-17-oic acid; (5) annoglabasin B; (6) *ent*-17-hydroxykaur-15-en-19-oic acid; (7) *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid and (8) asimicin. Substances 4 and 5 affected the elongation of wheat coleoptile with similar results compared with the commercial herbicide Logran™. The authors suggested that these substances can act as defense mechanisms in *A. glabra* protecting it from microorganisms that live in the same environment - flooded area (Matsumoto et al. 2014).

Traditionally, studies on plant allelopathy and toxicity to aquatic organisms (e.g., microalgae and cyanobacteria) use more frequently aquatic plants (e.g., macrophytes) than tree species as sources. The present research aimed at evaluating the effect of leaf extracts of *Annona glabra* on microalgae *Raphidocelis subcapitata* (Korshikov) and on oomycete *Pythium* strains.

MATERIALS AND METHODS

The adult plant of *A. glabra* and their healthy leaves were obtained from trees in the Massaguaçu River Estuary, Brazil (23º37'20''S and 54º21'25''W). We deposited the voucher specimen 7503 in the herbarium of Departamento de Botânica, at Universidade Federal de São Carlos. After collection, the leaves were dried in a greenhouse at 45°C for 48 h, ground and the powder stored in a freezer at -20°C until extraction.

Active compounds of *A. glabra* from 1.0 g of ground and dried samples were extracted using an ultrasound assisted extraction (UAE) procedure, following Firdaus et al. (2010). The extraction method recovers organic compounds, including those with allelopathic potential, such as alkaloids and flavonoids. The UAE conditions were: 25 mL acetone, 2 mm probe, cycle of 0.2, 30% amplitude of 200 W, and instrument potency of 24 Hz . These conditions were applied during 20 minutes at 5°C.

To evaluate the effects of leaf extracts of *A. glabra* on *R. subcapitata*, toxicity tests were performed with extract concentrations ranging from 25 to 400 mg L^{-1} (25, 50, 75, 125, 150, 175, 200, 300 and 400). After extraction procedure, we did a stock solution of extract in acetone (400 mg L^{-1}) and serial dilutions to obtain 50 mL of each test concentration (from 25 to 400 mg L^{1}), while the control had no extract addition, but 50 mL of acetone. We made three replicates for each treatment. Approximately 50 mL of the extract were placed in 150 mL Erlenmeyer flasks that were vacuum dried at 35ºC to volatilize the solvent. After complete dryness, when only the tested organic compound remained, 50 mL of exponentially growing *R. subcapitata* culture were added as inoculum.

Stock cultures of *R. subcapitata* were kept in LC Oligo culture medium (AFNOR 1980) under laboratory-controlled conditions of light intensity (130 µmol photons m^{-2} s^{-1}), light/dark cycle (16:8 h), and temperature (23 ± 2ºC). Hence, in the beginning of the experiment, each Erlenmeyer flask contained the dried extract and 5 x 10^4 cells mL^{-1}. Bioassays were incubated under the same growth conditions as the algae stock cultures. Daily aliquots (0.5 mL) were obtained up to 96 h of exposure to estimate algae biomass (cell mL^{-1}).

Algae cells were counted in an Improved Neubauer chamber under optical microscope and growth rates obtained by plotting the natural log of cell mL^{-1} against experimental time and calculating a linear regression for the exponential part of the growth curve. Growth rate values were calculated as the slopes of linear regressions. The inhibitory concentration that caused 50% (IC_{50}) of decrease in *R. subcapitata* biomass were estimated at 72 and 96 h of exposure.

Pure isolate of *Pythium aphanidermatum* strain CCMA 243 (Laboratório de Microbiologia Ambiental - Embrapa Meio Ambiente - CNPMA) was cultured in PDA (potato dextrose agar). Plates were disposed in culture chambers at 25 ± 2ºC, 12 h photoperiod, until the growth reached the edge of an 85 mm Petri dish plate. Disc diffusion test was used to evaluate the anti-oomycete activity. A 5 mm diameter plug of the PDA with *P. aphanidermatum* was transferred to the center of a Petri dish plate with PDA.

Six millimetres paper disks were soaked with 40 µL of the extract, dried and disposed equidistant from the centre of the plate (23 mm). Three groups of plates were established (A, B and C). Each group received 4 discs with different concentrations of the extract: A - 0; 0.97; 1.95 and 3.9; B - 7.8; 15.6; 31.2 and 62.5; C - 125; 250; 500 and 1000 µg $disc^{-1}$. The 0 µg disc was soaked with 40 µl of acetone and used as negative control. Four group replicates were applied and three PDA plates with an oomycete plug on the centre were prepared as growth control. The oomycete growth and inhibition zones (radius) were measured every 24 h for 15 days.

The final biomass of the treatments at 96 h of exposure of algae were contrasted through an ANOVA with a Tukey *post hoc* test, with the use of F statistics to compare growth rates. The oomycete growth after 15 days was fitted in Boltzmann equation and curves were compared with ANOVA. Inhibition radiuses were compared with ANOVA with Tukey's *post hoc* test.

RESULTS

Figure 2 shows cell density at 96 h of exposure as a function of extract concentration of *A. glabra*, as well as IC_{50} values for 72 h (135.6 mg L^{-1}) and 96 h of exposure (145 mg L^{-1}). The results show that up to 75 mg L^{-1} no effects of the leaf extract of *A. glabra* were detected on *R. subcapitata*. Above this concentration, inhibition increased gradually with extract concentration, until reaching null microalgal growth at 200 mg L^{-1} and higher. No statistical difference was detected for the three highest concentrations tested.

Figure 3 shows cell density as a function of experiment duration. Since no significant difference (F test) was detected in the growth rates among replicates, the data could be pooled together and a single slope was calculated for each treatment. Figure 3 shows that at the extract concentrations of 125, 150, and 175 mg L^{-1} there was a delay in the population growth of *R. subcapitata*, analogous to a *lag* phase in standard batch cultures. So, we calculated linear regressions for the growth curves of the treatments after 24 h of exposure for 125 and 150 mg L^{-1}, and after 48 h of exposure for 175 mg L^{-1}. For the treatments at the concentrations of 200, 300, and 400 mg L^{-1}, algae cells were unable to divide, then no growth was observed and cell densities (~ 5 x 10^4 cell mL^{-1}) were statistically similar (ANOVA $p > 0.05$).

The fitted curves of controls and tested groups (A, B and C) of *P. aphanidermatum* present no difference, with similar final growth and growth rate. No inhibition zones were noticed on A and B groups (data not shown). The C group started presenting inhibition zones on day 7, when the oomycete reached the discs and zones were measured, reaching the maximum inhibition in day 10.

The radius data from inhibition zones were pooled to calculate the average and standard deviation for ANOVA comparison. From the day

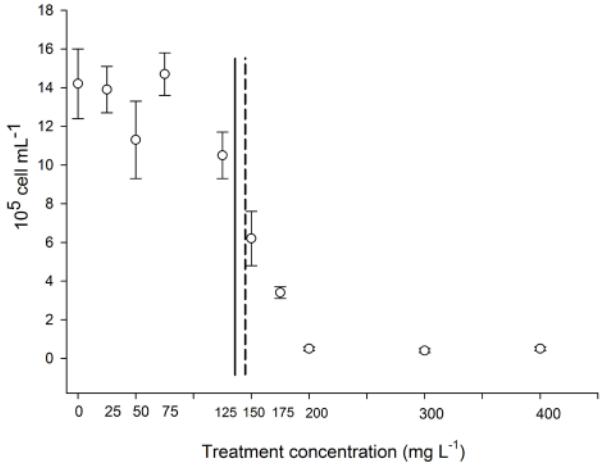

Figure 2 - *Raphidocelis subcapitata* culture density (cell mL^{-1}) at 96 h of exposure as function of extract concentration of *Annona glabra* (mg L^{-1}). Continuous line represents the half maximal inhibitory concentration (IC_{50}) at 72 h; dashed line represents IC_{50} at 96 h.

11 to the end of experiment, the oomycete has recovered and grew over the discs and dissipating the inhibition zones, then occupying the whole plate. ANOVA comparisons showed differences among the four concentrations of C group (125, 250, 500 and 1000 µg disc^{-1}), except between 500 and 1000, which present similar inhibition zones of 15.9 ± 0.6 and 16.4 ± 1.3 mm respectively (Fig. 4).

DISCUSSION

Several commercial herbicides and fungicides bring risks to the environment. Harmful and long-term effects on aquatic organisms have been related to exposure to commercial herbicides. The IC_{50} at 72 h of exposure of some of these particular products on *R. subcapitata* are between 270 mg L^{-1} and 100 mg L^{-1} (®Bayer 2010, 2012). An assessment of the impacts of herbicides on non-target aquatic plants and algae, including *R. subcapitata*, showed IC_{50} values ranging from 0.055 to 270 mg L^{-1} (Cedergreen and Streibig 2005). Commercial algaecides as Barquat low foam presents IC_{50} of 0.04 mg L^{-1} against *R. subcapitata* (®Lonza 2004).

Our results corroborate the use of extract of plants inhibiting the growth of microalgae (e.g. leaf litter on growth of *Chlorella vulgaris*), affecting interactions in phytoplankton community in the natural environment, reducing cyanobacteria dominance with the use of barley (Ridge et al. 1999). Cantrell et al. (2005) analyzed the effects of a terrestrial herbaceous plant (*Haplophyllum sieversii*) on the growth of *R. subcapitata* and obtained that the complete inhibition occurred at concentrations above 100 mg L^{-1}, while we observed the inhibition by the extract of *A. glabra* at concentrations above 200 mg L^{1}.

There are several *in situ* and laboratory studies showing that macrophytes also can act as algae growth inhibitor (Hilt and Gross 2008, Toporowska et al. 2008), for example, ethyl acetate fractions of the macrophytes *Potamogeton malaianus* and *P. maackianus* were toxic to the cyanobacteria *Microcystis aeruginosa* at the concentration of 60 mg L^{-1}, and reduced its biomass in ~ 54% and 58%, respectively (Wang et al. 2010). A study on the effects of aqueous extracts of five plant species on *R. subcapitata*, including Papaveraceae macrophytes, obtained IC_{50} at concentrations above 21 mg L^{-1}. *Stylophorum lasiocarpum* was the fourth most active species, with IC_{50} at the concentration of 114 mg L^{-1} (Jančula et al. 2007). By comparing our results to those of Jančula et al. (2007), we can assume that the extracts of *A. glabra*, which are toxic at the concentration of 145 mg L^{-1}, have a strong inhibitory effect. The IC_{50} of extracts of *A. glabra* on *R. subcapitata* in the present study was 135.6 mg L^{-1} and 145 mg L^{-1} for 72 h and 96 h of exposure. Hence, the plant *Annona glabra* is a promising species for the isolation of compounds with algaecide properties. As far as we know, this is the first study evaluating the algaecide effects of *A. glabra* extracts and those substances were not reported yet as algaecide. Table I summarizes the action of some extracts and pure products in tests on *R. subcapitata.*

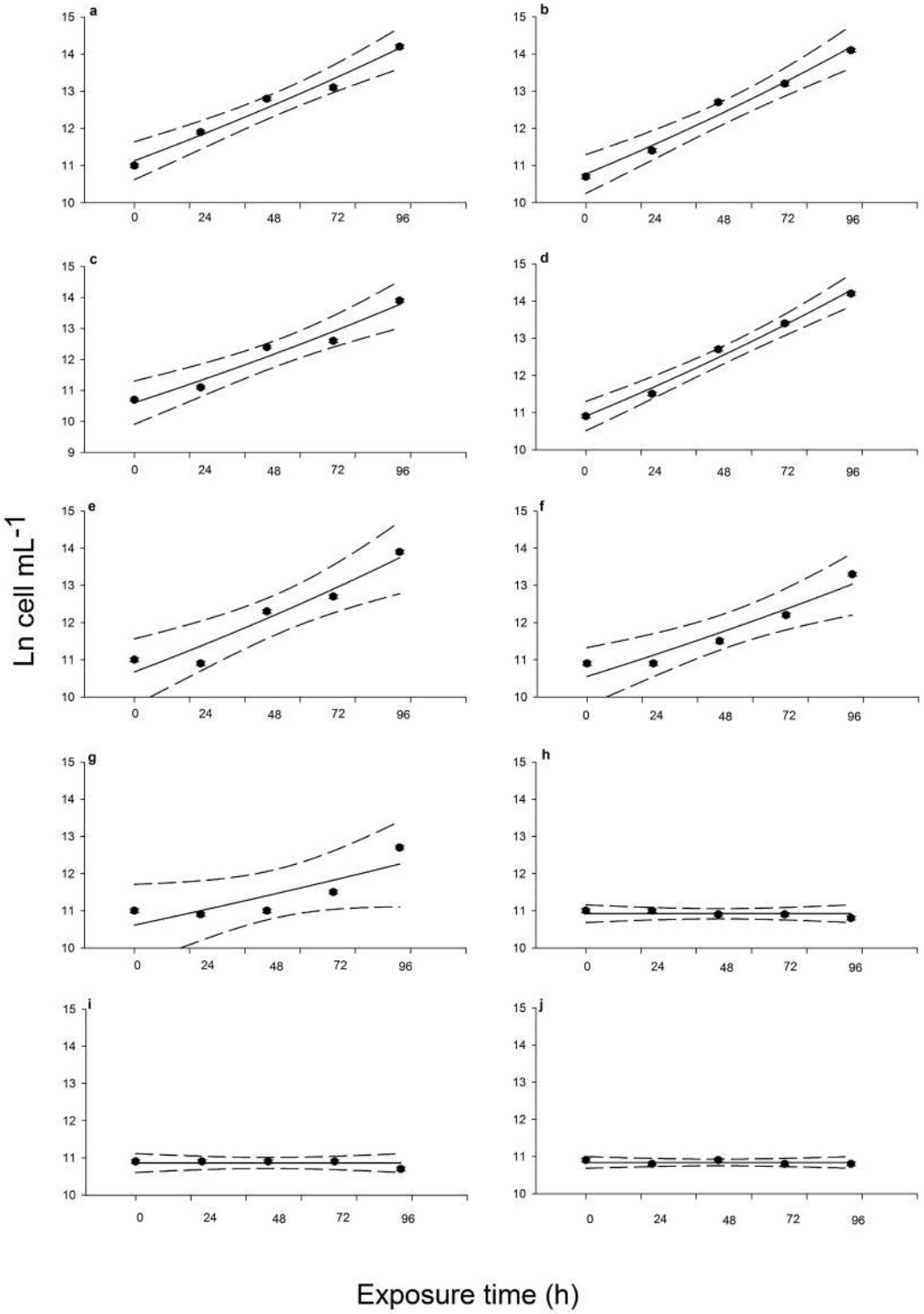

Figure 3 - *Raphidocelis subcapitata* density (cell mL^{-1}, ln) as a function of exposure time (h) for each treatment. Values represent average (± SD) of ln (cells mL^{-1}). (**a**) Control (0 mg L^{-1}); (**b**) 25 mg L^{-1}; (**c**) 50 mg L^{-1}; (**d**) 75 mg L^{-1}; (**e**) 125 mg L^{-1}; (**f**) 150 mg L^{-1}; (**g**) 175 mg L^{-1}; (**h**) 200 mg L^{-1}; (**i**) 300 mg L^{-1}; (**j**) 400 mg L^{-1} *Annona glabra* extract. Dashed lines represent 95% of confidence intervals.

TABLE I
Comparison of the half maximal inhibitory concentration (IC_{50}) of extracts and compounds on *Raphidocelis subcapitata* Korshikov in toxicity assays.

Donor Species/ Product type	Substances	Inhibition	mg L^{-1}	Reference
Annona glabra	Acetone extract	IC_{50} 72h	135.6	Present study
		IC_{50} 96h	145	
Haplophyllum sieversii	Crude extracts	100%	>100	Cantrell et al. 2005
	Flindersine	IC_{50} 96h	17.8	
	Haplamine	IC_{50} 96h	15.9	
Myriophyllum spicatum	Pyrogallic acid (PA)	IC_{50} 72h	5.45-8.45	Zhu et al. 2010
	Gallic acid		4.78-8.99	
	Ellagic acid		10.06-14.69	
	(+)-catechin		7.45-11.75	
Chelidonium majus	Aqueous extracts	IC_{50} 96h	60.87	Jančula et al. 2007
Dicranostigma lactucoides			21.27	
Macleaya microcarpa			868.09	
Sanguinaria canadensis			23.9	
Stylophorum lasiocarpum			114.1	
Fungicide	FLC+PCH SC 62.5+625 G	IC_{50} 72h	100	®Bayer 2010
	Lawn weedkiller ready to use2	IC_{50} 72h	270	®Bayer 2012
Herbicides	Glyphosate	IC_{50} 48h	270	Cedergreen and Streibig 2005
	Triasulfuron		0.405	
	Metsulfuron-methyl		0.677	
	Terbuthylazine		0.055	
	Bentazone		13.6	
	Mesotrione		6.78	
Algaecide		IC_{50} 72h	0.04	®Lonza 2004

According to NRA (2001), *R. subcapitata* presented lower growth rate and delayed growth when exposed to 10 mg L^{1} semduramicin (anticoccidial), whereas a total growth inhibition was observed at 39 mg L^{-1}. In our study, a similar delay was observed before the beginning of the exponential growth phase when the algae were exposed to concentrations of 125 - 175 mg L^{-1} of the extract of *A. glabra*. However, after such a lag phase, the two highest growth rates were observed. This may be related to a survival strategy under a stressing situation, with the algae adjusting its metabolism to the presence of the toxic agent (Stebbing 1982, 1987). This author linked a similar behavior to what is known as the hormesis effect, whereby the toxicant at low doses can stimulate the organism. Phytochemicals exist to protect plants from oxidative and other types of stress,

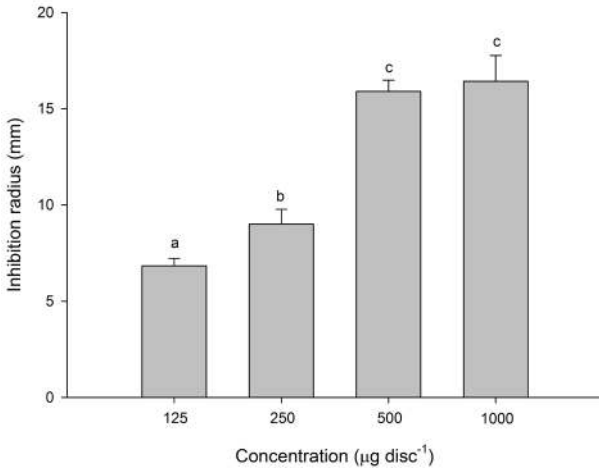

Figure 4 - Inhibition radius (mm) of *Pithyum aphanidermatum* exposed to different concentrations of *Annona glabra* extract (C group - 125, 250, 500 and 1000 µg $disc^{-1}$). At day 10, maximal inhibition zones were observed. Bars are means ± SD for *n*=3, same superscript letters are not significantly different ($p > 0.05$).

but as bioactive compounds, they can induce compensatory responses at the cellular level that can be beneficial. Standard algaecides such as $CuSO_4$ are not likely to cause hormesis because copper is a micronutrient to microalgae. However, it can cause it in other organisms, e.g. the presence of copper stimulated the reproduction and growth of a freshwater cladoceran (De Schamphelaere and Janssen 2004). In our study, statistically higher growth rates were obtained at the concentrations of 25, 125, and 150 mg L^{-1}, but not at the concentration of 75 mg L^{-1} of *A. glabra* extract, suggesting that a regulatory over-correction mechanism may have occurred. Nevertheless, more concentrations and higher exposure time should be tested to confirm the hormesis hypothesis for the organisms that we have tested.

Oomycetes were long considered a class within kingdom Fungi, but fundamental differences in physiology, biochemistry and genetics separate both groups (Latijnhouwers et al. 2003). Due to this separation, there are no specific anti-oomycetes substances developed and as an alternative, fungicides are used to control these organisms.

With the knowledge of some bryophytes such as *Thuidium* spp., *Bryum* spp. and *Plagiochasma appendiculatum* being effective against fungal strains (Bodade et al. 2008), as well the herbaceous plant *Verbascum thapsus* extracts inhibiting pathogenic fungi (Vogt et al. 2010), some recent studies tested different plant species extracts in oomycetes. Sánchez-Pérez et al. (2009) observed the inhibition of 50% of mycelial growth in the phytopathogenic *Phytophthora cinnamomi* in the presence of crude root extracts from Mexican avocado trees (*Persea americana*). In some studies using leaf extracts of 15 species of medicinal plants were observed the inhibition of *Pythium debaryanum* mycelia growth (Ambikapathy 2011, Pattnaik et al. 2012). Caruana et al. (2012) analyzed the activity of 24 crude plants extracts against *Saprolegnia australis*, a parasitic oomycete on fish and 12 extracts reduced growth at 100 mg L^{1}, while 3 were effective at 10 mg L^{-1}, while Jagtap et al. (2012) obtained inhibitions of 47% the mycelial growth of the phytopathogenic *Phytophthora nicotianae* using extracts of *Allium sativum* at 10% concentration.

Annona glabra extracts inhibited *P. aphanidermatum* in concentrations higher than 125 µg $disc^{-1}$, with an apparent oomycestatic effect, comparable to fungicides as Cycloheximide and Bifonazole. In our study, *A. glabra* extracts in concentarions from 125 to 1000 µg $disc^{-1}$ were more effective against the oomycete with 6 to 16 mm inhibition zones radiuses, higher than leaf extracts from five medicinal plants at 10 µg $disc^{-1}$ that inhibited *Pythium debaryanum* growth, with the higher values of 5 mm radius (Gomathi et al. 2011).

The *ent*-kaur-16-en-19-oic acid isolated from hexane extracts from *A. glabra* bark presented antifungal activity against 7 species (Padmaja et al. 1995), while stigmasterol and sitosterol isolated

from *Bulbine natalensis* inhibited 3 species of fungi (Mbambo et al. 2012), but there are no specific antioomycetes activities recorded for these substances. We believe that these substances were responsible for the inhibition of the oomycete growth, but more studies are necessary with them separately to check if only one substance is responsible for the inhibition or if there is some synergism between them.

Based on our results, we believe that the ultrasound extraction used in the present study was effective, resulting in the isolation of 8 compounds present in *A. glabra* extracts using low amounts of *A. glabra* and solvents. Some of these compounds have algaecide and fungicide activities, and should be considered for more studies to identify the specific compound(s) responsible for algae and oomycete growth inhibition. Besides the algaecide activity, it was observed a stimulation in algal growth in some concentrations, maybe a "hormesis effect", however, more studies are needed to confirm this hypothesis. Considering the fact that *A. glabra* and oomycetes share flooded habitats, such antioomycete activity obtained from the acetone extract is an important evidence of plant - pathogen interaction and a potential species for the prospection of new substances for oomycete control. If we consider the higher costs with water treatment when there are algae blooms and the losses caused by oomycetes and fungi in the crops, the study of the compounds of *Annona glabra* can be economically and environmentally profitable.

ACKNOWLEDGMENTS

Maria da Graça Gama Melão is acknowledged for providing the sonicator and laboratory facilities. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (process 302837/2012-4) granted a research fellowship (RSM), GSR is grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the grant no. 2009/15511-5. J.P.N. Ribeiro is acknowledged for the photographs.

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