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RESEARCH ARTICLE / *ARTIGO*

Antagonistic process of *Dicyma pulvinata* against *Fusicladium macrosporum* on rubber tree

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ABSTRACT

The interaction between *Dicyma pulvinata* and *Fusicladium macrosporum* was studied by scanning electron microscopy. Spores of *D. pulvinata* germinated on the surface of *F. macrosporum* lesions induced on artificially infected rubber plants were fixed 8 h after inoculation. *D. pulvinata* germ tubes seemed to elongate toward *F. macrosporum.* Close contact between the antagonistic fungus and *F. macrosporum* spores was verified 24 h after application of *D. pulvinata*. At the end of the process, spores of *F. macrosporum* seemed to have disintegrated and to be devoid of content. The hyperparasite grew completely over the pathogen. Six to seven days after application of the antagonistic fungus, *D. pulvinata* conidiophores were observed emerging from *F. macrosporum* structures with profuse sporulation. Studies have also shown the possibility of *D. pulvinata* producing hydrolytic enzymes, which could be associated with the control of plant pathogens. This information may help to elucidate some of the modes of action of *D. pulvinata*, a potential biological control agent for South American leaf blight of *Hevea* rubber plant.
Keywords: pathogen and antagonistic interaction, South American Leaf Blight, *Hevea brasiliensis*, biocontrol.

RESUMO

Processo de antagonismo de *Dicyma pulvinata* contra *Fusicladium macrosporum* em folhas de seringueira

Estudou-se a interação entre *Dicyma pulvinata* e *F. macrosporum* ao microscópio eletrônico de varredura. Esporos de *D. pulvinata* germinaram na superfície das lesões induzidas por *F. macrosporum* em plantas de seringueira (*Hevea brasiliensis*), infectadas artificialmente, fixadas 8 h após a inoculação do antagonista. Aparentemente, os tubos germinativos se alongaram em direção ao patógeno. O contato íntimo entre o hiperparasita e o patógeno foi verificado em amostras fixadas 24 h após a aplicação de *D. pulvinata*. Ao término do processo, os esporos de *F. macrosporum* aparentemente invadidos pelo antagonista mostraram-se desintegrados e esvaziados de seu conteúdo. *D. pulvinata* cresceu sobre as lesões, sobrepondo totalmente o patógeno. Seis dias após a aplicação, conidióforos do fungo antagonista foram observados emergindo das estruturas do patógeno, produzindo esporos em grande quantidade. Verificou-se, também, um possível envolvimento de enzimas hidrolíticas na associação antagonística entre *D. pulvinata* e o patógeno. Estas informações podem contribuir para elucidar o modo de ação de D. pulvinata, um potencial agente de controle biológico para o mal das folhas da seringueira.
Palavras-chave: interação patógeno e antagonista, mal-das-folhas da seringueira, *Hevea brasiliensis*, biocontrole.

INTRODUCTION

South American Leaf Blight of *Hevea* rubber plant (SALB), caused by *Microcyclus ulei* (Henn.) Arx (anamorphs *Aposphaeria ulei* Henn. and *Fusicladium macrosporum* Kujper) is one of the world's five most threatening plant diseases, and it is still epidemic to Central and South America. It was first recorded in 1900 on rubber trees in Brazil. Currently this disease extends from Southern Mexico (18^0 North latitude) to Sao Paulo State in Brazil (24^0 South latitude), covering Brazil, Bolivia, Colombia, Peru, Venezuela, Guiana, Trinidad and Tobago, Haiti, Panama, Costa Rica, Nicaragua, Salvador, Honduras, Guatemala and Mexico. The disease has been the main restraint on the development of rubber cultivation in Latin American countries.

According to Holliday (1970), the epidemiological process usually begins from the germination of *F. macrosporum* conidia, which are viable for a few days under natural environmental conditions and shade. Four to five hours of leaf-wetness is required for host penetration through the immature cuticle. Sporulation begins 5-6 days after infection; pycnidia are formed after 3-5 weeks, and ascocarps after a further 4-6 weeks.

In spite of the recommended control strategy of planting *Hevea brasiliensis* (Willd ex. A. Juss) Muell. in areas where climatic conditions are unfavorable to the epidemic development of the disease (escape zones),

experiments conduced by Gasparotto & Junqueira (1994) showed evidence of the existence of ecological races of *M. ulei* better adapted to adverse climatic conditions. This information was confirmed later (Rivano, 1997; Mattos *et al*., 2003; Romero *et al.*, 2006). Hence, difficulties in controlling the disease, even in escape zones, can be expected.

All improved *H. brasiliensis* clones, worldwide, are susceptible to SALB, although the disease is confined to South America. However, the possibility of the future spread of the disease should always be considered, even though natural rubber-producing countries have now adopted appropriate measures to prevent the introduction of the disease into their territories. It has been shown that two types of spores (conidia and ascospores) are responsible for disease dissemination, and it has been predicted that parts of the host plant (*Hevea*) infected can spread the disease over long distances.

Efforts have been made in order to control this disease, including the use of *Dicyma pulvinata* (Berk. & M. A. Curtis) Arx [syn. *Hansfordia pulvinata* (Berk. & M. A. Curtis) Hughes]. This fungus was first observed, colonizing stromatic lesions produced by *M. ulei*, in the Amazon Region, spreading from there to different geographic areas of Brazil. Results obtained from field trials (Junqueira & Gasparotto, 1991) have demonstrated the action of *D. pulvinata* against SALB in decreasing the inoculum potential of the parasite by killing hyperparasitized conidia on colonized lesions.

The mitosporic fungus *D. pulvinata,* which was first reported as a mycoparasite on *Isariopsis indica* and *Cercospora* spp. in India (Rathaiah & Pavgi, 1971; Krishna & Singh 1979), has been studied as a parasite of *Cladosporium fulvum* and *Cercosporidium personatum*, causal agents of tomato leaf mould, and late leaf spot of peanut, respectively (Peresse & Le Picard, 1980; Tirilly *et al.* 1983; Mitchell *et al*., 1987; Tirilly, 1991). Peresse & Le Picard (1980) suggested that this fungus could be used in the biological control of *C. fulvum* in greenhouse-grown tomatoes. Tirilly *et al.* (1983) isolated a fungitoxic metabolite (13-desoxyphomenome) from liquid cultures of *D. pulvinata* obtained from *C. fulvum* lesions in tomato. More recently, *D. pulvinata* was reported colonizing tissue of fruit bodies of Aphyllophorales (Basidiomycetes) in Japan (Watanabe & Kawano (2003).

According to Sharma & Sankaran (1986), organisms adapted to the same habitat as the pathogen are generally preferred for biological control purposes over those from other habitats, as the latter are less likely to survive for long periods in the environment and consequently would have to be reapplied to foliar surfaces more frequently. Based on this aspect, we have considered *D. pulvinata* as a potential candidate for biocontrol of SALB.

The objective of the present work was to investigate the interaction by scanning electron microscopy and to elucidate the possible involvement of hydrolytic enzymes in the antagonistic association between *D. pulvinata* and the plant pathogen.

MATERIALS AND METHODS

A survey was carried out from late February to late December of 1999 in different geographic areas across the country. *D. pulvinata* isolates were obtained from stromatic lesions of *Hevea* rubber and incorporated in Embrapa's collection of fungi for biological control of plant pathogen - CEN (Mello *et al*., 2005). A performance comparision of several isolates showed that at least seven could be used to control the disease: CG774 (CEN093), CG801 (CEN119), CG773 (CEN092), CG790 (CEN109), CG679 (CEN058), CG826 (CEN136) and CG682 (CEN061) (Mello *et al*., 2006).

Healthy potted rubber plants (*H. brasiliensis*, clone GT1) were inoculated by spraying a conidial suspension (10^6 conidia mL^{-1}) of *F. macrosporum* on leaflet surfaces. The leaflets were 6-8 days old, which corresponds to the B1 and B2 stages (Hallé *et al*., 1978). The conidia were originally obtained from artificially infected rubber plants, by washing lesions with sterile water and rubbing gently with a soft camel-hair brush. Conidia concentration was determined by using a Neubauer chamber before use. The inoculated plants were kept inside a growth chamber (Lab-line Instruments, inc.) adjusted for 24-h darkness (100% RH; 25 °C). After that, conditions were adjusted for 12-h darkness provided by fluorescent lamps. Five days after inoculation, when the leaf lesions were evident, plants were taken to the greenhouse for inoculation with the antagonistic fungus.

The *D. pulvinata* antagonist used in this study, isolate CEN093, was obtained from a survey (Mello *et al*., 2005) and stored at -180°C in the Embrapa Genetic Resources and Biotechnology fungus collection. Current cultures were grown at 25 to 27 °C on potato dextrose agar (PDA) and stored at 4 °C. In order to produce sporulating cultures for trials, mycelium discs from these stock cultures were inoculated on PDA plates and incubated under 12 h of alternating dark and light at 25°C.

The inoculum, obtained from 15-day-old cultures, was prepared by adding 2 mL of sterile distilled water + Tween 20 (0.02%) solution to each plate, and then sweeping the plates with a soft camel-hair brush to dislodge conidia. Conidia concentration was adjusted to 10^6 conidia mL^{-1} and the suspension obtained was sprayed on the surface of rubber plant leaves presenting *F. macrosporium* lesions. After inoculation, the plants were placed in previously moistened plastic bags, overnight.

Leaf samples were collected at 4, 8, 12 and 24 hours and 3, 4, 5, 6, 7 and 8 days after inoculation. The samples were fixed with a modified Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde in 0.05M cacodylate buffer, pH 7.2), post-fixed in 1% osmium tetroxide in the same buffer for 2 hours (Bozzola & Russel, 1998) and dehydrated in a graded acetone series. The specimens were then dried in an Elmitech Critical Point Drayer K 850, using CO_2 as transition fluid. The dried samples were glued onto specimen stubs and coated with gold in an Elmitech K

550 Sputter Coater. A ZEISS DSM 962 scanning electron microscope at 20KV was used to examine the samples.

For enzyme production essays, *D. pulvinata* was cultured in 50 mL of liquid medium (25 g L^{-1} glucose, 5 g L^{-1} yeast extract) at 28° C under agitation (150 rpm); after 72 hours it was collected in sterile distilled water and transferred to 50 mL of liquid culture medium containing (g L^{-1}) $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.6; KCl, 0.15; NH_4NO_3, 1.0;$FeSO_4.7H_2O$, 5.0 mg L^{-1}; $MnSO_4.H_2O$, 6.0 mg L^{-1}; $ZnSO_4.H_2O$, 4.0 mg L^{-1}; $CoCl_2$, 2.0 mg L^{-1}; crab shell chitin (0.5% and 0.1% [v/v]) trace elements (Fe^{2+}, Mn^{2+} and Co^{2+}), adjusted to pH 5.5. Cultures were then incubated for 24 h, 48 h and 72 h, at 28° C under agitation (150 rpm), in order to obtain enzyme production. After incubation, culture filtrates were collected by filtration (Whatman No. 1 paper) and stored at -20°C with sodium azide (0.02%).

Enzyme assays

β-1,3-Glucanase (EC 3.2.1.39) was assayed based on the release of reducing sugar from laminarin. Briefly, the reaction mixture contained 100 µL of laminarin dissolved in 50 mM sodium acetate buffer, pH 5.0 and a 100 µL substrate of enzyme solution. The reaction was allowed to proceed for 30 min at 37°C, after which the liberated reducing sugars were determined by dinitrosalicilic acid method (Miller, 1959) using a reference curve constructed with glucose as the standard. Enzyme and substrate blanks were also included. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the equivalent release of one µmol of glucose per minute under the described assay conditions. Chitinase activity (EC 3.2.1.14) was assayed using the colorimetric method described by Ulhoa & Peberdy (1992). The assay mixture contained 1 mL of 0.5% regenerated chitin (suspended in 0.05 M acetate buffer pH 5.2) and 1 mL of enzyme solution. The reaction mixture was incubated for a minimum of 6 h under agitation at 37°C and the reaction was blocked by centrifugation (5000 rev/ min) for 10 min and the addition of 1 mL of dinitrosalicylate reagent (Miller, 1959). The amount of reducing sugar produced was estimated using a reference curve constructed with *N*-acetylglucosamine (GlcNAc) as standard. One unit of enzyme activity (U) corresponded to the amount of protein necessary to release 1 µM of GlcNAc equivalent in 1 h at 37°C. Alternatively, the presence of GlcNAc as a product of chitinase activity was determined using the reagent *p*-dimethylaminobenzaldehyde (DEMAB). The *N*-acetylglucosaminedase (NAGase) activity (EC 3.2.1.30) was measured as described by Yabuki *et al*. (1986) using *p*-nitrophenyl-β-N-acetylglucosaminide (N*p*-GlcNAc) as the substrate. One unit of enzyme activity (U) was defined as the amount of the enzyme that releases one µmol of *p*-nitrophenol per minute under the described assay conditions. Protein estimation was performed by a simplification of the Lowry method (Peterson, 1977) and the proteases assay was based on Haran *et al.* (1996). All assays were run in triplicate.

RESULTS

Typical symptoms of SALB appeared on the abaxial surface of rubber plant leaves three days after *F. macrosporum* inoculation as small light green spots, subsequently becoming darker and larger. Samples of the lesions taken to examine under light microscopy showed profuse sporulation just before *D. pulvinata* application.

Conidial germination and germ tube growth of the antagonistic fungus was observed 8 h after application on all leaf surface tissues examined (Figures 1A-B). As the process progressed, *D. pulvinata* mycelium expanding from germ tubes grew on to the host structures (mycelium, conidiophores and conidia), surrounding and holding them (Figures 1C-F). Once in contact with *F. macrosporum* conidia, the hyperparasite produced appressorium-like structures which appeared strongly attached to the host and attached to the spores (Figures 1D and F). Occasionally the contact between host and parasite occurs without these types of structures (Figure 1E). Most *F. macrosporum* conidia seemed to be penetrated 72 hours after inoculation. No perforation and signs of physical deformation in the spore surface were observed, and therefore the event of penetration into the spores remains unclear. Even without enough evidence of penetration, conidiophores with conidia seemed to grow directly out of the pathogen structures, as observed in the samples fixed six days post-inoculation with the antagonist (Figure 2A). Invaded *F. macrosporum* conidia seemed deflated or devoid of content (Figures 2B-C). Colonization inside these structures was not studied, although the mycelium seemed to grow inside the host conidia (Figure 2C). In samples fixed seven days after inoculation, only *D. pulvinata* structures could be observed (Figure 2D). At this stage, entire foliar lesions induced by *F. macrosporum* were covered by the typical growth of *D. pulvinata* expressed as a peculiar whitish downy growth.

Examination of samples from non-infected rubber plant leaf tissue, taken after the eighth hour following the treatment with *D. pulvinata*, revealed the inability of the antagonistic fungus to survive in the absence of the pathogen. The determination of the total proteins secreted during a period of one week demonstrated increasing liberation of proteins during the whole induction period.

Substantial amounts of hydrolytic enzymes, such as NAGase (maximum in 48 h / 0.11 U) and Glucanases, were produced during the induction period, containing chitin (0.5%). The highest activity of endoglucanases occurred at 48 h (0.295 U) and 96 h (0,129 U), respectively, staying unaffected for a week after induction. The exoglucanase values indicated that the highest activity occurred at 48 h (0.037 U) and 72 h (0.023 U), respectively. After the reduction in enzyme activity, the values remained constant until the end of the enzymatic induction. The chitinase enzyme did not reveal activity; therefore, a high proteolytic activity was detected in the period of a week (0.075 U), at the end of induction.

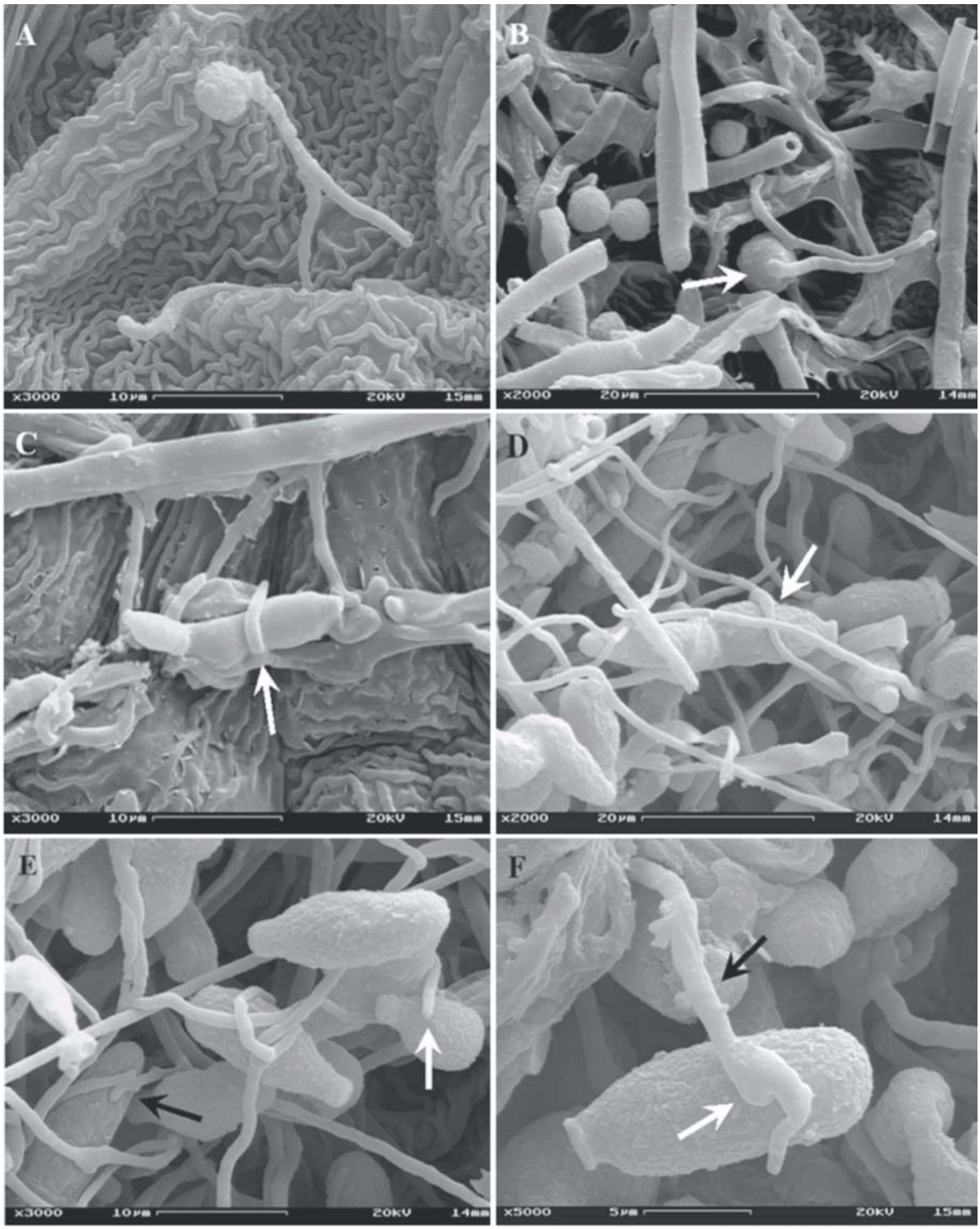

FIG. 1 - Scanning electron micrographs of the interaction of *Dicyma pulvinata* and *Fusicladium macrosporum* on rubber plant leaves. **A**. Germinating conidia of *D. pulvinata* on surface tissues without the pathogen and **B.** with the pathogen in sample fixed 8 h after inoculation (arrow). **C**. to **F**. *D. pulvinata* hyphae expanding, surrounding, and attacking the host structures with production of appressorium-like structures (F) or not (E).

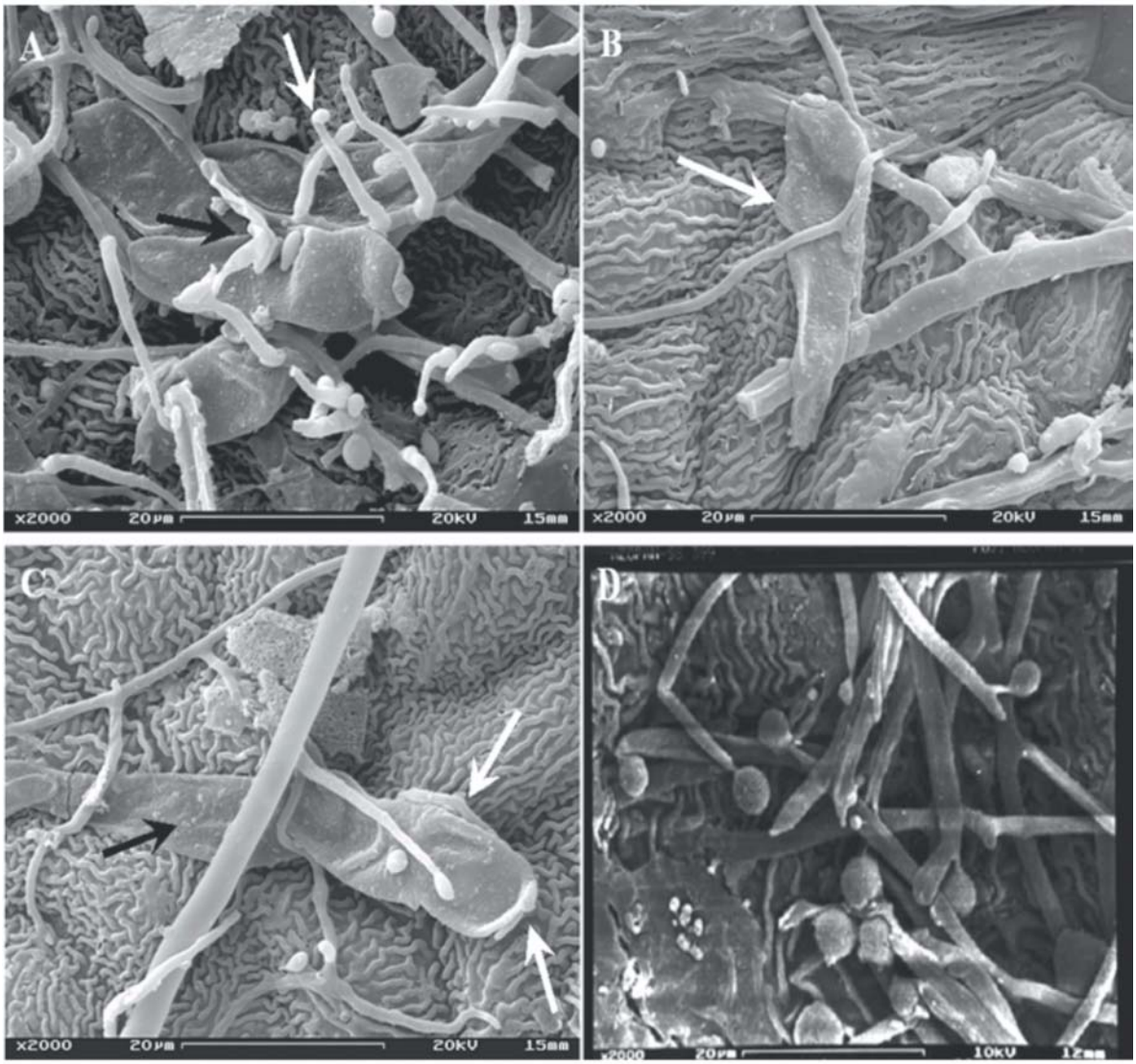

FIG. 2 - Scanning electron micrographs of the interaction of *Dicyma pulvinata* and *Fusicladium macrosporum* on rubber plant leaves. **A.** conidiophores emerging from the pathogen structures and its spores production 6 days after inoculation (arrow). **B.** The deflated aspect of spores and mycelium degrading of *F. macrosporum* colonizing by *D. pulvinata* (arrow). **C**. Details of the formation of invading hyphae after colonization (arrow). **D.** Tissue of rubber plant leaves surface covered by *D. pulvinata* after total destruction of the *F. macrosporum* structures.

DISCUSSION

Conidia germination and appressorium-like structure formation are considered as important events in antagonism for both phytopathogenic (Beckman & Payne, 1982) and entomopathogenic fungus (Hatzipapas *et al*., 2002). Thus, it should also receive special attention in the studies involving the action mode in hyperparasitic interaction. Here we present experimental results showing germination and formation of these types of infective structures in *D. pulvinata,* a hyperparasite of a foliar pathogen, for which the slow growth in artificial media makes investigation difficult in dual cultures. In controlled systems, the described method above is very useful and rapid for studying the antagonistic interaction process and may help to elucidate some of the modes of action of *D. pulvinata.*

Antagonism may be accomplished by different modes of action, such as competition, parasitism and antibiosis, which can act alone or in combination (Berto *et al.,* 2001). Our observations suggest that the efficiency of *D. pulvinata* can be from a direct effect caused by the attack on the pathogens destroying its spores. The aspects of the cell surface beneath the contact area do not show points of degradation in the host cell wall. However, fungal cell wall-degrading enzymes have been associated with degradation of hyphae of many pathogens (Berto *et al.*, 2001) and can be a mechanism involved in the digestion of wall-layers of *F. macrosporum* spores at the penetration point.

By using assays on liquid medium containing chitin, *D. pulvinata* revealed considerable activity of extracellular enzymes such as Glucanase, *N*-acetylglucosaminedase (NAGase), and proteases. The results have shown that along the time-line of *D. pulvinata* enzyme production in liquid medium containing chitin there was increasing activity from low levels in early stages of cultivation to higher levels at later stages. Nevertheless, the function of these enzymes' activity enhancement remains unclear. It could rest on the direct interaction between the antagonist and the pathogen fungus, but could also result in a metabolic process, leading to a cell wall degradation of either *M. ulei* or *D. pulvinata* itself as Berto *et al*. (2001) suggested for the *Ulocladium atrum* Preuss and *Botrytis cinerea* Persoon ex Fries interaction.

However, the nature of lytic enzymes and determinants of host specificity are not known and deserve further study (De Marco *et al.,* 2000). Probably, a chronological event of an antifungal activity is associated in a synergistic action of hydrolytic enzymes with the antagonistic properties (Lima *et al*., 1999). It is, therefore, likely that in nature the lytic enzymes act as a phytopathogen cell-wall-degrading factor following recognition and interaction of the antagonist with the phytopathogen and enzyme induction (Lima *et al.,* 2000).

On the other hand, a compound with fungitoxic activity has been obtained from a *D. pulvinata* isolate colonizing *C. fulvum* late leafspot lesions, and the 13-desoxyphomenone structure was proposed for that metabolite. As reported, this toxin might have a role in the tripartite system hyperparasite-parasite-host (Tirilly *et al.*, 1983).

The present study is the first report on the interaction by scanning electron microscopy, and to investigate the possible involvement of hydrolytic enzymes in the antagonistic association between *D. pulvinata* and the plant pathogen.

Our results confirmed the antagonistic effect of *D. pulvinata* against *M. ulei* on SALB lesions. This destructive effect can also be observed in tests carried out on stromatic lesions (*M. ulei*) from material collected in field (Mello *et al*., 2006). Such a reduction of inoculum by application of the antagonist can contribute to slowing down the SALB epidemic spread when the population of the pathogen develops independently of exogenous inoculum. On the other hand, the discovery that *D. pulvinata* cannot colonize rubber plant leaf tissue in the absence of the pathogen excludes preventive treatment as a biocontrol strategy for this tripartite system.

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