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

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RESUMO

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. Penetração foi verificada em amostras fixadas 24 h a após inoculação de *D. pulvinata*. Ao término do processo, os esporos de *F. macrosporum* 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 inoculação, conidióforos do fungo antagonista foram observados emergindo das estruturas do patógeno e produção de 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.

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ABSTRACT

The *Dicyma pulvinata* and *Fusicladium macrosporum* interaction was studied by scanning electron microscopy. Spores of *D. pulvinata* germinated on the surface of *F. macrosporum* lesions induced on rubber plants artificially infected, fixed 8 h after inoculation. Germ tubs seemed to elongate toward *F. macrosporum.* The penetration into the *F. macrosporum* spores was verified 24 h after *D. pulvinata* inoculation. In the end of the process, the *F. macrosporum* spores looked disintegrated and devoid of content. The antagonist completely overgrew the pathogen. Six to seven days after the inoculation with the antagonistic fungus, it was observed *D. pulvinata* conidiophores emerging from *F. macrosporum* structure, with profuse sporulation. Also, studies have appointed the ability of *D. pulvinata* to produce hydrolytic enzymes, which could be associated to the control of plant pathogens. This information may help elucidate the mode of action of *D. pulvinata*, a potential biological control agent to the South American Leaf Blight of *Hevea* rubber.

INTRODUCTION

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

Studies have appointed that usually the epidemiological process begins from conidia germinating, in an imperfect stage of the pathogen (*Fusicladium macrosporum* Kuyper, mitosporic), which occurs within 1 hr (optimum temperature near 24 C). Four – five hours leaf-wetness is required for hosp penetration wich is through the immature cuticle. Conidia are viable a few days under ambient temperature and shade. Sporulation begins 5-6 days after infection; pycnidia of *Aposphaeria ulei* P. Henn., another imperfect stage of the fungus, are formed after 3-5 weeks, and *M. ulei* ascocarps, after a further 4-6 weeks (HOLLIDAY, 1970).

In spite of the recommended control strategy of planting *Hevea brasiliensis* (Willd ex. A. Juss) Muell. in areas where the climatic conditions are unfavorable to the epidemic development of the disease (escape zones), experiments conduced by Gasparotto e Junqueira (1994) showed evidences on the existence of ecological races of *M. ulei*, better adapted in adverse climatic conditions. This information was confirmed later (RIVANO, 1997; MATTOS et al., 2003; ROMERO et al, 2006). Hence, it is predictable difficulties for controlling of this disease even in escape zones.

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 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. & Curt.) Arx [=*Hansfordia pulvinata* (Berk. & Curt.) Hughes]. This fungus at first observed in the Amazon Region, colonizing stromatic lesions produced by *M. ulei* spread up to different geographic areas from Brazil. Results obtained from field trials (JUNQUEIRA e GASPAROTTO, 1991) have demonstrated the action of *D. pulvinata* against SALB in decreasing the inoculum potential of the parasite by death of hyperparasitized conidia on colonized lesions.

The mitosporic fungus *D. pulvinata,* which was first reported mycoparasitic on *Isariopsis indica* (RATHAIAH e PAVGI, 1971) and *Cercospora* spp. in India (KRISHNA e SINGH, 1979), has been studied as a parasitic of *Cladosporium fulvum* Cooke and *Cercosporidium personatum* Earle, causal agents of tomato (*Lycopersicon esculentum* L.) leaf mould, and late leaf spot of peanut (*Arachis hypogaea* L.), respectively (PERESSE e LE PICARD, 1980; TIRILLY et al., 1983; MITCHELL et al.,1987; TIRILLY, 1991). Peresse e Le Picard (1980) suggested that this fungus could be used in the biological control of *C. fulvum* in grasshouse-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 e KAWANO, 2003).

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

A survey was carried out from late February to late December of 1999, in different geographic areas across the country. *D. pulvinata* isolates were harvest from lesions of *M. ulei* on leaves of *Hevea* rubber and incorporated to the Embrapa's collection of fungi for biological control of plant pathogen (MELLO et al., 2005). A performance comparision of several of these *D. pulvinata* isolates showed that at least the isolates CG774, CG801, CG773, CG790, CG679, CG826 and CG682 could be used to control the disease (MELLO et al., 2006). Antagonism may be accomplished by competition, parasitism, antibiosis, or by a combination of these modes of action (BERTO et al., 2001). The present study is the first report on the interaction by scanning electronic microscopy and to elucidate the possible involvement of hydrolytic enzymes in the antagonistic association between *D. pulvinata* and the plant pathogens.

MATERIALS AND METHODS

Healthy potted plants of rubber (*H. brasiliensis*, clone GT1) were inoculated by spraying a conidia suspension (10^6 conidia mL^{-1}) of *F. macrosporum* on the leaflet surface. The leaflet age was 6-8 days, which correspond to the B1 and B2 stage (HALLÉ et al., 1978). The conidia were originally obtained from rubber plants artificially infected, by washing lesions with sterile water and rubbing gently with a soft camel's hair brush. Conidia concentrations were determined by neubauer chamber counts before use. The inoculated plants were kept inside a growth clamber (Lab-line Instruments, inc.) adjusted for 24-h darkness (100% RH; 25 °C). After that, the chamber was adjusted for 12-h

darkness provided by fluorescent lamps. Five days after inoculation, when the leaf lesions had formed, the plants were taken to the greenhouse for inoculating with the antagonistic fungus.

The *D. pulvinata* antagonist used in this study, isolate CG 774, was obtained from a survey (MELLO et al., 2006) and stored at -180 ºC on the Embrapa Recursos Genéticos e Biotecnologia fungus collection. Current cultures were grown at 25 to 27 ºC on potato dextrose agar (PDA) home medium and storage at 4 ºC. In order to produce of sporulating cultures for trials, mycelium disc from these stock cultures were inoculated on PDA plates and incubated under 12 h of alternating dark and light at 25 ºC.

The inoculum was obtained from 15-day-old cultures. It was prepared by adding 2 mL of sterile distilled water + Tween 20 (0.02%) solution to each plate that then was swept with a soft camel's hair brush to dislodge conidia. Conidia concentration was adjusted for 10^6 conidia mL^{-1} and the suspension obtained thus was sprayed on the surface of rubber leaves presenting *F. macrosporium* lesions. Post inoculated, the plants were placed into plastic bag overnight. Bags were moistened by spraying water inside prior to insertion of plants.

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 e RUSSEL, 1999) and dehydrated in a graded acetone series. The specimens were then dried in an Oryer Emitech Critical Point K 850, using CO_2 as transition fluid. The dried samples were glued onto specimen stubs and coated with gold in an Emitech K 550 Sputter Coater. ZEISS DSM 962 AT scanning electron microscope at 20KV was used to examine the samples.

For enzyme production essays, the *D. pulvinata* isolate was cultured in 50 mL of liquid medium (25 g L^{-1} glucose, 5 g L^{-1} yeast extract) at 28º C under agitation (150 rpm) and after 72 hours it was collected in sterile distilled water and transferred to 50 mL of liquid culture medium contained (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 28º C under agitation (150 rpm), in order to obtain enzyme production. After incubation for time periods, culture filtrates were collected by filtration (Whatman No. 1 paper) and stored at -20ºC 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 37ºC, 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 e 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 37oC. 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 measure 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 proteases assay was based on the written paper by Haran et al. (1996). In general, all assays were run in triplicates.

RESULTS

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

Conidial germination and germ tub growth of the antagonistic fungus was observed 8 h after inoculation on all leaf surface tissues examined (Figures 1A-B). As the process progressed, *D. pulvinata* mycelium expanding from germ tubs grew to the host structures (mycelium, conidiophores and conidia), surround and held them (Figures 1C-F). Once on contact with *F. macrosporum* conidia, the hyperparasite produced appressorium-like structures which invaded them (Figures 1D and F) despite none perforations in the host cells were observed. However, occasionally the penetration seemed occur without these kind of structures (Figure 1E). Most of *F. macrosporum* conidia looked to be penetrated 72 hours after inoculation. Conidiophores with conidia emerged from the pathogen structures was observed in the samples fixed six days pos inoculated with the antagonistic (Figure 2A). *F. macrosporum* conidia invading looked deflated or devoid of content (Figures 2B-C).

Colonization into these structures was not studied, although the mycelium could be noted to be growing 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 (Figure 3).

Exam of samples from rubber leaves tissue no infected taken longer eighth hour after the treatment with *D. pulvinata* revealed the incapability of the antagonistic fungus for surviving in the absence of the pathogen.

Aiming to elucidate the possible involvement of hydrolytic enzymes in the antagonistic association between *D. pulvinata* and the plant pathogens, we have undertaken studies on characterization of the enzymes produced by this antagonist. The determination of the total proteins secreted during a period of one week demonstrated growing liberation of proteins during the whole induction period. Substantial amounts of hydrolytic enzymes as NAGase (maximum in 48 h / 0.11 U) and Glucanases was produced during the induction period, containing chitin (0.5%). The endoglucanases indicated the highest activity in 48 h (0.295 U) and after that, in 96 h (0,129 U), staying unaffected until a week of induction. The exoglucanases indicated the highest activity in 48 h (0.037 U) and in 72 h (0.023 U). After the reduction in the activity, this stayed constant until the end of the enzymatic induction. The chitinase enzyme did not reveal activity, therefore, it was detected a high proteolytic activity in the period of a week (0.075 U), at the end of induction.

DISCUSSION

Conidia germination and appressorium-like structures formation are important antagonism determinants in pathogenic fungus and 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 kinds of infective structures in *D. pulvinata,* a hyperparasite of foliar pathogens. The above-described in controlled system is a very useful and rapid method to study the antagonistic interaction process and may help elucidate the mode of action of *D. pulvinata*, a potential biological control agent to the South American Leaf Blight of *Hevea* rubber.

Antagonism may be accomplished by different modes of action, as competition, parasitism and antibiosis which can act each alone or combined (BERTO et. al., 2001). Ours observations suggest that the efficiency of *D. pulvinata* can be from a direct effect traduced by the attack to the pathogens destroying its spores. The aspects of the cell surface beneath the penetrated area do not showed 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 appointed that the time course of enzymes production of *D. pulvinata* in liquid medium containing chitin showed activity increased from low levels in early stages of cultivation to higher levels at latter stages. Nevertheless, the function of this enzymes activity enhancement remains unclear. It could rest on the direct interaction between the antagonist and the pathogens fungi, but could also result in a metabolic process, leading to a dead cell wall degradation of either *M. ulei* or *D. pulvinata* itself as Berto et. al. (2001) opined for the *Ulocladium atrum* and *Botrytis cinerea* interactions.

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 fashion 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 have obtained from a *D. pulvinata* isolate colonizing *C. fulvum* late leaf spot lesions and was proposed the 13-desoxyphomenone structure for that metabolite. As reported, this toxin would be possibly a role in the tripartite system hyperparasite-parasite-host (TIRILLY et al., 1983). Our work showed the death of the spores in advance of hyphal penetration that suggest the action of one or more fungitoxic compound.

Our results confirmed the antagonistic effect of *D. pulvinata* destroying the spores on necrotic leaves. This effect destructor also can be observed in stromatic lesions (*M. ulei*) exams from material collected in field (MELLO et. al., 2006). Such reduction of inoculum by application of the antagonistic can contribute to slow down the SALB epidemy spread when the population of the pathogen is developing independently of exogenous inoculum. On the other hand, the finding that *D. pulvinata* can not colonize rubber leaf tissue in the absence of the pathogen excludes preventive treatment as a biocontrol strategy for this tripartite system.

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Table 1. Extracellular enzymes production by *Dicyma pulvinata* in liquid medium induced by chitin showed the higher activity period and staying constant until the end of the enzymatic induction.

Enzymes	Assay	Period of Activity	Enzymatic Activity
NAGASE	*p*-Nitrophenol	48 hours	0.11 U
Endoglucanase DNS		48 hours	0.295 U
Endoglucanase DNS		96 hours	0.129 U
Exoglucanase	Glicose Oxidase	48 hours	0.037 U
Exoglucanase	Glicose Oxidase	72 hours	0.023 U
Proteases	Casein	120 hours	0.075 U
Chitinase	DNS/ DMAB	120 hours	----------

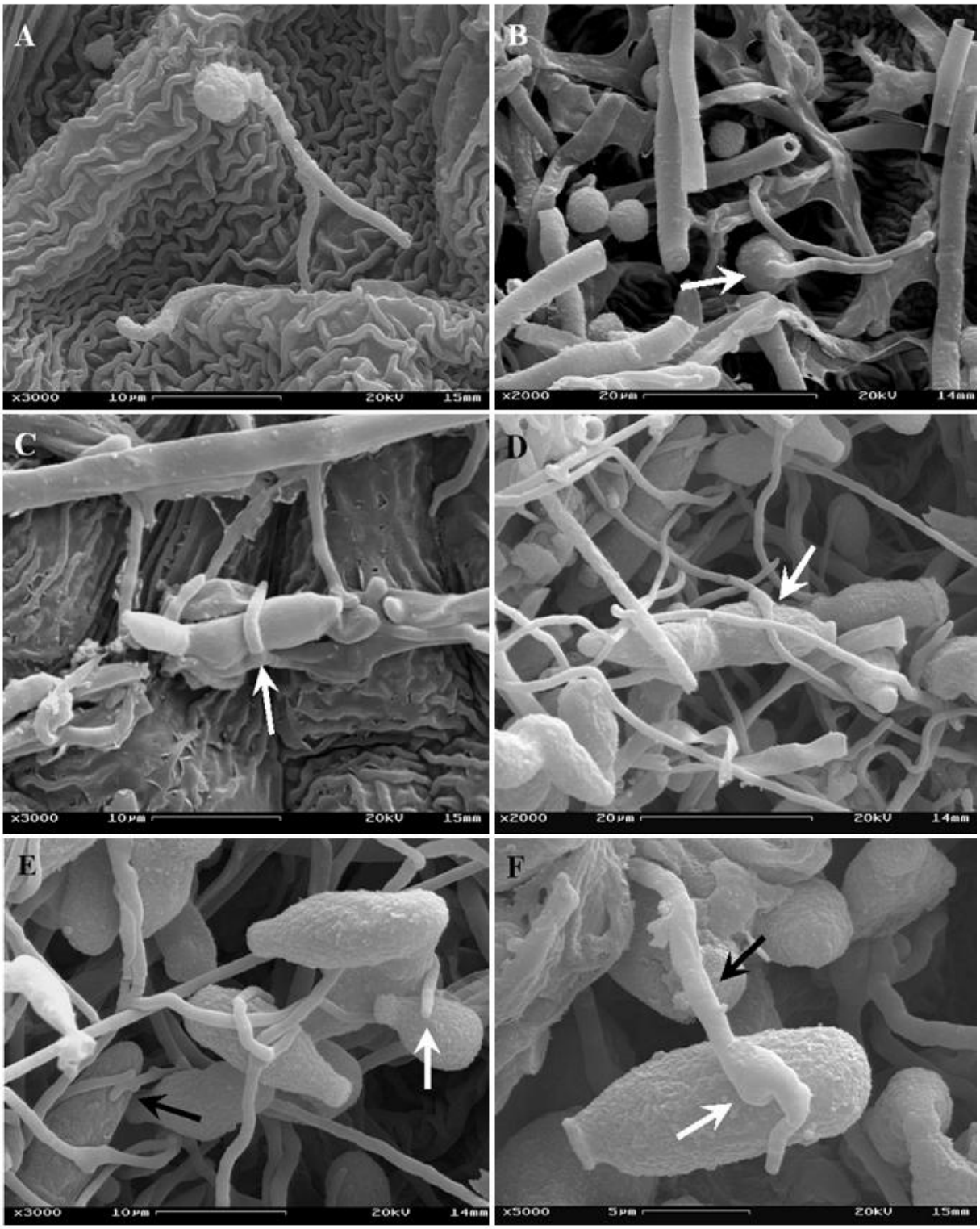

FIG. 1 - Scanning electron micrographs of the interaction of *Dicyma pulvinata* and *Fusicladium macrosporum* on rubber leaves. **A**. Germinating conidia of *D. pulvinata* on surface tissues without the pathogen and **B.** with the pathogen in sample fixed 72 h after inoculation. **C**. to **F**. *D. pulvinata* hyphae expanding, surrounding, and attacking the host structures with production of appressorium-like structures (F) or not (E).

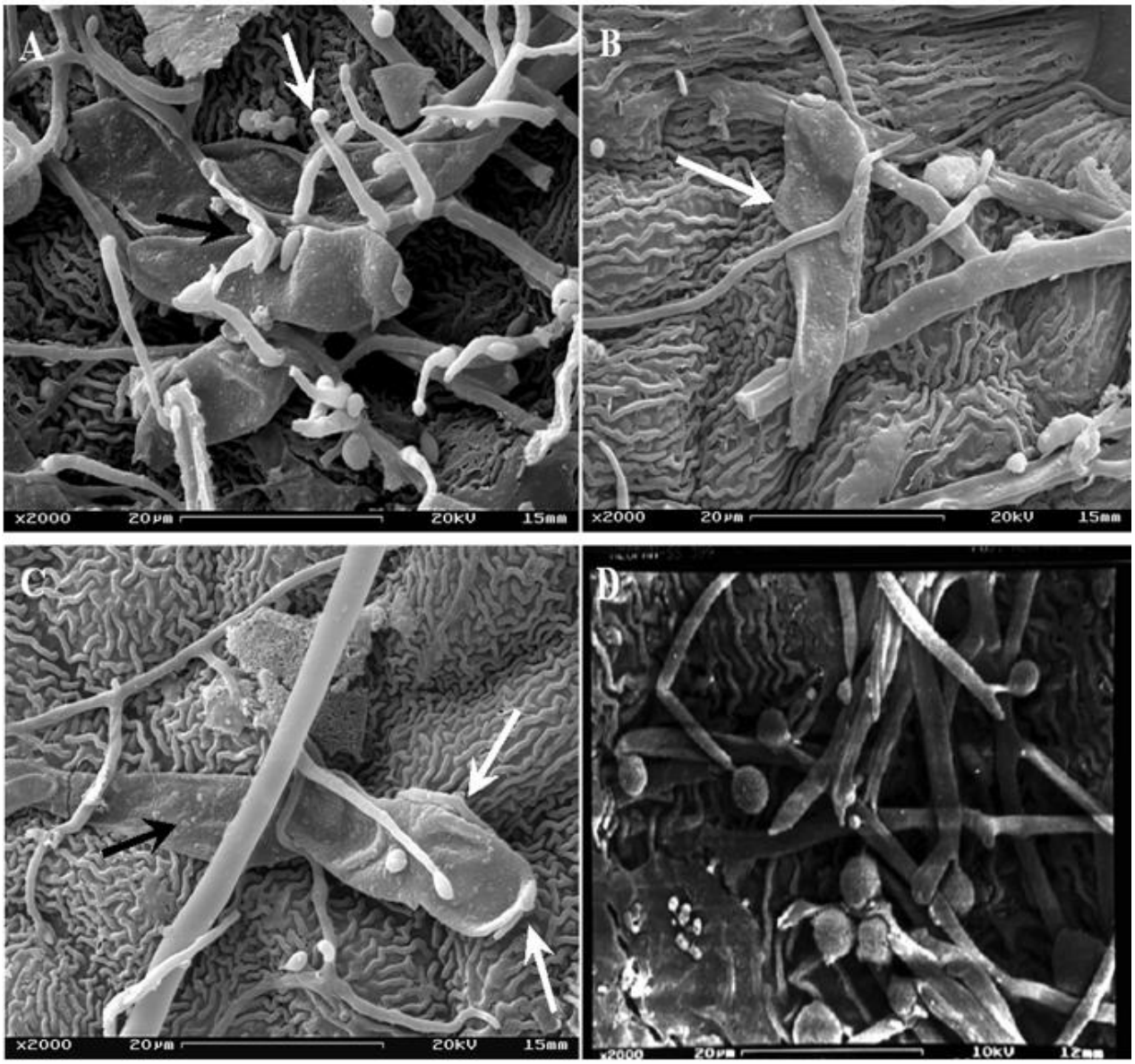

FIG. 2 - Scanning electron micrographs of the interaction of *Dicyma pulvinata* and *Fusicladium macrosporum* on rubber leaves. **A.** conidiophores emerging from the pathogen structures and spores production 6 days after inoculation. **B.** The deflated aspect of spores and mycelium degrading of *F.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

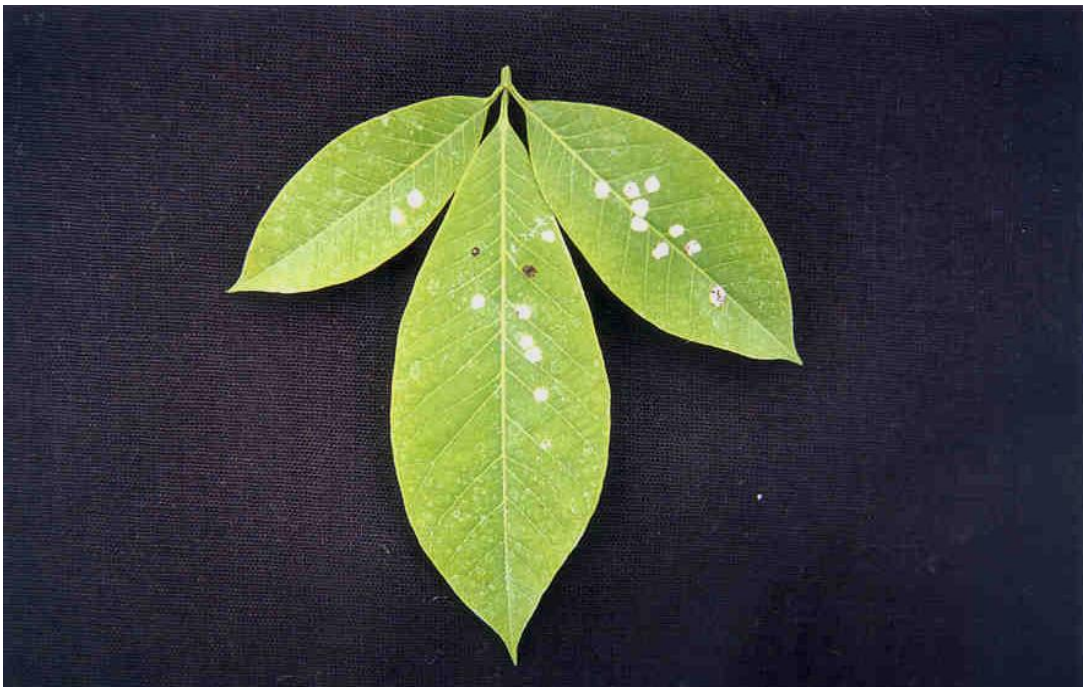

FIG. 3 - *F. macrosporum lesions* on rubber leaf covered by the typical whitish downy growth of *D. pulvinata* in sample fixed 7 days after inoculation.