COMMUNICATION

BIOCHEMICAL COMPOSITION OF *Theobroma grandiflorum* LEAVES INFECTED BY *Crinipellis perniciosa*[1]

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ABSTRACT- Cupuaçu (*Theobroma grandiflorum* Schum.) has fruit production severely affected by the development of the witches' broom disease, caused by the fungus *Crinipellis perniciosa* (Stahel) Singer. In order to investigate the biochemical alterations in infected plants, healthy and infected leaves were compared regarding to the contents of soluble sugars, starch, proteins, chlorophyll, soluble phenolics and tannins. In general, healthy leaves showed the highest contents of the analyzed compounds.

Additional index terms: chlorophyll, cupuaçu, fungi, phenolic compounds, plant pathogen interaction, protein, soluble sugars, starch, tannin, witches' broom.

COMPOSIÇÃO BIOQUÍMICA E ENZIMAS OXIDATIVAS EM FOLHAS DE CUPUAÇU INFECTADAS POR *Crinipellis perniciosa*

RESUMO- Cupuaçu (*Theobroma grandiflorum* Schum.) tem a produção de frutos bastante afetada, quando atacada pela doença vassoura de bruxa, causada pelo fungo *Crinipellis perniciosa* (Stahel) Singer. Com o intuito de se estudar as alterações bioquímicas em plantas infectadas, folhas sadias e doentes foram analisadas quanto ao conteúdo de açúcares solúveis, amido, proteinas, clorofila, fenóis solúveis totais e taninos. De modo geral, os maiores teores desses compostos foram encontrados em folhas sadias.

Temos adicionais para indexação: açúcares solúveis, amido, clorofila, fenóis, fungo, interação planta-patógeno, proteína, *Theobroma grandiflorum,* vassoura de bruxa.

INTRODUCTION

Today, witches' broom represents the major problem limiting cocoa (*Theobroma cacao*) production in Brazil. The basidiomycete fungus *Crinipellis perniciosa* (Stahel) Sing. is the causal agent of this disease and it is also found infecting other species of *Theobroma* and *Herrania*, endemic in the forests of the Amazon basin (Baker & Holliday, 1957). The life cycle of the pathogen can be divided into two well-defined phases, parasitic and saprophytic. The basidiospores germinate and penetrate unhardened tissues, mainly through stomata, fallen/collapsed multicellular trichomes and wounds, causing hypertrophy and hyperplasia of stems, flowers and pods. The mycelium grows only intercellularly and proliferation occurs exclusively in active growing tissues. the fungus persists as a saprophyte, producing basidiocarps for long periods (Evans, 1980; Calle *et al.*, 1982; Frias *et al.*, 1991). At present, a few cocoa cultivars displaying partial resistance to *C. perniciosa* are known. So far, information on the biochemical basis of the resistance in these cultivars are scanty. Recent findings have indicated that condensed tannins might be involved in the resistance of cocoa to witches' broom (Brownlee et al, 1990).

Cupuaçu (*Theobroma grandiflorum* Schum.) is an indigenous tree of the Amazon basin and the pulp covering the seeds is appreciated for its pronounced aroma and flavor. The tree is not economically exploited on a large scale and the fruits are harvested from native trees growing in the forest by small local farmers. The pulp is mostly used for consumption as juice.

[1]Received 30/12/1996 and accepted 08/06/1997. This work received financial support from Japan International Cooperation Agency and Embrapa-CPATU.

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Cupuaçu is also infected by witches' broom. Vieira (1942) considered that, compared to cacao, the production of fruits in this plant is more affected by the disease, since fruit set occurs only in the flushes. However, the isolates of *C. perniciosa* infecting cacao do not infect cupuaçu (Bastos, 1986), showing specificity for the host. So far, to our knowledge, there is no cultivar of cupuaçu displaying resistance to witches' broom nor any report on the physiological behavior of this plant when infected by the pathogen. Most of the information available on pathogen-plant interactions relates to cacao.

Thus, this papper is concerned with the interaction between cupuaçu and *C. perniciosa*, where infected and healthy leaves were compared with regard to some chemical constituents.

MATERIALS AND METHODS

Cupuaçu has alternate leaves. The first and second, the fourth and the eighth leaves were collected from infected and healthy flushes of 12 year old cupuaçu plants growing at Centro de Pesquisa Agroflorestal da Amazônia Oriental, Belém (CPATU-Embrapa). Since there was no way to control the infection of these plants, for each leaf type (healthy or infected) fifteen to twenty leaves were collected from several branches of five plants and mixed. Then, they were divided in two samples, and three replicates were taken from each one. After harvesting, the leaves were kept on ice until they were taken to the laboratory, where they were measured in length and width, for determination of leaf area, weighed and immediately used for the extractions. Ten leaves with the same physiological age were used to obtain the fresh and dry matter.

Segments of five healthy and infected stems, between the first and eighth leaves, were collected from the same cupuaçu trees. After removal of the leaves, they were weighed and their volumes were obtained by recording the displacement volume of a mixture (80 ml of 50% aqueous ethanolic solution in a 100 ml graduated cylinder. The dry matter was obtained after one week at 70^{o}C.

Approximately 1g of leaves were covered with 25 ml of ethanol 80% and disrupted in a Virtis "45" homogeneizer (Virtis Company, New York). A second extraction was made with the same volume of ethanol and the combined extracts transferred to 250 ml Erlenmeyer flasks.

After covering with aluminum foil, the flasks were maintained in a water bath (80^{o}C) for one hour, with occasional agitation. After cooling the debris were filtered off and the filtrate was used for the determination of soluble sugars according to Dubois et *al.* (1956). Sucrose was used as standard.

The starch content was measured in the recovered debris after overnight digestion with 10ml of 35% perchloric acid. The extracts were centrifuged at 1500 g and the glucose residues were quantified (Dubois et al, 1956). Glucose was used as standard.

The same ethanolic extract used for the determination of soluble sugars was also used for phenolic (Swain & Hillis, 1959) and chlorophyll determinations (Arnon, 1949). For phenolics, phenol was used as standard.

Tannins were twice extracted with boiling distilled water (1g/ 2 X 25ml). The leaves were disrupted in a Virtis homogenizer and incubated for one hour in a boiling water-bath. The debris were filtered off and tannin concentration was measured by the precipitation of methylene blue in phosphate buffer (Okuda et *al.*, 1985).

Soluble proteins were determined only in the first and second and fourth leaves. The main vein was removed and the leaf blades were ground in a mortar with cold 250 mM sodium phosphate buffer, pH 7.0, containing 5% ascorbic acid. The extracts were centrifuged at 1500 g, 4^{o}C and the supernatant recovered for protein determination (Bradford, 1976). Bovine serum albumin was used as standard.

RESULTS AND DISCUSSION

Surprisingly, the fourth infected leaf differed significantly from the others, showing in infected plants a lower accumulation of dry mass (Fig. 1B) associated with a faster leaf expansion (Fig. 1A), and, as a result, the dry mass per leaf area for this leaf was three times lower than for the healthy one (Fig. 1C). For this reason, the contents of the chemical constituents shown in Table 1 were expressed as kg.m^{-2}.

The percentage of dry mass was higher for healthy tissues (23.4%) when compared to infected ones (17.8%). When the density was calculated using the fresh matter, no difference was observed (0.931 leaves kg.L^{-1} for healthy and 0.972 kg.L^{-1} for infected). However,, using dry mass the healthy stems showed higher density (0.218 kg.L^{-1}) than diseased ones (0.174 kg.L^{-1}), indicating that they accumulated more dry matter per volume.

Cupuaçu leaves infected with *C. perniciosa* showed reduction in the contents of phenolic compounds and tannins (Table 1). This data, associated with the knowledge that infected cocoa tissues become swollen and the cells disorganized as a response to increased phytohormone levels (Isaac, 1992), leads us to speculate that in order to facilitate its growth in the apoplast, in some way the fungus changes the hormonal balance in the leaves, affecting the accumulation of cell wall constituents. In addition, it is

known that increased levels of auxin may induce an increase in elasticity of the cell wall due to chemical alterations of its composition (Salisbury & Ross, 1992). Since in plants lignin would account for 20-35% of the cell wall (Monties, 1989), acting therefore as strong sink of phenolic acids, a low accumulation would cause a lowered demand of these compounds in cupuaçu leaves infected with *C. perniciosa*.

TABLE 1- Chemical composition of healthy and infected cupuaçu leaves.

	Leaf position			
		First + second	Fourth	Eight
Sol.Sugars	Healthy	5.34 a	1.12 a	1.00 a
	Infected	2.97 b	0.62 b	0.52 b
Starch	Healthy	4.08 a	0.72 a	0.30 a
	Infected	5.16 a	0.31 b	0.55 a
Proteins	Healthy	0.84 a	0.21 a	nd
	Infected	0.62 b	0.08 b	nd
Chlorophyll	Healthy	0.062 a	0.013 a	0.028 a
	Infected	0.026 b	0.005 b	0.020 b
Phenolics	Healthy	9.13 a	1.67 a	1.24 a
	Infected	4.83 b	0.86 b	0.89 a
Tannins	Healthy	8.75 a	1.16 a	0.38 a
	Infected	3.21 b	0.64 b	0.40 a

Data are means of three replicates. Different letters indicate statistical differences between healthy and infected leaves (Duncan, P ® 0.05).

Data expressed as X 10^{-2} kg.m^{-2}.

There is little information about the effects of fungal infection on photosynthesis in plants and the consequent effect on sugar content. Some studies reported increased (Klecan & Buchanan, 1988) or decreased (Manners, & Gay, 1982) starch levels in some plant/pathogen combinations, and photosynthetic rates not necessarily accompany the increase of sugars in infected tissues (Coghlan & Walters, 1992).

Here, infected leaves showed a decrease in the contents of soluble sugars, starch, and chlorophyll (Table 1). Healthy leaves almost always showed the highest values for all the compounds analyzed. Similarly, the first/second leaves had the highest contents. Soluble proteins also decreased in infected leaves, which might be a consequence of the altered CO_2 assimilation.

C. perniciosa grows exclusively in the intercellular space (Frias et *al.*, 1991). Therefore, the nutrients used for its growth must be available in the apoplast. It has been shown in cocoa tissue culture and protoplasts that the fungus causes leakage of electrolytes (Isaac, 1992), suggesting that nutrients may migrate from inside to outside the cells, as demonstrated for other plants attacked by pathogens (Aked &Hall, 1993). Increase of extracellular invertase activity was also detected (Aked & Hall, 1993).

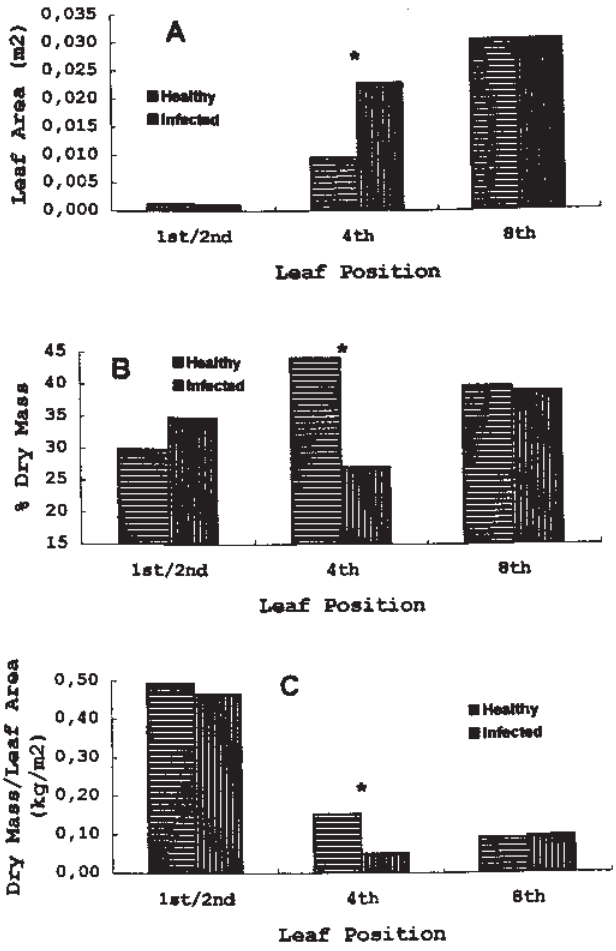

FIGURE 1- Leaf area - m^2 (A), % dry mass (B) and dry mass/leaf area - kg.m^{-2} (C) in the first/second, fourth and eighth healthy and infected leaves. Data are means of ten replicates and the asterisk indicates statistical difference (Duncan, P 0.05) between healthy and infected leaves at the same leaf position.

Our results indicate a general metabolic disturbance in leaves of cupuaçu infected by the fungus. It seems likely that due to a reduced chlorophyll content, photosynthesis is affected in these leaves and, consequently, the level of carbohydrates. However, considering that *C. perniciosa* grows in the intercellular space, further studies on the chemical composition of leaf apoplastic washout, and invertase activity are necessary and should provide new insights on the physiology of this plant-pathogen interaction. Whith respect to the variations of phenolics and tannins, a more detailed study should be conducted, investigating the nature of these compounds, the lignin content, and ultrastructural alterations of the cells in diseased stems and leaves.

ACKNOWLEDGMENTS

To Cleo Marcelo de Araújo Souza for his technical assistance and Dr. Jorge Vega for helpful comments.

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