



Neem (*Azadirachta indica* a. Juss) components: Candidates for the control of *Crinipellis pernicioso* and *Phytophthora* ssp.

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Accepted 6 June 2006

KEYWORDS

Witches' broom;
Pod rot;
Crinipellis;
Phytophthora;
Neem tree;
Biological control

Summary

Witches' broom and pod rot are the two most devastating diseases of cocoa in South America and Africa, respectively. Their control by means of phytosanitation and chemical fungicides is labor-intensive, costly and, in many cases, environmentally undesirable. Therefore efforts are made in order to identify alternative, environmentally safe and cost-efficient methods for the control of these pathogens. Promising candidates are components of the neem tree (*Azadirachta indica*), that have been used for centuries in Asia as insecticides, fungicides, anticonceptionals in popular medicine. Here we report about tests on the effect of various concentrations of extracts from neem leaves on growth of mycelia of *Crinipellis* and *Phytophthora* and on germination of spores of *Crinipellis*. We show a 35% growth reduction of mycelia of *Phytophthora* on neem leaf extract media, whereas growth of mycelia of *Crinipellis* was not affected, even at the highest concentration of neem leaf extracts used (35%). However, the most dramatic effect of neem leaf extracts is observed on *Crinipellis* spore germination, here the extracts (20–35%) reduced germination almost completely. Based on these results, we believe that the neem tree might be a source for the production, on small and medium scale, of an effective and cheap formulation for the control of *Crinipellis* and *Phytophthora*.

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Introduction

Witches' broom and pod rot are the two most devastating diseases affecting cocoa production. The first disease is caused by the basidiomycete *Crinipellis pernicioso* [(Stahel) Singer, 1943] and affects cocoa plantations specifically in South America while pod rot, caused by *Phytophthora* *ssp.*, is ubiquitous and affects cocoa plantations in Africa as well.

Due to the action of *Crinipellis*, cocoa production in Brazil decreased about 50% from 1985 to 2000 (FAO 2002) and in Africa, pod rot caused production losses to amount to 50–80% (ICCO site). Control of witches' broom has, so far, been tried through means of phytosanitation, signifying the removal of affected shoots, flower cushions and pods from the cocoa tree. In order to increase its efficiency, this method is used in combination with chemical methods, mostly involving copper compounds. The latter are also used for the control of *Phytophthora*-induced diseases. Both methods, however, are labor-intensive and expensive or environmentally undesirable and the results obtained so far are below expectations.

For these reasons and out of growing concern about the general degradation of the environment, more attention has been given, in recent years, to possibilities of biological control of these diseases. In the case of *Crinipellis* and *Phytophthora* moderate successes have been obtained by using antagonistic fungi of the genus *Trichoderma* (Holmes 2004). Methods that involve natural organic products are alternatives to the use of life antagonistic organisms in order to control these diseases. Within this scope neem, *Azadirachta indica* (A. Juss) is a promising candidate. This tree, originally cultivated in India, belongs to the *Meliaceae* family and occupies a prominent space in Asian (and recently in Euro-American) popular medicine as well as in corporal hygiene. Among its many attributed properties range antimicrobial activities and therapeutic effects in cases of digestive disorders, of diabetes, of high cholesterol and in cases of breast and gastrointestinal cancer and it has been shown that components of neem oil could, due to their spermicidal activity, be used as contraceptives (SaiRam et al. 2000; Sinha et al. 1984). Scientific data to support these claims, however, are only recently beginning to emerge (see for instance Bramachari, 2004; Singh and Singh, 2003). The consensus is that the general antimicrobial activity of neem extracts is mainly due to azadirachtin, a molecule belonging to the class of tetranortriterpenoids (Salehzadeh et al., 2003). While the antimicrobial activity of neem-derived products is

uncontested (Pai et al. 2004; SaiRam et al. 2000), only few reports are available on neem's action against fungi, mostly belonging to the phylum *Ascomycota* (Bhonde et al. 1999; Mossini et al. 2004). Since we are interested in alternatives allowing for the control of basidiomycetae and oomycetae, pathogenic to cocoa, we tested extracts from neem and, as a control, cocoa leaves with respect to their activity on growth of mycelia of *C. pernicioso* and *Phytophthora* spp. and with respect to inhibitory effect on *Crinipellis* spore germination.

Material and methods

Leaf extracts

Leaves were obtained from neem and cocoa trees, both grown at "Embrapa Recursos Genéticos e Biotecnologia" in Brasília, Brazil. The leaves were collected, washed in distilled water and ground to powder in liquid N₂. Subsequently water was added in order to obtain a 40% (w/v) extract. Since these extracts, in particular those from cocoa leaves, are extremely rich in mucilage, the extracts were filtered through two layers of household towels and then stored at –20 °C until use.

Culture media

Mycelia were grown and germination of spores was tested in solid "YG" medium (0.4% yeast extract, 2.5% glucose, 2% agar). For the tests, leaf extracts from neem and cocoa were added to YG medium to a final concentration of 3, 5, 10, 15, 25 and 35% (v/v). Subsequently the media were distributed into Petri dishes (Ø: 90 mm).

Mycelia and spores

Spores of *Crinipellis* were obtained from basidiocarps grown at the farm "Almirante Cacau" in Itabuna, Bahia, Brazil and kept as suspension in 22% glycerol. The concentration of spores was determined by counting aliquots in a Neubauer chamber and their viability was determined by counting the germinated spores after 4 h on 1% agar in H₂O. In order to obtain monosporic mycelia, the spores were germinated on solid "YG" medium and a mycelium originating from a single spore was then isolated and grown at room temperature until 100% confluent.

Mycelia from *Phytophthora* were obtained from the farm "Almirante Cacau", where they were produced from fungi collected from infected cocoa fruits.

Effect of extracts from neem and cocoa leaves on spore germination

Spores from *C. pernicioso* were diluted to a concentration of 700 viable spores/ml in H₂O. Hundred microliter of this solution were plated out on 2% agar in "YG" containing leaf extracts in the concentrations described above. The dishes were kept at 26 °C and a photoperiod of 14D/10N and the germinated spores were counted after 7 days. Each concentration was done in triplicate and the standard deviation determined according to the unbiased formula.

Effect of extracts from neem and cocoa leaves on mycelial growth

Mycelial discs (\varnothing : 8.0mm) from *Crinipellis* and *Phytophthora* were deposited on 2% agar plates in "YG" containing 3, 5, 10, 15, 25 and 35% of neem or cocoa leaf extracts. The dishes were kept at 26 °C and a photoperiod of 14D/10N and the diameter of the mycelia was determined at 2 days intervals during ten days. Each concentration was done in triplicate.

Results and discussion

Here we report the effect of leaf extracts from neem and cocoa on mycelial growth of *Crinipellis* and *Phytophthora* and on spore germination of *Crinipellis*. These organisms are the causative agents of witches' broom and pod rot in cocoa. Both diseases are responsible for disastrous economic and ecologic damages, the latter because the uncontrolled use of cupric fungicides and the deforestation brought about by impoverished farmers.

The rationale of testing components of neem was based on its traditional use, in Asia, in popular medicine against a broad spectrum of microbial diseases. Recently these claims have been supported by scientific facts, amongst which are some reports on neem seed extracts being antagonistic to fungi as described in: <http://www.organicconsumers.org/patent/neemtree030905cfm>.

Mycelial growth

The effect of neem and cocoa leaf extracts

In the neem tree the occurrence of bioactive substances is not restricted exclusively to the seeds; bioactive terpenoids are also present in the leaves. Therefore we tested aqueous neem leaf extracts in the concentrations indicated in "Material and methods" on mycelia of *Crinipellis* and *Phytophthora*. Since there are no reports on the occurrence of antimicrobial substances in cocoa, we included extracts of cocoa leaves as a control. The effect of neem leaf extracts on mycelial growth of *Crinipellis* (Fig. 1a) and *Phytophthora* (Fig. 1b) over a 10-day period are shown. In the case of *Crinipellis* and at the concentrations used, the growth of mycelia is not affected at all, but in the case of *Phytophthora*, a 35% inhibition is observed at 35% extract present in the media. With extracts from cocoa leaves (Figs. 2a and b), on the other hand, the picture is different: the presence of up to 15% cocoa extract in the medium stimulates mycelial growth slightly in *Crinipellis* (Fig. 2a) and for *Phytophthora* (Fig. 2b) inhibitory effects are observed at concentrations between 25% and 35%.

Germination of *C. pernicioso* spores

Effect of leaf extracts from neem and cocoa

The life cycle of *C. pernicioso* involves a biotrophic and saprotrophic phase and infection starts with the biotrophic stage when basidiospores germinate on meristems of the host plants. They subsequently form mycelia which invade the host tissue and are responsible for broom formation. After the dead of the broom, the saprotrophic phase sets in and ends with the formation of new basidiocarps producing the next spore generation. From this it follows that the crucial phase in infection is the germination of the spores once they have settled on the host's surface. Consequently an effective control of the pathogen could be obtained by inhibiting spore germination. Therefore we tested the effect of leaf extracts on spore germination. The results shown in Fig. 3 document that extracts from neem leaves are inhibiting germination at concentrations between 15% and 35%. With leaf extracts of cocoa, on the other hand, an amazing picture emerges: in the concentration range of 3–10% cocoa leaf extract, spore germination is stimulated. As outlined in "Material and methods", the spore concentration was determined by counts in a Neubauer chamber and spore viability was subsequently determined by counting

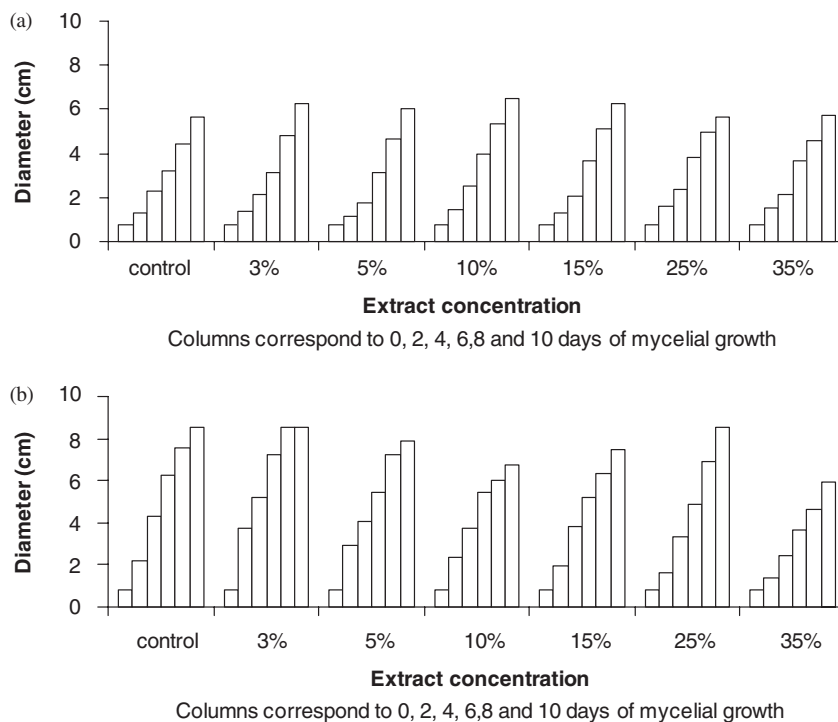


Figure 1. Effect of Neem Leaf Extract on Growth of Mycelia. X-axis: Days of mycelial growth and extract concentration; Y-axis: Diameter of mycelia (mean value of three experiments).

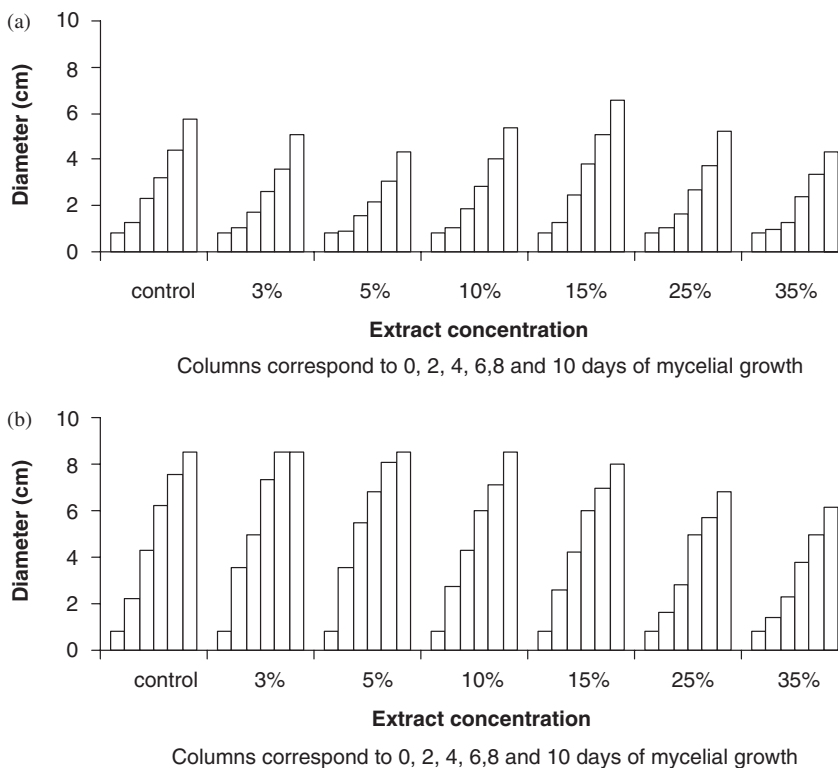


Figure 2. Effect of Cocoa Leaf Extract on Growth of Mycelia. X-axis: Days of mycelial growth and extract concentration; Y-axis: Diameter of mycelia (mean value of three experiments).

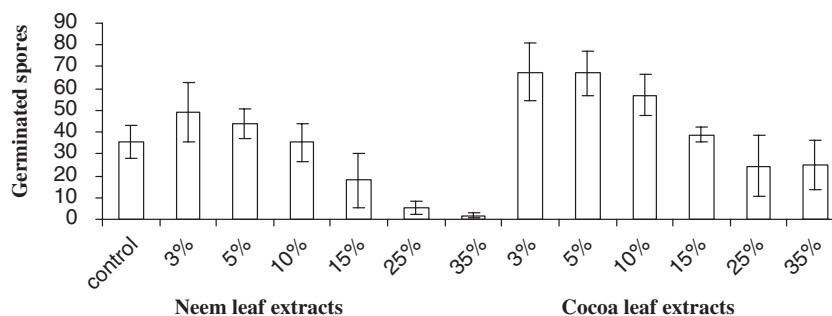


Figure 3. Effect of Neem Leaf and Cocoa Leaf Extracts on Germination of *Crinipellis* Spores. X-axis: Concentrations of extracts; Y-axis number of germinated spores, vertical bars: Standard deviation.

the percentage of spores capable to germinate. For the experiments, aliquots containing 70 viable spores, independent of the actual number of spores present, were plated out. The fact that, with extracts of cocoa leaves, the germination rate of spores almost doubles means that cocoa extracts can restore germinative bioactivity. Actually this interpretation is supported by the other observation i.e. that cocoa leaf extracts seem to stimulate mycelial growth (Fig. 2a) at a concentration of 15%. From an evolutionary point of view this makes sense since the patosystem *Crinipellis*/cocoa must be extremely fine-tuned towards the needs of the pathogen.

Conclusions

Taken our results together, we believe that they point to the potential of neem leaf extracts, as bioactive components useful for the control, alone or in combination with traditional methods, of witches' broom and of pod rot of cocoa. Both diseases have devastating effects on economy as well as on ecology and the methods actually used are not efficient or economically satisfying. In particular in Brazil, a secondary effect of witches' broom is of alarming ecological importance: cocoa was traditionally grown in the shadows of the Atlantic rain forest. Since *Crinipellis* abolished almost all cocoa trees and, with that, a fairly easy and risk-less income, the farmers started selling the rain forest trees. It is possible that the neem tree might represent a major component in a strategy that aims at the reforestation and simultaneous revival of the cocoa plantations in areas affected by witches' broom or pod rot. Botanically it is a close relative of mahogany (*Swietenia mahagoni*) with very tough and termite-resistant wood. It is a tropical evergreen, grows fast up to 30m and thrives even in poor and exhausted soil.

These characteristics make it a candidate to be useful in establishing environmental sustainability and economic profitability for small- and medium-holder cocoa producers. Not only would it provide the necessary shadow for the cocoa trees and commercially high-value wood, but it also would provide the "*materia prima*" for the preparation of ecologically safe and easy to produce formulations for the control of cocoa's most important diseases.

Acknowledgments

This study was supported by the Brazilian National Research Council (CNPq). ARR was supported by a grant from Coordination for the Improvement of Higher Education Personnel (CAPES). We thank Dr. Stephan Nielen for critical reading of the manuscript.

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