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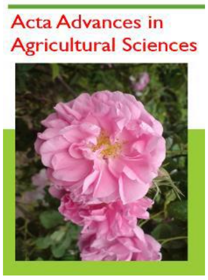

Research Article

In vitro inhibition of *Xanthomonas axonopodis* pv. *phaseoli* by crude ethyl acetate extracts of endophytic fungi isolated from *Piper hispidum* Sw.

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ABSTRACT

The chemical control of the causal agent of common bacterial blight of bean, *Xanthomonas axonopodis* pv. *phaseoli*, is not completely successful. Therefore, it is important to search alternatives for the control of this disease. Endophytic fungi, that inhabit the interior of plants without causing apparent harm to them, have been described as a possibility for the biological control of phytopathogens. The medicinal plant *Piper hispidum* Sw. has several properties, including its action against some species of *Xanthomonas*. In view of this, the present study evaluated the inhibitory effect of four crude extracts excreted by endophytic fungi isolated from *P. hispidum* against *X. axonopodis* pv. *phaseoli*. Results obtained for cup plate assay showed the formation of inhibition halos with diameters between 4.83 to 10.50 mm. The analysis of variance emphasized the results obtained for the extract from endophyte *Lasiodiplodia theobromae* JF766989, tested in a concentration of 61.4 mg/ml. However, the other three extracts used also presented positive results. Data corroborated with a previous study regarding antibiotic action of these same extracts against human pathogenic bacteria, reinforcing their antibacterial potential. Considering that a similar property can be found in a medicinal plant and in its endophytes, these results confirmed the expectation of find endophytic candidates for the biological control of this bacterium.

Introduction

The common bean (*Phaseolus vulgaris* L.) is the most important grain legume for human consumption, being an important dietary component especially in some developing countries, such as Brazil and Mexico, where it is a major source of protein (Gepts et al. 2008). One of the most worldwide destructive diseases which significantly reduces bean crops is the common bacterial blight caused by *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Vauterin, firstly described as *X. campestris* pv. *phaseoli* (Smith)

Dye (He 2010; Vale et al. 1997; Vauterin et al. 1995).

Although it is considered primarily a foliar disease, common bacterial blight of bean can also occur in stems, pods and seeds. Initial symptoms are small water-soaked spots that enlarge and become necrotic and bordered by a yellow zone in case of leaf spots. Individual lesions may grow together causing plants to look burned. Spots on pods are usually circular and brownish red, while infected seeds develop yellow to brown spots and show weak vigor and germination (Gilbertson and Maxwell 1992). Its control is difficult, since the use of chemical agents is usually inefficient (Maringoni 1990). According to Mirik et al. (2007), copper-containing compounds have been routinely applied to the control of some pathovars of *X. axonopodis*, but it has not been totally effective.

Alternatives to obtain better results in disease control and less damage to the environment have been searched, such as biological control and induction of plant resistance by natural products (Schwan-Estrada and Stangarlin 2005; Silva et al. 2008). The use of microorganisms that inhibit or antagonise those phytopathogens can be applied in this type of control (Azevedo et al. 2000) and the selection of antagonists can be based on the *in vivo* and *in vitro* bioassays in greenhouse or at laboratory conditions (Mariano 1993).

Fungal endophytes have been pointed out as effective antagonists against phytopathogens (Campanile et al. 2007; Hastuti et al. 2012; Rocha et al. 2009). These microorganisms can be defined as those that, during at least a period of their life cycle, colonize the living internal tissue of their host plants without causing apparent harm to them, establishing mutual relationships (Petrini 1991). Moreover, these fungi have been reported as producer of several biologically active compounds (Flores et al. 2013; Garcia et al. 2012; Hormazabal and Piontelli 2009; Orlandelli et al. 2012b; Phongpaichit et al. 2007; Rhoden et al. 2012; Specian et al. 2012; Sumarah et al. 2010; Tayung et al. 2011; Xu et al. 2008).

The medicinal plant *Piper hispidum* Sw. (Piperaceae family) has pantropical distribution, including all Brazilian geographic regions (Guimarães and Giordano 2004). Its properties include the use as astringent, diuretic, stimulant, for removing hepatic obstructions and stopping hemorrhages (Roíg y Mesa 1945), for the treatment of malaria (Milliken 1997), cutaneous leishmaniasis and the healing of wounds (Estevez et al. 2007). Also, its extracts are active against some *Xanthomonas* species (Nair and Burke 1990).

The term “bioprospecting” is frequently used to describe the screening of biological organisms for commercial purposes. The bioprospecting studies of endophytes include the search of useful microbial compounds with antibacterial, antifungal, antiviral, insecticidal, anticancer, antioxidant and other activities (Strobel and Daisy 2003; Synnes 2007). It is knowledge that *P. hispidum* harbors a diversity of fungal endophytes (Orlandelli et al. 2012a), including some active against human pathogenic bacteria (Orlandelli et al. 2012b). However, there is little information about bioprospecting of fungal endophytes isolated from this shrub for biological control. This current study is aimed at evaluating the inhibitory effect of extracts produced by endophytic fungi against *X. axonopodis* pv. *phaseoli* in order to verify whether extracts produced by these endophytic fungi are active against *Xanthomonas* genus, as related for the host plant *P. hispidum*.

Materials and Methods

Endophytic fungi and crude ethyl acetate extracts (CEAEs)

Lasiodiplodia theobromae (JF766989), an endophyte from Diaporthales order (JF767007) and two non-identified isolates (named by codes G33-73 and G53-83) were isolated from leaves of *P. hispidum* Sw. (Orlandelli et al. 2012a). The sequences of those molecularly identified are deposited in GenBank. These endophytic fungi belong to the fungal culture collection of Laboratory of Microbial Biotechnology, State University of Maringá, Brazil.

The CEAEs produced by these endophytes were previously obtained by Orlandelli et al. (2012b), using ethyl acetate P.A. as solvent, followed by resuspension in absolute methanol P.A., at the final concentration of 61.4 mg/ml ($CEAE_{Lasiodiplodia}$), 50.1 mg/ml ($CEAE_{Diaporthales}$), 19.9 mg/ml ($CEAE_{G33-73}$) and 24.0 mg/ml ($CEAE_{G53-83}$).

Phytopathogenic bacterium and culture media

The strain of the Gram-negative phytopathogenic bacterium *Xanthomonas axonopodis* pv. *phaseoli* I227 was provided by EMBRAPA Genetic Resources and Biotechnology (CENARGEN), Brazil. The culture media Luria Bertani Agar (LBA) and Luria Bertani Broth (LBB) were prepared according to Sambrook and Russel (2001).

In vitro inhibitory effect of CEAEs against Xanthomonas axonopodis pv. phaseoli

This assay was performed by qualitative biological analysis using cup plate technique. Phytopathogenic bacterium was grown in LBA and LBB for 24 h and spread (100 µl in concentration of $1x10^6$ cells/ml) on Petri dishes (9 cm) containing LBA. Each dish received, equidistantly, four 6 mm disks of sterile filter paper (Whatman no. 4) inoculated with 10 µl of CEAEs, autoclaved distilled water, absolute methanol P.A. (negative controls) or antibiotic tetracycline (Sigma) (the positive control, in the same concentration of each extract). Dishes were incubated at 37º C for 24 h and inhibition halos were measured in mm.

Tests were carried out using completely randomized design (CRD) with three replications and were analyzed by ANOVA (Analysis of Variance). In order to verify the efficiency of CEAEs, means were compared by Tukey test ($p<0.05$) using statistical program SAS (2001).

Results and Discussion

Endophytic microorganisms are antagonists to plant pathogens and they inhabit a similar ecological niche in the interior of host plants. Endophytes can be used for biological control by competition for nutrients, parasitism of pathogens, production of antimicrobial substances or induction of plant resistance to diseases (Azevedo et al. 2002). According to Rocha et al. (2009), the inhibition halos visualized by *in vitro* experiments are indicators of the antibiosis caused by antagonist substances. In view of this, the current study used the cup plate technique for evaluation of the antimicrobial bioactivity of CEAEs of *P. hispidum* fungal endophytes against *X. axonopodis* pv. *phaseoli*.

Table 1. *Antibacterial activity represented by inhibition halos of crude ethyl acetate extracts (CEAEs) of endophytic fungi isolated from Piper hispidum against Xanthomonas axonopodis pv. phaseoli.*

Treatments and Controls	Concentration (mg/ml)	Inhibition halos* (mm) (mean±standard deviation)
$CEAE_{Lasiodiplodia}$	61.4	10.50 ± 0.75^{b}
$CEAE_{G33\text{-}73}$	19.9	06.08 ± 3.45^{c}
$CEAEA_{Diaporthales}$	50.1	05.25 ± 1.75^{c}
$CEAE_{G53\text{-}83}$	24.0	04.83 ± 1.88^{c}
Distilled water^{c-}	-	00.00 ± 0.00^{d}
Absolute methanol^{c-}	-	00.00 ± 0.00^{d}
Tetracycline^{c+}	61.4	28.08 ± 1.63^{a}
	19.9	25.17 ± 2.10^{a}
	50.1	25.83 ± 0.76^{a}
	24.0	26.25 ± 1.00^{a}

*Means in the same column followed by the same letter are not significantly different at $p<0.05$ according to Tukey test. $^{c-}$ = negative control; $^{c+}$ = positive control

Data published by Nair and Burke (1990) showed that some compounds isolated from *Piper aduncum* and *P. hispidum* have strong antimicrobial activity against *X. campestris* pv. *campestris* and *X. campestris* pv. *carotae*. Firáková et al. (2007) pointed that is questioned whether bioactive compounds of medicinal plants are really produced by plant itself or it is a consequence of the endophyte-plant association, since some endophytes may have developed genetic systems allowing for the transfer of information between themselves and hosts. Considering that a similar property can be found in a medicinal plant and in its endophytes, this current study expected to find new candidates for the biological control of *X. axonopodis* (= *campestris*) pv. *phaseoli*.

As results, cup plate technique allowed to verify the presence of inhibition halos with diameters between 4.83 and 10.50 mm, with best result showed by *L. theobromae* (Table 1). Some inhibition halos are shown in Figure 1.

Researches about *in vitro* tests with compounds produced by fungal endophytes as candidates for the biological control of plant pathogenic microorganisms have been published. Xu et al. (2008) tested the antibacterial activity of extracts produced by endophytic fungi isolated from rhizomes of Chinese medicinal herb *Dioscorea zingiberensis*. The authors obtained positive results against *Xanthomonas vesicatoria*, with minimal inhibitory concentration varying between 0.0625 to 2.00 mg/ml^{-1} for extracts produced by *Acremonium* sp., *Fusarium* sp., *Fusarium oxysporum*, *Penicillum* sp. and two other non-identified endophytes.

Hormazabal and Piontelli (2009) tested the extracts produced by endophytic fungi from Chilean gymnosperms against the phytopathogenic bacterium *Erwinia carotovora*. Data revealed that when 100 µg extract/disk were used, the inhibition of *E. carotovora* varied between 12% and 64%, with best result observed for the extract produced by the endophytic fungi *Penicillium janczewskii*. Positive results are also observed for isolates from genera *Acremonium*, *Alternaria*, *Aureobasidium*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Malbranchea*, *Microsphaeropsis*, *Stegosnosporium*, *Triblidiopycnis* and other *Penicillium* isolates.

Promising results for biological control were observed when Devaraju and Satish (2011) tested the antimicrobial activity of non-identified secondary metabolites produced by endophytic

Fusarium sp. isolated from the stems of *Mirabilis jalapa* against three *Xanthomonas* species. The most expressive diameters of inhibition halos were 17.33, 14.00 and 20.66 mm for *X. campestris* pv. *vesicatoria*, *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *malvacearum*, respectively.

Using a similar methodology to the presented herein, Specian et al. (2012) observed that the CEAE produced by *Diaporthe helianti* isolated from *Luehea divaricata* generated inhibition halos with 7.7 mm against *X. axonopodis* pv. *phaseoli*. Although the authors pointed that human pathogenic bacteria (*Enterococcus hirae*, *Escherichia coli*, *Micrococcus luteus*, *Salmonella typhi* and *Staphylococcus aureus*) were also inhibited by some fractions of the crude extract, including the compound tyrosol, it was not possible to identify the fraction responsible for the inhibition of the phytopathogenic bacterium.

Recently, a study conducted by Flores et al. (2013) showed that fractions of the secondary metabolite, including 3-nitropropionic acid, from *Phomopsis* (= *Diaporthe*) *longicolla*, associated with *Trichilia elegans*, produced halos varying from 0.33 to 2.33 mm against the same species of *X. axonopodis pv. phaseoli*. The same authors also related the inhibition of phytopathogenic fungi (*Guignardia citricarpa*, *Moniliophthora perniciosa* and *Fusarium* sp.) and human pathogenic bacteria (*S. typhi* and *M. luteus*).

Herein, ANOVA emphasized the result obtained for CEAE produced by *L. theobromae*, with 10.50 mm inhibition halos. This data corroborated with the previous study regarding antibacterial action of these four same extracts (Orlandelli et al. 2012b), where $\text{CEAE}_{\textit{Lasiodiplodia}}$ was also more effective against the most of human pathogenic bacteria tested (*E. hirae*, *E. coli*, *M. luteus* and *S. aureus*). The ascomycete *L. theobromae* represents the asexual (= anamorphic) state of *Botryosphaeria rhodina* (Mohali et al. 2005), an important plant pathogenic fungus for both tropical and subtropical regions, causing leaf spots, necrosis, gummosis and even the death of many plants (Encinas 1996; Encinas and Ahmad 1999). However, many studies have shown their endophytic association with the host plant (Cardoso et al. 2009; Hanada et al. 2010; Linnakoski et al. 2012; Mohali et al. 2005; Rubini et al. 2005; Slippers and Wingfield 2007).

Considering that the selection of effective antagonists is the first step in biological control (Kamalakannan et al. 2004), this current study presented an important information about potential candidates for future investigations regarding *in vivo* biological control of *X. axonopodis* pv. *phaseoli*. The choice of endophytes from a medicinal plant (*P. hispidum*) for the screening of potential candidates for the biological control of common bacterial blight of bean is based on the search of genetic variants (different strains of microorganisms) with the capacity of antagonize phytopathogens.

Owing to the fact that *X. axonopodis* pv. *phaseoli* causes disease on bean crops, it is possible that endophytes which naturally colonize *P. vulgaris* are not effective in the competition and inhibition of this pathogen inside host plant. Thus, the search of antagonists isolated from other plants is important. *P. hispidum* is described as producer of compounds biologically active against *Xanthomonas* genus (Nair and Burke 1990); therefore, the results presented herein suggest that the ability of inhibiting *Xanthomonas* strains is also related with some *P. hispidum* endophytes.

Similarly, the search of variants with antagonist potential was conducted by Kumar and Kaushik (2013), showing that endophytic fungi from *Jatropha curcas* (a perennial plant with antimicrobial and pesticidal activity, also extensively studied due to its potential as biofuel) can be potential candidates for helping a variety of plants in protecting against pathogens, since

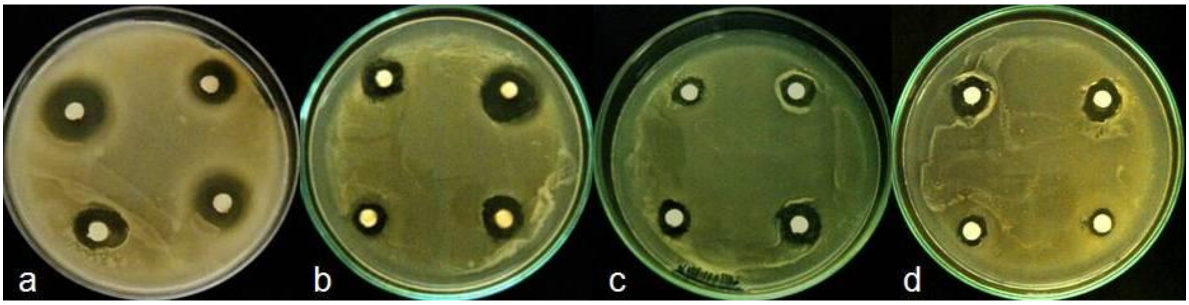

Figure 1. Inhibition halos generated by crude ethyl acetate extracts (CEAEs) produced by *Piper hispidum* endophytes a) *Lasiodiplodia theobromae* against *Xanthomonas axonopodis* pv. *phaseoli*: a) $CEAE_{Lasiodiplodia}$, b) $CEAE_{G33-73}$, c) $CEAE_{Diaporthales}$ and d) $CEAE_{G53-83}$

these endophytes are active against *F. oxysporum*, *S. sclerotiorum* and *Rhizoctonia solani*, causal agents of losses in important food crops such as rice, maize, wheat, and chickpea.

Conclusion

The results obtained suggest that CEAEs produced by some fungal endophytes from *P. hispidum* can be candidates for the biological control of the phytopathogenic bacterium *X. axonopodis* pv. *phaseoli*. Data indicated a best result for *L. theobromae*, but positive results were observed for other extracts tested, including the one produced by an endophyte from Diaporthales order. Regarding the fact that the control of this phytopathogen using chemical products is not completely successful, studies as the presented herein are important in order to demonstrate new possible antagonists for the control of this bacterium.

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