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Assessment of *Phaseolus vulgaris* L and *Vigna unguiculata* (L.) Walp leaves for antifungal metabolites against two bean fungal pathogens *Colletotricum lindemuthianum* and *Phaeoisariopsis griseola*

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The antifungal potential of hydro-ethanolic leaf extracts of bean varieties was analyzed by bioautography, assessing the contribution of defense molecules of proteic nature against two bean fungal pathogens *Colletotricum lindemuthianum* and *Phaeoisariopsis griseola*, also, the Rf values and relative activities of separated compounds were determined; these compounds were in a range of medium polarity to high polarity. The bioactivity expressed by the studied bean varieties could be correlated with the presence of proteic nature compounds, in joint action with secondary plant metabolites. There was some similarity in the chemical composition of the components of the extracts. The varieties G18350, G14241, G1320 CIAT and *Vigna ungiculata* were the most promising for isolating antifungal compounds. The results demonstrate the value of bioautography as a simple and cheap method to examine plant extracts with antifungal activity.

Key words: *Phaseolus vulgaris L*, *Vigna unguiculata* (L.) Walp, bioautography, peptides, secondary metabolites, *Colletotricum lindemuthianum, Phaeoisariopsis griseola,* antifungal defense.

INTRODUCTION

Beans are the most important grain legumes for direct human consumption in the world. Bean represents one third of the total world production of pulses (19.3 Mt/year; FAOSTAT, 2007) and the total production exceeds 23 million metric tonnes (MT) of which 7 million MT are produced in Latin America and Central Africa where it is a staple food for many people due to its energy, protein, dietary fiber and minerals content (Haytowitz et al., 1981; Norton et al., 1985; Broughton et al., 2003; Bitocchi et al., 2012).

One of the relevant factors associated with significant poor yield in most areas of bean production is phytopathogen attack (Santoyo et al., 2010). More than 200 species of phytopathogens that cause plant disease

have been reported, nonetheless only some of them cause considerable economic losses (Venegas, 2002). Despite the environmental implications associated with the excessive use, chemical fungicides remain the first line of defense against fungal pathogens, this problem involves many researchers to seek viable, safe and effective alternatives in controlling pests and diseases affecting crop plants of commercial interest, which has led to the introduction of improved varieties. To produce these varieties, the investigation has been focused in searching natural compounds with biological activity.

From this perspective, legumes have been extensively studied phytochemically finding out several types of compounds such as alkaloids, non-protein amino acids, amines, flavonoids, isoflavonoids, coumarins, phenylpropanoids, anthraquinones, terpenes, cyanogenic glycolsides, protease inhibitors, chitinases and lectins (Carlini and Grossi-de-Sa', 2002; Wink and Mohamed, 2003). Other researchers have detected proteins that protect legumes from pathogens and predators, such as arcelin, α-amylase inhibitor, vicilins, trypsin inhibitor, canatoxin, soybean cystatins, chymotrypsin inhibitors, lysozymes, ribosome inactivating proteins, antifungal proteins, and small cysteine-rich proteins, including plant defensins (Chen et al., 2002; Ye and Ng, 2003; Wang et al., 2005, 2007, 2012; Wong et al., 2012).

Among the species of the genus *Phaseolus*, there are representative phytoalexins like isoflavones, isoflavans, pterocarpans (phaseollin), coumestans and isoflavanones (kievitone), substances that are produced as a consequence of microorganism attacks (Mazid et al., 2011), therefore are considered as one of the most important defensive mechanisms in plants, considering that, Durango et al. (2002) demonstrated a link between phytoalexin accumulation and resistance/susceptibility to pathogenic microorganisms. The identification of high levels of arcelin in the seeds of the bean line called RAZ-2 shows *Phaseolus* as a potential source of defensins that could be explored for different applications (Pusztai et al., 1993; Montoya et al., 2010).

Although there have been a number of investigations related to the bean, little is known about the preventive management of pathogens. Considering that there have been scarce reports about biological activity in the leaves of beans, and that the studies have been done mainly in seeds, this study sought to evaluate the antifungal potential of hidro-ethanolic extracts of bean leaves; assessing the contribution of defense molecules of proteic nature and understanding the immune system of plants would allow farmers to create better yielding crop plants.

MATERIALS AND METHODS

Plant material

Phaseolus vulgaris seeds varieties G5734; G5747; G18350; G5694; G5732; G51094; G14241; G1320; G2233; G5038; G6030; G22164, G2333 and G10474 were provided by the Germplasm Bank of the International Center for Tropical Agriculture (CIAT). These varieties have been previously described as resistant to Angular leaf spot, Anthracnose and Rust diseases (Wahome et al., 2011). Cowpea bean was supply by local farmers.

Microorganisms

Colletotricum lindemuthianum (Cl 600 andean, Cl 242 Mesoamerican) and *Phaeoisariopsis griseola* (Pg 286 andean, Pg 305 Mesoamerican) fungi, were provided by the CIAT, these fungi were recovered by transferring them into a Petri dish (10 cm of diameter) with 20 ml of culture medium potato dextrose agar (PDA) for *C. lindemuthianum* and V8 agar for *P. griseola*, (de Oliveira et al., 2011) leading to incubation at 20 and 24°C, respectively.

Propagation of the seeds

There was a planting of all the varieties keeping a permanent control of growth. The spread of seeds was conducted on a greenhouse separating the material to avoid unwanted crossing between them (Tinivella et al., 2009). Eight days after planting, the number of germinated seeds was counted and fifteen days after, it was established which of them had continued to mature, thereby obtaining the percentages of germination and seed maturation. Analyses were performed in duplicate.

Considering the germination, maturation and plant resistance to the pathogenicity test, the best seeds (Cowpea, *Phaseolus vulgaris* varieties G18350, G5732, G5747, G51094, G14241, G10474, G22164 and G2333) were propagated for obtain sufficient material, this propagation was developed under field conditions, separating each cultivar. Only varieties that developed successfully were used in subsequent trials.

Pathogenicity test

When the plants were around one month old the pathogenicity test with *C. lindemuthianum* and *Phaeoisariopsis griseola* was performed (Bugeme et al., 2009). For this assay, leaves of healthy plants were collected; each one of them was disinfected with sodium hypochlorite (2%) for 2 min, and washed with sterile distilled water. At the same time circles of absorbent paper were cut, they were moistened with sterile distilled water and placed in the bottom of each Petri dish, together with the leaves who were then inoculated with the sprinkling of a spore suspension (2.4×10^5CFU/mL). The Petri dishes were sealed and incubated at 28 ±

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Abbreviations: MTT, Methyl thiazolyl tetrazolium; **C. l,** *Colletotricum lindemuthianum;* ***P. g,*** *Phaeoisariopsis griseola;* **PDA,** potato dextrose agar; **CIAT,** International Center for Tropical Agriculture.

Table 1. Germination and maturing rates of bean varieties with resistance to pathogens.

Varieties	Resistance to	Average germination rate (%)	Average maturing rate (%)
G5734	*U. phaseoli*	90	100
G5747	*U. phaseoli*	80	75
G18350	*U. phaseoli*	100	100
G5732	*U. phaseoli*	60	100
G5694	*P. griseola*	30	100
G51094	*P. griseola*	90	100
G14241	*P. griseola*	90	88.8
G10474	*P. griseola*	100	100
G1320	*C. lindemuthianum*	60	100
G2233	*C. lindemuthianum*	80	100
G5038	*C. lindemuthianum*	40	25
G6030	*C. lindemuthianum*	70	57.14
G22164	*C. lindemuthianum*	70	100
G2333	*C. lindemuthianum*	100	100
Cowpea	*nd*	100	100

nd: not determinate. The coefficient of variation were minor or equal to 10%

2°C in the dark, throughout the incubation period. Each test was performed in duplicate.

Metabolite extraction of beans leafs

Leaves of the *Phaseolus vulgaris* varieties G18350, G14241, G1320 and cowpea were exposed to cleaning treatment (sodium hypochlorite 2%), and each g of left in contact with the mixture extractor ethanol/water 2:5 used 10 mL of the extraction mixture submitting to mechanical agitation

Bioautography

Chromatograms were performed on Thin Layer Cromatography (TLC) plate silica gel 60 F_{254} (Merck 2 × 6 cm), each plate was loaded with 5 µl of each extracts. The solvent systems dichloromethane-methanol (8:2) and water-propanol (5:5) gave good results for the fraction of secondary metabolites and peptides, therefore, were used to further study. All chromatograms were developed from the extracts under the same working conditions; a group of them was used to perform the bioautography, while the others served as controls for the identification of active compounds after application of the revealing agents: vanillin, Liebermann-Burchard and Shinoda test for secondary metabolites and ninhydrin/butanol/acetic acid for peptides.

Once obtained, the chromatography plates were dried to completely remove the solvent. Each chromatogram was placed on a Petri dish and inoculated culture prepared with tetracycline (0.1%) and the fungi object of study, at a concentration of 2.2 x 10^5 UFC/ml, was sprayed over all the plate; then *Colletotricum lindemuthianum* were incubated at 20°C for and 24°C and *Phaeoisariopsis griseola*, both for 24 h time after which the aqueous solution of methylthiazolyltetrazolium (MTT) was sprayed to detect the dehydrogenase activity. The inhibition was evaluated for 24 or 48 h after spraying with MTT (Schmourlo et al., 2005). This test was run in triplicate.

Proteins and secondary metabolites determination

The presence of peptide bonds was verified by the biuret test (Gornall et al., 1949). The detection of primary and secondary amines was performed by Ninhydrin test (Kaiser et al., 1980). Other tests were the Shinoda test (Mallikharjuna et al., 2007), and the Liebermann-Burchard reaction (Coelho and Alves, 1946) obtaining positive results for flavonoids and steroids, respectively. These assays were realized for observation if there were secondary metabolites that could be involved in the antifungal defense of the plant.

The protein content was determined from the extracts by the Lowry method, with bovine serum albumin as the standard, following the methodology proposed by Rutten et al. (1987). This method is consists of the complex made by the phenolic group of tyrosine and trytophan residues (amino acid) in a protein with Folin-Ciocalteau; this method is sensitive down to about 10 µg/ml (Sreekumar, 2010).

Data analysis

For those data related for the germination rate, including the maturity of different varieties, the coefficient data of variation and standard deviation for each of the measurements were made with the zones of inhibition measured in the autobiogram report.

RESULTS AND DISCUSSION

Performance and endurance of the seeds

Table 1 showed the germination and maturation rates of resistant bean varieties. The values evidence that the varieties resistant to *P. griseola* showed better germination reaching maturity as well. However, the Cowpea bean was the most remarkable, not only for its high germination and maturity (Gómez, 2011) but also because of its sturdiness comparing to other varieties that showed chlorosis and their stems were thin.

These results could be explained due to the wide adaptation of cowpea, which is an important grain legume

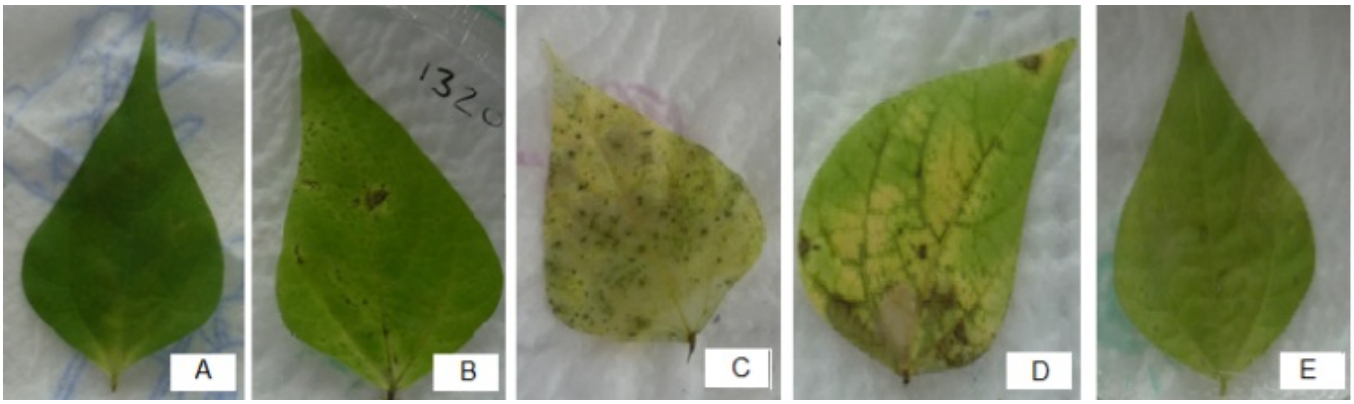


Figure 1. Pathogenicity test performed with *C. lindemuthianum.* A: blank; B: *P. vulgaris* variety G1320; C: *P. vulgaris* variety G18350; D: *P. vulgaris* variety G14241; E: cowpea.

throughout the tropics and subtropics, covering Asia, Africa, and Central and South America, as well as parts of Southern Europe and the United States (Maredia and Raitzer, 2006). The crop has considerable adaptation to high temperatures and drought, it is also tolerant of low fertility, due to its high rates of nitrogen fixation, effective symbiosis with mycorrhizae and its ability to withstand acid and alkaline soil conditions, at the same time it is shade-tolerant (Ehlers and Hall, 1997). In all cases the error rate associated with germination and mature rats did not exceed 10%.

Compared to cowpea, the varieties provided by the CIAT are not known to have any resistance to biotic or abiotic stresses; even some of them came from areas that present quite different climatological characteristics from Colombia, which explains the reason why these seeds were not adapted to neither greenhouse nor field conditions in Ibagué city.

The varieties G18350, G14241, G1320 and cowpea, showed the highest production, best morphological characteristics, great resistance to pests and developed more foliage, reasons why they were chosen for continuing the search for antimicrobial activity of the peptides. It is also worth mentioning that each of them has resistance to a particular fungus.

Pathogenicity test

In this assay, the varieties resistant to *U. phaseoli* were the ones who showed further deterioration, being strongly affected when evaluated against *C. lindemuthianum*. Still, as it was expected, the varieties described by the CIAT as those resistant to *C. lindemuthianum*, although were affected by the fungi, showed less damage compared to other varieties. In spite of this, cowpea showed the highest resistant to the microorganism attack even if it is compared to the resistant varieties provided by the CIAT. This could be explained by the presence of defensins, such as ɣ-thioninas present in cowpea seed involved in antibacterial and antifungal activities (Carvalho et al., 2001; Franco et al., 2006). Figure 1 exhibits the results of pathogenicity test performed with C*. lindemuthianum* only for the chosen varieties.

Figure 1 shows pathogenicity test performed with C*. lindemuthianum.* A: blank; B: G1320; C: G18350; D: G14241; E: Cowpea.

The Figure 2 shows the pathogenicity test performed with *P. griseola* for the elected varieties. The variety G18350 showed the highest resistance of all, although it was not expected to have that property, opposed to this variety G14241 which was considered having resistance to this fungus, was very affected. Once again, cowpea exhibited high resistance to the fungi.

Figure 2 shoes pathogenicity test performed with *P. griseola.* A: blank; B: G1320; C: G18350; D: G14241; E: Cowpea.

Protein determination

The protein content in leaves was evaluated in the varieties that showed increased resistance to field and laboratory conditions, even if the bioactivity was evident on single fungal specie. The results obtained allow to sort the varieties according to their protein content as follows: G18350 (4.16 ± 0.25 mg/g) > G1320 (2.41 ± 0.11 mg/g) > G14241 (2.29 ± 0.05 mg/g) and cowpea (1.75 ± 0.09 mg/g).

It is notorious that although the G18350 variety expressed the highest protein content its bioactivity was only against *P. griseola*, whereas Cowpea revealed the lower content of this metabolite, having resistance to both of the fungal species tested, this means that the bioactivity could be not only the result of the protein activity but also of secondary metabolites contained in these bean species, such as flavonoids and anthocyanins that are related to protection from attack by

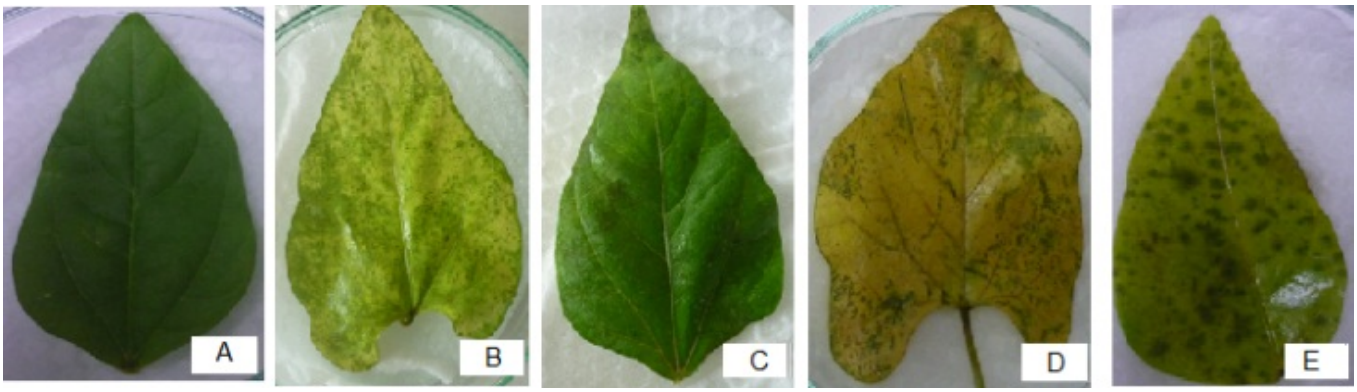


Figure 2. Pathogenicity test performed with *P. griseola.* A: blank; B: *P. vulgaris* variety G1320; C: *P. vulgaris* variety G18350; D: *P. vulgaris* variety G14241; E: cowpea.

pathogens and insects that have been reported in previous studies (Lattanzio et al., 2000; Makoi et al., 2010).

The antimicrobial activity of plant peptides from bean species and others plants has been widely demonstrated (Lipkin et al., 2005; Loeza et al., 2008; Wu et al., 2011; Chan et al., 2012), in proteins the bioactivity has been detected mostly in seeds, as evidenced by the work of Chen et al., (2002), Louis et al. (2007) and Wua et al. (2011). However, thus far, few studies have been interested in finding this functionality in leaves, making this work one of the first developed for this purpose; the interest increases if it is considered that this research was focused on the search of proteins in leaves of several bean varieties, one of the most widely consumed legume in developing countries.

Bioautography results

Bioautography was used to separate the antifungal compounds to obtain more information on the diversity of antifungal compounds present in hidro-ethanolic extracts of different bean varieties. Inhibition zones were observed as white spots on a purple-blue background, indicating where reduction of MTT to the coloured formazan did not take place due to the presence of compounds inhibiting the growth of tested fungi.

In some cases organisms did not grow too well, being difficult to detect inhibition zones. The most likely available explanation is that the growth of *P. griseola* and *C. lidemuthianum* at laboratory conditions in Ibagué city is very slow. In other cases there were growth, but apparently there was no inhibition, these could be explained by the disruption of synergism between active constituents caused by TLC. In spite of the differences observed in the zones of inhibition and the high values of diversions standard obtained, it is of highlight that the results were consisting of the different replies and in all the cases they presented zones of inhibition that were associated with a value of certain Rf.

As shown in the Table 2, in all cases, the four extracts developed in dichloromethane-methanol presented metabolites with Rf values of 0.1; 0.24 ± 0.005; 0.67 ± 0.01 and 0.82 ± 0.016, showing antifungal compounds that were very active against *P. griseola* on a range from 0.67 ± 0.01 to the solvent front.

On the other hand, the metabolites present on the bioautography test against *C. lindemuthianum* did not exhibit a similar pattern among varieties, presenting inhibition zones with Rf values of 0.56 (G1320); 0.92 (G14241) and 0.84 (cowpea). In this case G18350 did not evidence any antifungal activity, which is consistent with the results revealed by this variety on the pathogenicity test against *C. lindemuthianum,* where it was the most affected variety. Figure 3 shows the bioautograms of the secondary metabolites against *P. griseola* and *C. lindemuthianum*.

Table 2 shows antifungal activity of hidro-ethanolic leaf extracts of bean varieties by direct bioautography.
C. l = *C. lindemuthianum,* P. g = *P.griseola*

Figure 3 shows bioautography of bean varieties extracted with water-ethanol separated by dichloromethane-methanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

In what is related to the bioautography test developed in water: propanol for the separation of peptide compounds, it was observed that all the extracts presented Rf values of 0.55 ± 0.01; 0.65 ± 0.02; 0.81 ± 0.05 and 0.91 ± 0.01, the last one was not observed in G18350. All the varieties were active against *P. griseola* on a range from 0.66 ± 0,005 to the solvent front, except for G14241; this is in line with the pathogenicity test where this variety was highly affected.

It is interesting to note that all the varieties presented different patterns against *C. lindemuthianum*, displaying

Table 2. Antifungal activity of hidro-ethanolic leaf extracts of bean varieties by direct bioautography.

Parameter	Rf value	G1320		Rf value	G18350		Rf value	G14241		Rf value	COWPEA	
		X̄ +/- ð Diameter of inhibition zone (mm)			X̄ +/- ð Diameter of inhibition zone (mm)			X̄ +/- ð Diameter of inhibition zone (mm)			X̄ +/- ð Diameter of inhibition zone (mm)	
		C. l	P. g		C. l	P. g		C. l	P. g		C. l	P. g
	0.08			0.04			0.04			0.1		
	0.1			0.1			0.1			0.24		
	0.24			0.14			0.18			0.34		
	0.28			0.26			0.26			0.68		20 +/- 5.3
	0.4			0.4			0.4			0.74		
Dichloromethane-methanol	0.56	25 +/- 7.1		0.5			0.42			0.84	10 +/- 3.4	
	0.68		16 +/- 4.6	0.6			0.52					
	0.76			0.66		16 +/-3.6	0.6					
	0.82			0.72			0.64		18 +/- 6.1			
	0.9			0.76			0.68					
				0.82			0.8					
				0.88			0.92	5 +/- 1.3				
	0.54	0.5		0.56			0.52			0.36		
	0.6			0.6			0.56			0.56	20 +/- 5.8	21 +/- 4.9
Water-propanol	0.66		17 +/- 5.1	0.66	15+/- 4.3	17+/- 5.4	0.63			0.68		
	0.74			0.88			0.8	13 +/- 4.2		0.76		
	0.8	10 +/- 2.8					0.92			0.92		
	0.9											

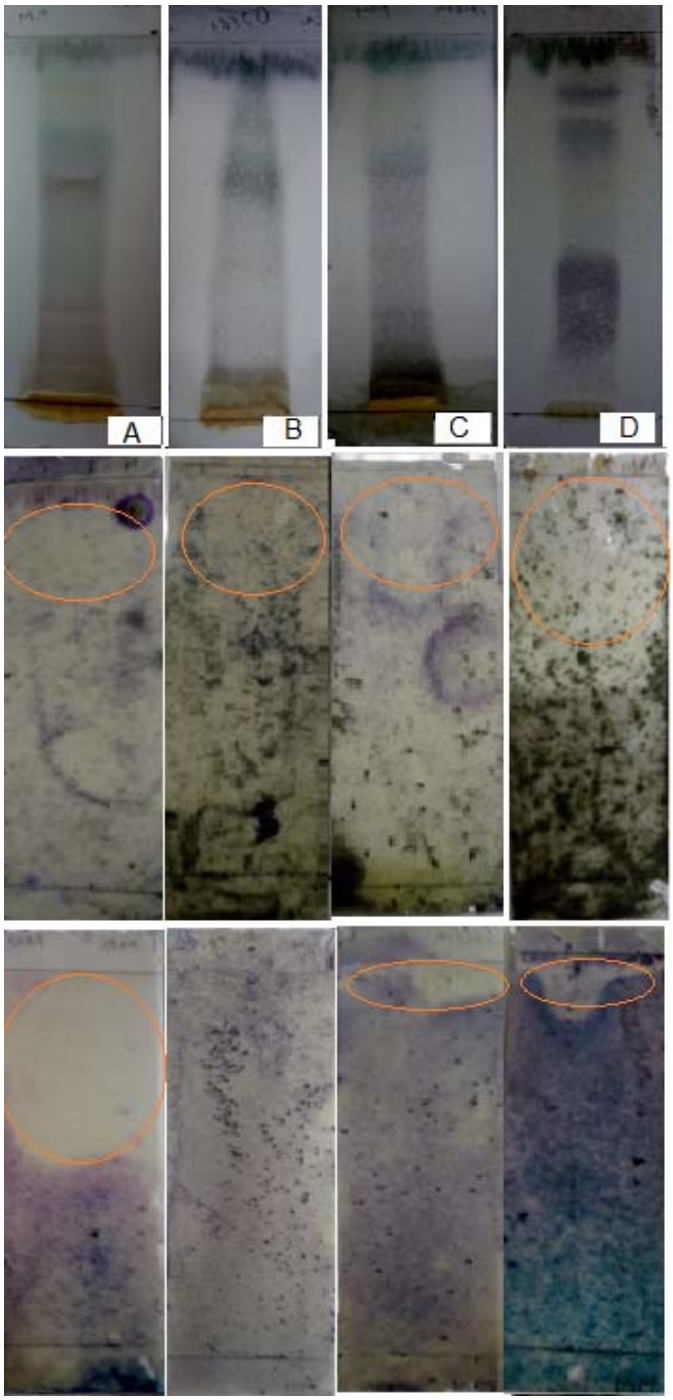
C.l = *C. lindemuthianum,* P.g = *P.griseola*

inhibition zones with Rf values of 0.66 (G18350) 0.8 (G14241) and 0.56 (cowpea). However, unlike the others, G1320 showed three inhibition zones (Rf values: 0.1; 0.54 and 0.8); of them, the lowest (0.1) did not show the characteristic coloration of peptides revealed with ninhydrin, although it showed inhibition against *C. lindemuthianum* (Figure 4), indicating that it is a non-protein character compound. The high inhibition on this variety could be related to the results revealed on the pathogenicity test against *C. lindemuthianum* (Figure 1), where it did not show much damage. Figure 4 shows the bioautograms of the peptides against *P. griseola* and *C. lindemuthianum*.

Another important observation is that Rf values of 0.66 in G18350 and 0.56 in cowpea presented antifungal compounds with bioactivity in both fungal species.

Figure 4 shows bioautography of bean varieties extracted with water-ethanol separated by water:propanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

Some researchers (Hancock and Chapple, 1999; Reuter et al., 2009) argue that the fractions of a positively charged peptide, bind to the negative portion of the bacterial cell membrane (phosphatidylglycerol and cardiolipin,



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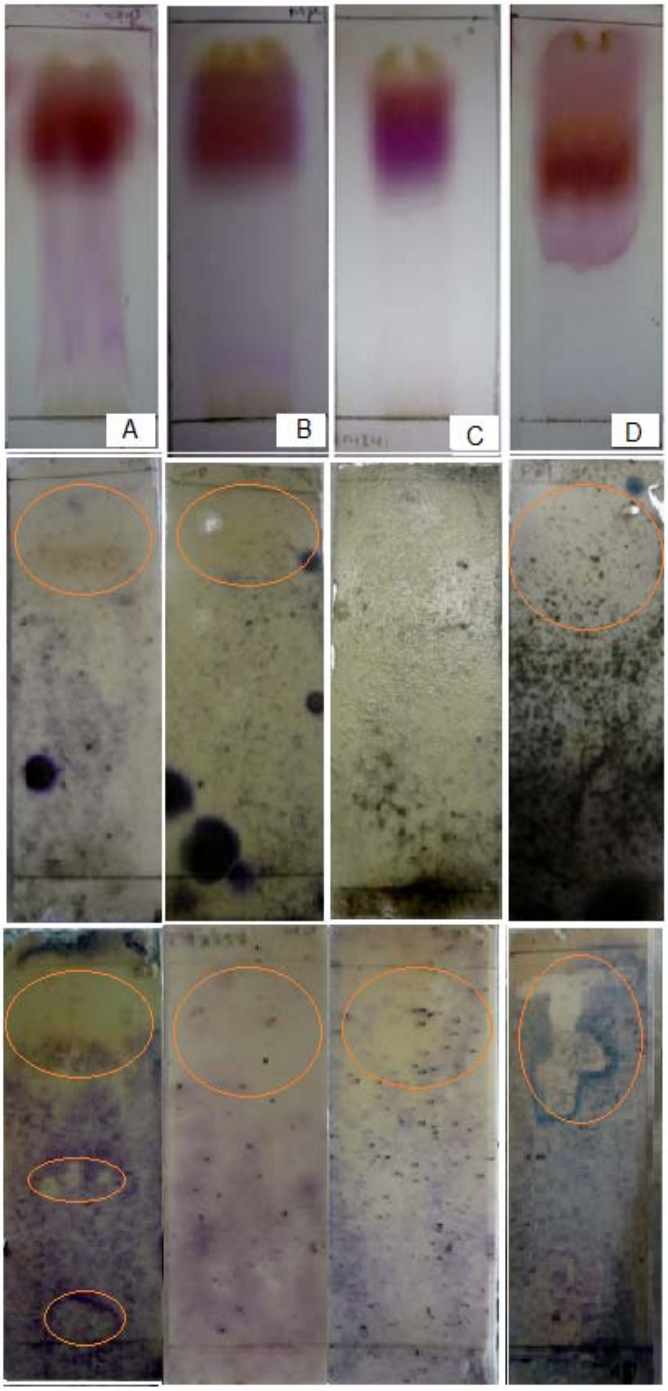

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among others) causing pores resulting in the death of the microorganism. A similar mechanism may also occur in case of fungi inclining to induced hyperbranching of fungal hyphae, plasma membrane permeabilization

and induction of apoptosis (Thevissen et al., 2003; Sagaram et al., 2011; Katrijin et al., 2011; Wilmes et al., 2011).

In general, the low activity of the samples using bioautography assay could be explained by a weak selectivity of the extract components against the microorganisms chosen for this study, or by the very low concentration of the active compounds in the crude extract under the tested conditions. The results obtained in the bioautography test suggested that the bioactive compounds of the varieties studied are in a range of medium polarity (dichloromethane 8: methanol 2) to high polarity (water:propanol).

Although the bioautography assay has not been of wide application in our country, this methodology has certain advantages when compared with other trials to test the antimicrobial activity of extracts or compounds of plant origin, such as, direct isolation of active constituents in complex mixtures using small amounts of material, greater sensitivity, and it can provide useful information about the nature of the active compounds.

Conclusions

The bioactivity expressed by the studied bean varieties could be correlated with the presence of protein nature compounds, perhaps in joint action with secondary plant metabolites, resulting in the varieties: G1320, G14241, G18350 and cowpea, as the most promising interest in the search for bioactive compounds, all of which would constitute a scientific basis for a better understanding of the bean immune system, allowing inroads to improving varieties of study.

This study is constituted as one of the few developed in the country, using the bioautography as a method for the determination of bioactive compounds in plants, this being a simple and economic technique that saves time and requires no sophisticated equipment. This work, besides being pioneer in the search for bioactive protein compounds in bean leaves the results obtained through it may contribute to decrease the difficulties present in the screening of antifungal compounds from plants.

Conflict of interests

The author(s) have not declared any conflict of interests.

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