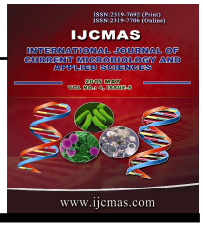
Int.J.Curr.Microbiol.App.Sci (2015) 4(5): 596-606

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 596-606**
http://www.ijcmas.com



Original Research Article

In-vitro Fungitoxic Effect of Cold and Hot Water Extracts of Three *Ocimum* Species on Fungi Causing Common Bean (*Phaseolus vulgaris* L.) Root Rot in Cameroon

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Keywords

Common bean root rot, *F. solani*, Plant extracts, Antifungal

A B S T R A C T

In this study the effects of leaves extracts of *Ocimum basilicum* (*O. basilicum*), *Ocimum gratissimum* (*O. gratissimum*) and *Ocimum canum* (*O. canum*) on spore germination and mycelial growth of the most frequently found fungal pathogen that causes common bean root rot were investigated. The leaves cold and hot water extracts were tested using the "poisoned food technique". *Fusarium solani* f. sp. *Phaseoli* (*F. solani*) was the most isolated fungus. Most of the extracts significantly inhibited its growth at tested concentrations. The cold water extract of *O. basilicum* exhibited good anti-fungal activity (100% mycelial growth inhibition at a concentration of 20%) and conidia germination at 10%. For the hot water extracts, *O. canum*s howed the best effect on inhibiting mycelial growth (100% at a concentration of 20%) while *O. basilicum* and *O. gratissimum* performed best with regards to conidia germination, with 65,4% and 69,3% reduction respectively at 20% concentration. None of the hot water extracts of the spices succeeded in achieving 100% inhibition of the pathogen conidia germination. The cold water extracts were generally more effective than the hot water extracts in controlling the pathogens and might be further investigated as natural alternatives to synthetic fungicides for the control of the root rot disease of common bean.

Introduction

Root diseases of beans (*Phaseolus vulgaris* L.) are widespread and often cause significant yield losses, thus reducing profitability of dry bean production

worldwide. Fungi are the most frequent pathogenic agents and represent the major threat to this crop since they attack the root parts and destroy the proper functioning of the plant intaking up water and other nutrients (Abawi and Pastor-Corrless, 1990). The diseases can be caused by a single soil-borne pathogen or by a combination of several pathogens, resulting in disease complexes (Abawi and Pastor-Corrless, 1990; Ul-Haq *et al.,* 2012). *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *phaseoli*, *F. solani* f. sp. *phaseoli*, *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Meloidogyne* spp. are among the major pathogens known to impact bean production in many countries in Latin America and Africa (Abawi and Pastor-Corrless, 1990; Ul-Haq *et al.,* 2011). However, little is known about the disease and its related fungi in Cameroon.

The above-ground symptoms of the disease include poor emergence and stand establishment, damping off, unthrifty growth, chlorosis (especially of lowerleaves), wilting, premature defoliation and lower yield. Symptoms on roots and lower stem tissues are variable depending on the pathogen (s) involved (Abawi and Pastor-Corrless, 1990). The possible involvement of several soil-borne pathogens with different mechanisms of pathogenicity has made it difficult to develop effective disease management strategies.

However, antimicrobial chemicals such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are often successfully used in the control of plant diseases in agriculture (Ashaand Tiwari, 2009). Besides, the worldwide trend towards environmentally safe methods of plant disease control in sustainable agriculture calls for reducing the use of these synthetic chemicals since they have shown major drawbacks such as their lack of long-term efficacy due to the development of resistance by plant pathogens and the non-target environmental impacts. Therefore during the last two decades there has been a resurgence of interest in biofungicides that are environmentally safe and easily biodegradable (Gnanamanickam, 2002). The investigation of plants containing natural antimicrobial metabolites has thus been identified as a desirable method of disease control (Rai and Carpinella, 2006; Seema *et al.,* 2011; Seint and Masera, 2011; Dwivsdi and Neeta, 2012).

A literature review reveals that many research projects have been carried out regarding the use of plant extracts for controlling plant diseases. Nevertheless, only 4 -10 % of the 250,000 plant species constituting the biodiversity of the world's flora have been examined chemically for antimicrobial activity (Earnsworth, 1990; Pallant, 2010). A huge potential therefore exists in this regard especially in tropical areas like Cameroon where we find a wide range of plant genera, including *Ocimum* sp.

Belonging to the Lamiaceae (Labiatae) plant family, *Ocimum* is a genus of annual and perennial herbs and shrubs found in the tropical and sub-tropical regions of Asia, South America and Africa (Darrah, 1988). The genus comprises 65 species which are currently the subject of phytochemical attention because of their biological and chemical diversity and essential oil production (Paton *et al.,* 1999). Several reports investigating the biological potentials of *Ocimum* species have been restricted to the volatile oil concentrations (Janine *et al.,* 2005; Orafidiya *et al.,* 2006; Tatsadjieu *et al.,* 2003; Ali and Setzerb, 2013; Agbogidi *et al.,* 2014). Therefore, the aim of this study was to (1) identify the fungal species causing common bean root rot in a selected common bean field, to (2) screen for the antifungal activity of cold and

hot water extracts of three *Ocimum* species on the pathogenic agent (s).

Materials and Methods

Isolation and identification of the pathogenic fungus (i)

Bean plants exhibiting typical symptoms of root rot or damping-off were collected from common bean fields in Yaoundé, Centre-Cameroon. Diseased plants were carefully uprooted and rinsed under running tap water for 10 min to remove soil and other debris from the field. Sections were bisected longitudinally and then cut into 0.5 cm pieces. They were then surface-sterilized in 95% ethanol, soaked in 10% Clorox for 5 min, and rinsed 2 to 3 times with sterile distilled water (Mwang'ombe *et al.*, 2007).

The sterilized fragments were dried on a sterile paper towel, placed in Petri dishes containing chloramphenicol (200 mg/l)-amended Potato Dextrose Agar (PDA: 39g Difco PDA per litre) and left under continuous light. Four to five days later, mycelia emanating from plant sections were removed at the growing point and transferred onto fresh chloramphenicol amended PDA until pure cultures were obtained. The resulting isolates were then subcultured on PDA slants and conserved at 4°C.

Isolates were identified as *Fusarium* species based on cultural and morphological characteristics. Each isolate was transferred onto Carnation Leaf Agar (CLA) and antibiotic-amended PDA, and cultures were incubated for 7-20 days at room temperature under fluorescent light.

They were each then examined microscopically and identified to species according to the system of Leslie and Summerell (2006).

Pathogenicity test

To assess the pathogenicity and aggressiveness of a representative isolates, clean colonies of the pathogen were subcultured onto new PDA plates and allowed to grow for up to 21 days. Thereafter, the plates were flooded with sterile water and the mycelia scraped off the PDA media using sterile cover slips into more sterile water. The resulting slurry was then filtered through a double layer of muslin cloth.

The inoculum concentration was adjusted to 2×10^5conidia/ml using a haemocytometer, The resulting inocula were used to inoculate a previously sterilized (121°C; 60 min) soil-sand mix (3:2) (Abawi and Pastor-Corrales, 1990), that was then filled into plastic pots (25-cm diameter) and irrigated every second day for 1 week before sowing. Common bean seeds from a local susceptible line (GLP 190) were surface-sterilized in 2% NaOCl for 5 min and planted in each pot. Soil without inoculum served as control. The trial was replicated three times.

Twenty-eight (28) days after planting, the disease incidence was assessed by counting the plants exhibiting root rot symptoms as compared to the total number of standing plants. The disease severity on the roots and stems was assessed on a 1-9 rating scale (Abawi and Pastor-Corrales, 1990) where: 1 = no visible symptoms; 3 = light discoloration either without necrotic lesions or with approximately 10% of the hypocotyl and root tissues covered with lesions; 5 = approximately 25% of the hypocotyl and root tissues covered with lesions but tissues remain firm with deterioration of the root system; 7 = approximately 50% of the hypocotyl and root tissues covered with lesions combined with considerable softening, rotting and reduction of root

system and; 9 = approximately 75% or more of the hypocotyl and root tissues affected with advanced stages of rotting combined with severe reduction in the root system.

Plant sample collection

The samples of medicinal plants, made up of the above ground part of *O. basilicum, O. gratissimum,* and *O. canum,* were collected from the local area of Messassi, Yaoundé, Cameroon. They were identified and authenticated at the National Herbarium of Cameroon, where voucher specimens are deposited. In the laboratory, the samples were washed under running tap water followed by distilled water to remove dust particles. They were then air-dried (28°C) for 14 days and ground using a grinder.

Cold water extract preparation

The cold water extracts were prepared by weighing out 50 grams of each powdered sample into different conical flasks, adding 500 ml of cold water in each, stirring vigorously with a glass rod and leaving the mixtures for 24 hours. They were then filtered through sterile filter paper (Whatman No 1) into clean conical flasks and the filtrates were dried in a hot air oven for one week. The cold water extracts thus obtained were kept at 4^{o}C until use.

Hot water extract preparation

The hot water extracts were obtained by separately mixing 50g of each ground plant material with 500 ml of distilled water in 2 litre conical flasks. The flasks were then placed in a water bath at 90^{o}C for 1h. Thereafter, the suspensions were filtered 5 times through sterile muslin cloth and dried in a hot air oven. The hot water extracts thus obtained were kept at 4^{o}C until use.

Antifungal activity assays

Mycelial linear growth

The effects of the plant extract son the mycelial growth of *F. solani* were studied by food poison technique (Nene and Thaplyal, 1979). PDA with 2, 4, 8, 10 and 20% concentrations of both cold and hot water extracts were prepared for each test plant; namely, *O. basilicum, O. gratissimum,* and *O. canum*. About 15 ml of the medium were poured into Petri dishes and allowed to solidify. Five (5) mm discs of seven-day old cultures of *F. solani* were placed at the center of the Petri dishes and incubated at 25 ± 2°C for seven days. PDA medium without the extract served as control. For each treatment three replicates were done. After the incubation period, radial colony growth was measured (in mm) and recorded for each treatment. The fungal toxicity of the extracts in terms of % inhibition of mycelial growth was calculated using the following formula:

$$\% \text{ Inhibition} = dc - dt / dc \times 100$$

where dc = average increase in mycelia growth in control,

dt = average increase in mycelial growth in treatment.

Conidia germination assay

The spore germination assay of the tested extracts was performed using the M38-A2 microdilution method (CLSI, 2008) with minor modifications**.** A hundred (100) µL of Potato Dextrose Broth (PDB; Sigma Ltd) were each introduced into 96-well microtiter plates. Then 100 µL of stock solutions of each extract were added separately into the first line of wells. The resulting mixture was homogenised thoroughly before transferring

100µL each into the wells of the second line. Six two-fold serial dilutions of the test samples were done from line A up to line F. Furthermore, 100 µL of *F. solani* conidia (4×10^5 conidia/mL) prepared in PDB were introduced into all the wells containing the test substances except for the columns for the blanks which constituted the sterility controls. The concentrations ranged from 20 to 1.25 %. After 16 hours of incubation (25± 2°C), a loop-full drop was taken from each of the wells and examined microscopically. The percentage inhibition was determined for each concentration as compared to the germination in the control wells.

Statistical analyses

The data collected were analyzed with the Sigmaplot Statistical ANOVA Software (version 11.0). All the tested parameters were submitted to ONE WAY, and differences between means were tested using the Duncan's Multiple Comparison Test at 5% level of significance. The Pearson Correlation was used to study the relationships between tested parameters.

Results and Discussion

Isolation and identification

By the end of the isolation tests for the causal agent of Fusarium root rot from diseased common bean roots, 18 isolates were obtained, excluding fast-growing fungi. Grown on PDA, all the isolates produced aerial (dense and floccose) whitish mycelia. From the reverse of the Petri dishes it could be seen that some isolates produced yellowish pigmentations (Figure 1b) (FS1 and FS4). These colours remained with time while others were mostly cream-white at a young age but changed into purple (FS2) or remained whitish (FS3) with time. The rowth rate of the overall isolates was between 2.5 and 5.0 cm after 4 days. On CLA, all the isolates had slightly sickle-shaped macroconidia, with foot-shaped basal cells and had 3-5 septates moderately curved 27x5.0 µm. Microconidia produced on long monophialids were abundant, oval-shaped and mostly two-celled (Figure 1b). The above characteristics designated the fungus to be *Fusarium solani* f.sp *phaseoli.*

Pathogenicity and aggressiveness

The pathogenic ability of four representative isolates to induce common bean root rot was tested. All the tested fungal isolates were able to provoke root rot at different degrees on a susceptible common bean cultivar. A disease incidence of 100 % was registered from all the isolates, indicating their high infectivity potential (Table 1). The disease severity that shows how severe are the fungus, ranged from 8 to 4.09 according to the scale defined by the International Centre of Tropical Agriculture (CIAT). *Fusarium solani* isolate FS4 provoked a significantly high disease severity (8) while the FS1 isolate was the least severe (4.09). Thus, *Fusarium solani* isolate FS4 (Figure 2), the most aggressive isolate, was selected for antimicrobial assays.

Effect on mycelial growth

The antifungal assay showed that all the tested extracts could significantly reduce the mycelial growth of *F. solani* as compared to negative controls ($P < 0.05$). The Pearson test revealed that the percentage of mycelial growth inhibition of the overall extract depended on the concentration used for treatment. The comparison between the two groups of extracts (hot and cold) in inhibiting the mycelial growth of the fungus showed that it varied with plant species (Table 2).

Hot water extracts: At the lowest concentration (2%), 2.9%, 6.1% and 22.4% mycelial growth inhibitions were obtained from *O. gratissimum, O. basilicum,* and *O. canum* extracts respectively. No growth occurred on the culture media treated with *O. canum* when the concentration reached 20%. Consequently, the most active of the hot water extracts was *O. canum* while the least active one was that from *O. gratissimum.*

Cold water extracts: The mycelia linear growth inhibition seemed to be greater with cold water extracts as compared to hot water extracts at a concentration of 2% though not significantly ($P>0.05$). In fact, 14.6%, 15.9%, and 24.4% mycelial growth inhibitions were registered with *O. canum, O. basilicum* and *O. gratissimum* respectively. The extract from *O. basilicum* totally inhibited the *F. solani* growth at a 20% concentration while at the same concentration, *O. canum* and *O. gratissimum* exhibited 60,9% and 76,5% mycelial growth inhibition respectively. Globally, *O. basilicum* was more active than the others, followed by *O. gratissimum* and lastly *O. canum.*

Effect on spore germination

The antifungal potential of the extracts tested on *F. solani* conidia germination showed that, independent of the extraction mode, the overall extract significantly reduced the germination of the conidia as compared to the distilled water control except for the hot water extract of *O. canum* that had no inhibitory effect. The Duncan's Multiple Comparison Test revealed a generally greater inhibitory effect of cold than hot water extracts that was statistically significant:

Hot water extracts: At concentrations below 5% (2,5 % and 1,25%) no inhibition of conidia germination was observed. However, at the highest concentration (20%), 65.4%, 0 and 69.3% conidia germination inhibition was registered with extracts from *O. basilicum, O. canum* and *O. gratissimum* respectively. This indicated that no total inhibition of spores was observed with this extraction mode. Extracts from *O. basilicum* and *O. gratissimum* were most active.

Cold water extracts: Inhibitions of spore germination of 87.7% ; 63.2% and 0% were recorded at 1,25% concentrations with *O. basilicum*, *O. canum* and *O. gratissimum* respectively. A total inhibition (100 %) was observed in wells treated with 20% extracts of the overall plant species. Generally, *O. basilicum* was the most effective extract but was placed in the same statistical group with *O. canum*. *O. gratissimum* was not as effective as the two others with respect to the conidia inhibitory effect of its cold water extracts.

Plant pathogens are of serious concern to food security since they cause huge damage to economic and food crops. *Fusarium* spp. are well known for their pathogenicity and cause seed abortion, vascular wilt, damping off, die back, stunting and stem, seedling, seed and root rots in a variety of host plants (Ahmad *et al.,* 1994; Sharfun-Nahar *et al.,* 2005; Bashir and Tahira, 2012). In Cameroon, root rot causes serious damage in common bean fields (unpublished data) but up to date no reports focusing either on the related fungi or on the control strategies have been published. Perhaps, synthetic chemicals might successfully control the disease but their application is against the logic of eco-organic agriculture. Hence the exploration of alternative antifungal agents, especially plant extracts, has merits (Ul-Haq *et al.,* 2014). In fact, the use of plant-derived products as disease control agents has been studied, since they tend to have low

mammalian toxicity, fewer environmental effects and there is widespread public acceptance of the development of environment-friendly alternatives to synthetic fungicides for the control of fungal plant diseases (Price, 2000; Aba AlKhail, 2005; Mamdouh and Ewies, 2007; Dwivedi and Neeta, 2012, El Mohamedy *et al.,* 2013; Nana *et al.,* 2015; Muneera farooq *et al.,* 2015). In the present study, we attempted to isolate and identify the pathogenic fungi related to root rot in common bean fields and to assess the antifungal potential of the hot and cold water extracts of three *ocimum* species (*O. basilicum*, *O. canum* and *O. gratissimum*) on its mycelial linear growth and conidia germination. Based on their macroscopic and microscopical features as compared to those provided by the Leslie and Sumarell (2006) monography, the isolated fungi were identified as *F. solani* f.sp *phaseoli.* Four representative isolates, namely FS1, FS2, FS3 and FS4, were tested for their capability to induce the disease pathogenicity under greenhouse conditions after which the FS4 isolate was found to be the most pathogenic, resulting in 100% disease incidence and a display of the highest severity scores (8). The variations in the pathogenicity and morphology of the isolates confirmed the variability among the strains of *F. solani* occurring in Uganda (Tusiime, 2003; Mukankusi, 2008).

In addition, Mukankusi (2008) recognized the common bean (*Phaseolus vulgaris* L.) as the main host of *F. solani* f.sp *phaseoli* on which it causes Fusarium root rot disease. The author also claimed that several strains of *Fusarium solani* f. sp. *phaseoli* that cause Fusarium root rot occur in nature, with some being more pathogenic than others.

For the fungitoxic activity, both types of extracts (hot and cold water) drastically reduced the growth of *F. solani*. However, the effect was found to be variable depending on the concentrations and plant species i.e. higher concentrations caused more inhibition than lower concentrations. The hot water extract of *O. canum* did not show any significant difference in its potency against the inhibition of *F. solani* conidia. Of the two extraction methods, considering both the mycelial growth and the conidia germination inhibition, the cold water extracts were generally more effective in controlling the pathogens. Similarly, Kurucheve *et al.* (1997) tested the cold and hot water extracts of thirteen plant species against *Rhizoctonia solani*, the pathogen of sheath blight of rice and found that the maximum inhibition of mycelial growth was observed with the cold water extract of *Prosopis juliflora*.

Table.1 Pathogenic ability of different *F. solani* isolates to induce *Fusarium* rot disease on common bean seedling under greenhouse conditions

Fusarium isolates	Disease parameters	
	Disease incidence	Disease severity
F. solani (FS1).	100	4,09[b]
F. solani (FS2).	100	5,7[c]
F. solani (FS3).	100	6,2[d]
F. solani (FS4).	100	8[e]
Control	0,00	1[a]

Mean values within columns followed by the same letter are not significantly different (p= 0.05)

Table.2 Effect of cold and hot water extracts from *O. basilicum, O. canum* and *O. gratissimum* on the mycelial growth of *F. solani*

[1]Conc. (%)	Mycelia growth inhibition (%)					
	O. basilicum		*O. canum*		*O. gratissimum*	
	Hot water extract	Cold water	Hot water extract	Cold water extract	Hot water extract	Cold water extract
Control	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]
2	6,1 ± 3,6[b]	15,9 ± 0,9[b]	22,4 ± 0,9[b]	14,6 ± 0,9[b]	2,9 ± 2,7[b]	24,4 ± 0,0[c]
4	15,6 ± 2,2[c]	21,1 ± 0,9[c]	41,6 ± 3,2[c]	16,6 ± 0,0[b]	4,8 ± 5,5[bc]	20,9 ± 0,0[b]
8	36,8 ± 2,7[d]	49,5 ± 2,3[d]	57 ± 7,3[d]	34,5 ± 4,1[c]	6,8 ± 2,7[c]	39,4 ± 0,0[d]
10	47,8 ± 0,0[e]	54,7 ± 5,0[e]	74,2 ± 0,4[e]	28,9 ± [bc]	15,3 ± 0,0[d]	52,7 ± 0,0[e]
20	88,2 ± 1,8[f]	100 ± 0,0[f]	100 ± 0,0[f]	60,9 ± 1,8[d]	23,7 ± 2,7[e]	76,5 ± 3,6[f]

Mean values within columns followed by the same letter are not significantly different (p= 0.05), 1= Concentration

Table.3 Effect of cold and hot water extracts of *O. basilicum, O. canum* and *O. gratissimum* on *F. solani* conidia germinaton

Conc (%)	Inhibition of conidia germination (%)					
	O. basilicum		*O. canum*		*O. gratissimum*	
	Hot water extract	Cold water extract	Hot water extract	Cold water extract	Hot water extract	Cold water extract
Control	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]
1,25	0 ± 0.0[a]	87,7 ± 4,7[b]	0 ± 0.0[a]	63,2 ± 1,9[b]	0 ± 0.0[a]	0 ± 0.0[a]
2,5	0 ± 0.0[a]	90,8 ± 1,4[bc]	0 ± 0.0[a]	81,6 ± 2,1[c]	0 ± 0.0[a]	22,4 ± 2,8[b]
5	23,4 ± 0,9[b]	95,9 ± 1,4[c]	0± 0.0[a]	93,8 ± 4,2[d]	46,9 ± 4,2[b]	41,8 ± 9,8[c]
10	59,2 ± 1,9[c]	100 ± 0.0[d]	0 ± 0.0[a]	100 ± 0.0[e]	47,94± 2,1[b]	66,3 ± 6,3[d]
20	65,4 ± 4,5[d]	100 ± 0.0[d]	0 ± 0.0[a]	100 ± 0.0[e]	69,3 ± 1,4[c]	100 ± 0.0[e]

Mean values within columns followed by the same letter are not significantly different (p= 0.05); 1=Concentration

Fig.1 Macroscopic and microscopic characteristics of *Fusarium solani* f. sp *phaseoli*: a: colony feature on PDA; b: microconidia, macroconidia and chlamydospores on CLA

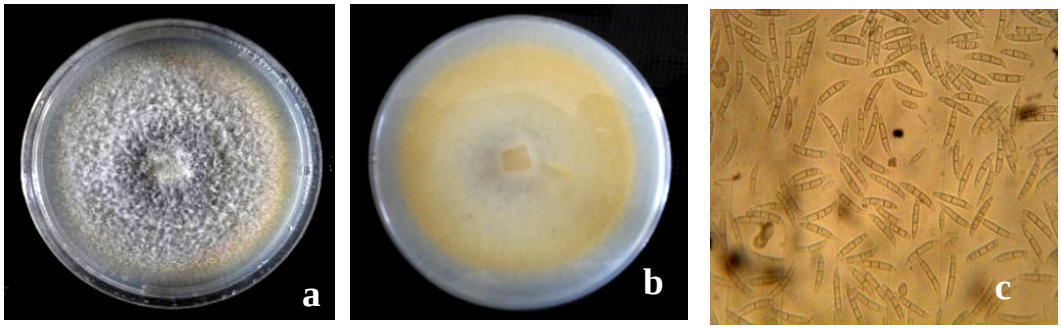

Fig.2 Differential common bean root rot symptoms induced by *F. solani* isolates (FS1 to FS4) on above-ground part (A) and root system (B)

In fact, the active principles present in plants are influenced by many factors which include the age of the plant, the extraction solvent, the time of harvest of the plant materials and the method of extraction (Qasem and Abu-Blan, 1996; Amadioha and Obi, 1999; Okigbo and Ajalie, 2005; Okigbo *et al.*, 2005; Okigbo and Ogbonnaya, 2006). Moreover, the difference observed in fungitoxic activity between the hot and cold water extracts is likely to be due to the thermosensitivity of the active compound(s) when extracted at high temperature or thepresence of inhibitors to the fungitoxic principle as stated by Amadioha (2000) and Okigbo and Ogbonnaya (2006). The present report seems to be the first study evaluating the antifungal efficacy of *Cameroonian ocimum* extracts against *F. solani* that causes common bean root rot in the country. According to our results, the cold water extracts of *O. basilicum* and *O. canum* can be, prior to formulation, used by indigenous farmers as less hazardous and cheaper natural plant products for controlling this disease since the plants are currently used as spices. Furthermore, additional research is required before we can consider the industrial application of these plant extracts, including the evaluation of their efficacy at a larger scale and under field conditions as well as the evaluation of potential health and environmental component risks associated with their application.

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