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Antifungal potential of lemongrass essential oil as affected by plant age and Arbuscular Mycorrizal fungi inoculation.

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Abstract: *Improving plant productivity still a challenge for researchers and administrations because yield losses caused by pathogenic fungi attacks of plant remains. This situation unfortunately highlights several problems, including food security. Phytopathogenic fungi are responsible of a range of diseases on a wide variety of herbaceous plant species. This study was addressed to test the influence of harvest periods alongside with Essential oil (Eo) from Arbuscular Mycorrhiza (AM) fungi lemongrass plant on the growth inhibition, susceptibility rates and enzymes activity on three phytopathogenic fungi in vitro. Poisoned food technique was used to determine inhibition capacity of mycelial growth, minimum inhibitory concentration (MIC), minimum fungicidal concentration, susceptibility rate and enzymes inhibition potential of Eos on the tested pathogens. Lemongrass Eos exhibited mycelial growth inhibition at various rates in regard of the AM fungi status and the harvest period of the plant used for Eos production. Otherwise, the biological activity addressed to Eos was shown to be fungi species dependent, with Cf more susceptible and F35 more resistant. The best MIC values were recorded with Eos from ten months old mycorrhizal plant, ranging as 0.5, 0.33, 0.32 µl/ml for F35, Hel, Cf respectively. Pectinase activity was significantly reduced in pectin base media after 5 days of growth for Hel fungus in the presence of lemongrass Eos. The best activity was recorded with lemongrass Eos obtained with AM plant. Flagrant biocontrol substances from lemongrass plant origin are therefore an alternative to fight against phytopathogenic fungi impacting plant growth. AM fungi appear as a good indicator to improve pharmacological properties of flagrant substances from plant origin and therefore a safe protection of crops for better production.*

Key words*: fungi; lemongrass; essential oil; pectinase; cellulase; mycorrhiza.*

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I. Introduction

Fighting against crops pathogen like mycosis is an important contribution to drop down the growing demand for food security (Strange and Scott, 2005). Food security is a complex item including availability, physical and economical access, and utilization of food. The primary focus of researchers since the last century is to reduce yield losses by improving crops protection from biological and non-biological causes. Despite hard work and strategies addressed that way, yield lost caused by various form of plant attack by fungi still remain a problem. Moreover, post-harvest losses due to fungal infection with possible accumulation of toxins during storage highlight the above issue. Fungi are amongst plant pathogens of major importance, causing diseases on a wide variety of woody and herbaceous plants (Teng and Krupa, 1980; Oerke, 2006; Savary et al., 2012).

Fungus from *Colletotrichum* genera are of great contribution, affecting common and unusual plant pathogenic genera (Gautam, 2014). They are distributed primarily in tropical and subtropical regions, and are responsible for anthracnose of a wide range of host plants worldwide (Sutton, 1992; Hyde et al., 2009). They are responsible of the symptom known as anthracnose, include sunken necrotic lesions on leaves, stems, flowers and fruit, as well as crown and stem rots, seedling blight etc. (Waller et al., 2002; Agrios, 2005). All members of the genus cause major economic losses, especially in fruits, vegetables, and ornamentals. *Colletotrichum falcatum* Went. belong to the genera *Colletotrichum* and is responsible for major economic

losses caused by red rot in sugarcanes. In a country like India, Red rot disease caused by *C. falcatum* is one of the most serious threats to sugarcane cultivation (Beniwal et al., 1989 and Satyavir, 2003).

Fungi from Fusarium genus is ubiquitous, filamentous and commonly found in soils, aerial plant parts, and plant debris (Burgess, 1981; Hue et al., 1999). They are pathogenic fungi causing vascular diseases in plants, such as watermelon, cucumber, tomato, pepper, muskmelon, beans and cotton (Gordon and Martyn, 1997; Gullino et al., 2015; Hashem et al., 2010). Most of these fungi are toxigenic and are able to produce deleterious secondary metabolites mostly in stored grains (Maheshwar et al., 2009). Pathogenic strategies of those fungi include the damage of host plants through the penetration of hyphae into host vascular tissues, secretion of hydrolytic enzymes related to pathogenesis, and cellular apoptosis of host plant cells as the infection progresses due to mycotoxin production (Bacon et al., 1996; Abbas et al., 1997; Pavlovkin et al., 2004). The pathogen has the ability to persist for a very long period in the soil without a host plant (Larena et al., 2003). As earlier stated, Fusarium wilts are caused mainly by *Fusarium oxysporum*. This fungus includes many important pathogenic species which are able to infect different plants causing wilt in crops of economic importance. Among them, *Fusarium oxysporum* f.sp. lycopersici responsible for Fusarium wilt of tomato, significantly contribute to decrease the yields of this plant (Miller et al., 2011).

Previously known as *Bipolaris oryzae* Subr, *Helminthosporium oryzae* is a fungus belonging to the genus Cochliobolus. Fungi from this genus generally produces inter and intra-cellular mycelium, which develops as greyish-brown to dark-brown mat on the infected tissues. In culture media, their mycelium is grey to olive or black in colour (Sunder et al., 2014). Diseases caused by this fungus has been reported in rice growing countries like Japan, China, Burma, Sri Lanka, Bangladesh, Iran, Africa, South America, Russia, North America, Philippines, Saudi Arabia, Australia, Malaya, India and Thailand (Ou, 1985; Khalili et al., 2012). The disease is of great importance in several countries and has been reported to cause enormous losses in grain yield (Ghose et al., 1960; Mew and Gonzales, 2008). The disease occurs especially in environments where water supply is scarce combined with nutritional imbalance, particularly lack of nitrogen (Baranwal et al., 2013).

The major way used; to fight against plant pathogen since decays is the use of synthetic chemicals since it gives appreciable results. However, growing rates of resistant pathogenic microorganisms previously under control are progressively recorded. An alternative is the use of plants derived substances like Eos, which could contribute to solve the problem. Eos are concentrated volatile liquids substances from plant origin, highly diversified in their origin. They were commonly used since early civilizations, first in the East and the Middle East, then in North Africa and Europe (Pawar and Thaker, 2007; Macwan et al., 2016). Eos can represent one of the most promising natural products for fungal inhibition (Hu et al., 2007, Kalemba and Kunicka, 2003). In fact, many kinds of Eos obtained from different plants or herbs exhibited intense antifungal properties (Bakkali et al., 2008; Prakash et al., 2012; Hu et al., 2007; Lang and Buchbauerl., 2012). The remarkable biological activity of Eos against phytopathogens has previously been reported (Arici et al., 2013; Tejeswini et al., 2014).

The aim of this work is to test the influence of the harvest periods alongside with AM fungi inoculation of lemongrass Eo on the growth inhibition, susceptibility and enzymes production of three phyto-pathogenic fungi in vitro. The effective active concentrations will be screened, and the minimum inhibition/fungicidal concentration checked, as well as the inhibition test of enzymes activities in respond to the Eos.

II. Materials and Methods

2.1. Lemon grass Essential Oil.

Lemongrass essential oil, originally steam distilled from plant leaves was graciously provided by the Soil Microbiology Laboratory of the Biotechnology Centre of the University of Yaoundé I in Cameroon. Oils were of two categories in four samples with the characteristic described in table 1. Two samples NAM10 and NAM14 extracted from none mycorrhizal plant of 10 and 14 months old and the other two AM10 and AM14 extracted from mycorrhizal plant of 10 and 14 months old. Chemical composition of oils exists in Fokom et al., 2019.

Table 1: Description of the lemongrass Essential oils.

Codes	NAM_{10}	AM_{10}	NAM_{14}	AM_{14}
Producing plant age	10 months	10 months	14 months	14 months
Color	Yelow-Brun	Yelow-Brun	Yelow-Brun	Yelow-Brun

NAM= Eo from none mycorhizal plant,
AM= Eo from mycorrhizal plant.

2.2. Description and Retrieval of Fungal Test Pathogens.

The fungi used in this work were provided by the Rhizosphere Research Laboratory, Department of Biological Sciences, College of Basic Sciences and Humanities, G.B. Pant University Pantnagar, India. They include: *Fusarium oxysporum* f.sp. lycopersici (F35), *Helminthosporium oryzae* sp. (Hel) and *Colletotrychum*

falcatum sp. (Cf). Prior to the study, the fungal strains were grown twice at 28 °C on Potatoes Dextrose Agar media (PDA) for five days to obtain an active grown fungus ready for use.

2.3. Determination of the Antifungal Activity.

Assessment of the antifungal activity of the Eo was carried out using the poisoned food technique as described by Adjou et al., 2012. Eos was first diluted in DMSO before use.

Specific initial concentrations of 0,1; 0,2; 0,4; 0,6; 0,8 and 1 µl/ml were prepared by adding appropriate amount of Eo solution to sterilized cooled molten PDA containing 0.5% (v/v) Tween 80, followed by manual rotation in a sterile Erlenmeyer flask to ensure well mixing of Eo with the medium. 20 milliliters of the medium was dispensed into sterile 9 cm diameter Petri plates with enough care taken to avoid trapping of air bubbles. The medium was allowed to solidify at room temperature. Equal amounts of DMSO and Tween 80 were added to the control plates. 1 cm of agar discs with mycelia were cut from the periphery of actively growing regions of a 5 day old pure culture and aseptically inoculated at the center of the Petri plates. Control plates (without the essential oil) were inoculated following the same procedure. Three replicates were maintained for each treatment and the plates incubated at 28°C. Appreciation of the radial growth of fungal colonies was done after five days of incubation. The inhibition percentage of mycelial growth of the fungus was calculated using the formula proposed by Philippe et al., 2012.

Inhibition of mycelial growth (%) = $dc{-}dt/dc\times 100$,

dc = diameter of colony record in the control plate. dt = diameter of colony record in the treated plate.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC).

MIC was defined as the lowest concentration of essential oil at which no growth occurred. To establish whether the essential oil had biocide effect on the test fungi, MFC was assessed. The inhibited fungal discs from the Eo treated plates were re-inoculated into freshly prepared PDA Petri plates and their growth revival observed after incubation for 72hours at 28°C. Minimum fungicidal concentration was taken as the lowest concentration of the Eo at which no growth occurred on the plates after sub culturing (Adjou et al., 2012).

2.5. Cellulase production

Fungal strain was grown on Czapek-Dox agar media, supplemented with 1,5% Carboxymethyl Cellulose (CMC) and 0,2 µl/ml Eo. Control Petri plate with 1,5% CMC and no Eo was simultaneously made to follow up the experiment. After 7 days of incubation, the plate was flooded with an aqueous solution of Congo red (1 % w/v) and incubated for 15 min. The Congo red solution was poured off, and the plate was further treated by flooding with NaCl solution (1 M) for another 15 min. The formation of a clear zone of hydrolysis around colonies indicated cellulose degradation. The diameter of the clear zone around colony was measured in order to classify the Eo according to their capacity to affected cellulase activity. The largest diameter was assumed to contain the highest activity (Ariffin et al., 2006).

2.6. Pectinase production

Fungal strain was grown on Czapek-Dox agar media, supplemented with 1.5 % pectin and 0,2 µl/ml Eo. Control petri plate with 1.5 % pectin and no Eo was made simultaneously to follow up the experiment. After 7 days of incubation, the plate was flooded with an aqueous iodine solution (1 % w/v) and incubated for 15 min. The iodine solution was poured off after incubation before screened for the pectinolytic activity (Okafor et al., 2008). Clear zone formed around the fungal colony indicate reaction between the enzyme secreted by the fungi and chromogenic substrates. The diameter of the clear zone around colony was measured in order to classify the Eo according to their capacity to affected pectinase activity.

2.6. Data Analysis.

Data were subjected to analysis of variance (ANOVA) using a general linear model with mixed effects and balanced design. Prior to ANOVA, the Levene's test was applied to data for homogeneity. Means were compared by Tukey's test ($p \leq 0.05$), using the SPSS statistical package, version 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

III. Results

3.1 Antifungal Activity of lemongrass Eo.

Eo of lemongrass exhibited mycelial growth inhibition on the tested fungus F35, Hel and Cf. The mycelial growth inhibition rate was governed by the age and symbiotic status of the plant used to produce Eo (AM and NAM), the concentration of the tested Eo and the species of the fungi used for the test (table 2 and 3). With

respect to Eo from ten months old plants, AM_{10} showed better activity compare to NAM_{10} but the difference was significant only for Cf fungi at the concentration of 0.2 µl/ml ($p<0.05$). Between concentrations of 0.2 and 0.6 µl/ml, there was an exponential improvement of the mycelial growth inhibition potential of the overall tested Eo which was recorded for each fungi species Complete mycelial growth inhibition was observed at Eo concentration of 0.8 µl/ml for F35 fungi with NAM_{10} Eo and a concentration of 0.6 µl/ml for the same fungi with AM_{10} Eo. Similar observation was noted for the fungi Hel at a concentration of 0.6µl/ml for NAM_{10} Eo, and 0.4µl/ml for AM_{10} Eo. For the fungi Cf, total mycelial growth inhibition was recorded at a concentration of 0.4 µl/ml for the two Eo tested (table 2).

Table 2: Percentage of mycelial growth inhibition of *Fusarium oxysporum* sp, *Helminthosporium oryzae* and *Colletotrychum falcatum* by essential oils from 10 months old lemongrass plant.

Fungi	Eo (µl/ml)									
	NAM_{10}					AM_{10}				
	0,2	0,4	0,6	0,8	1	0,2	0,4	0,6	0,8	1
F35	32.5^{c}	73^{b}	98^{a}	100^{a}	100^{a}	32^{c}	74^{b}	100^{a}	100^{a}	100^{a}
Hel	64^{b}	98^{a}	100^{a}	100^{a}	100^{a}	68^{b}	100^{a}	100^{a}	100^{a}	100^{a}
Cf	58^{c}	100^{a}	100^{a}	100^{a}	100^{a}	62^{b}	100^{a}	100^{a}	100^{a}	100^{a}

Data in line at the concentration range of 0.2 to 1 µl/ml, followed by the same letter are not significantly different at $p<0.05$.
F35= *Fusarium oxysporum*
Hel= *Helminthosporium oryzae*
Cf= *Colletotrychum falcatum*
NAM= Eo from none mycorhizal plant,
AM= Eo from mycorrhizal plant.

Related to Eo from plants of fourteen months old, AM_{14} showed better activity compare to NAM_{14} but the difference was significant only for Hel fungi at a concentration of 0.2 µl/ml ($p<0.05$) (table 3). Between ~~the~~ concentration of 0.2 and 0.6 µl/ml we record an exponential improvement of the mycelial growth inhibition potential of the overall tested Eo on each fungi species. Complete mycelial growth inhibition was observed at the Eo concentration of 0.8 µl/ml for F35 fungi with NAM_{14} Eo and at a concentration of 0.6 µl/ml for the same fungi with AM_{14} Eo. Similar observation was noted for the fungi Hel at a concentration of 0.6µl/ml for NAM_{14} Eo, and 0.4µl/ml for AM_{14} Eo. For the fungi Cf, total myceliall growth inhibition was recorded at a concentration of 0.2 µl/ml for the two Eo tested (table 3). The susceptibility rates of the tested fungi followed the same rate for the tested Eo, with Cf more susceptible than F35. The MICs recorded with NAM_{14} Eo were 0.6, 0.4, 0.3 µl/ml for F35, Hel, and Cf respectively while those recorded with AM_{14} Eo were 0.48, 0.3, 0.32 µl/ml for F35, Hel, and Cf respectively.

Table 3: mycelia growth inhibition of *Fusarium oxysporum* sp, *Helminthosporium oryzae* and *Colletotrychum falcatum* by essential oils from 14 months old lemongrass plant.

Fungi	Eo (µl/ml)									
	NAM_{14}					AM_{14}				
	0,2	0,4	0,6	0,8	1	0,2	0,4	0,6	0,8	1
F35	32.5^{c}	73^{b}	98^{a}	100^{a}	100^{a}	44^{c}	73^{b}	100^{a}	100^{a}	100^{a}
Hel	63.4^{b}	98.2^{a}	100^{a}	100^{a}	100^{a}	74^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Cf	61^{b}	100^{a}	100^{a}	100^{a}	100^{a}	66^{b}	100^{a}	100^{a}	100^{a}	100^{a}

Data in line at the concentration range of 0.2 to 1 µl/ml, followed by the same letter are not significantly different at $p<0.05$.
F35= *Fusarium oxysporum*
Hel= *Helminthosporium oryzae*
Cf= *Colletotrychum falcatum*
NAM= Eo from none mycorhizal plant,
AM= Eo from mycorrhizal plant.

2.2 Inhibition of lytic enzymes by lemongrass Eo.

F35 and Hel: fungi less susceptible to the inhibition of mycelial growth by the studied Eo, were used to test their ability to produce two lytic enzymes in two different culture media. Whether or not the CMC media contained Eo, no halo zone was recorded around colonies and therefore no activity of cellulase enzyme for the Hel fungi. However a halo zone appeared around the colonies in pectin media with a variable diameter depending on the type of the Eo for the same fungi (table 4). The largest halo diameter was recorded in the control treatment and the lowest in the treatment with Eo from AM fungi origin. In contrast, whether or not pectin media contained Eo, we did not record any halo zone around the colonies and therefore no activity of

pectinase enzyme with the F35 fungi. Halo zone appeared around the colonies in CMC media for the control treatment and nothing around colonies of fungi growth in CMC media supplemented with the Eos.

Table 4: Diameters of clear zone formed by cellulase and pectinase activity of two pathogenic fungi (Fusarium *oxysporum* sp (F35) and *Helminthosporium oryzae* (Hel) growth on Eos enriched CMC and pectin media.

	Hel		F35	
Eos	CMC (cm)	Pectin (cm)	CMC (cm)	Pectin (cm)
AM_{10}	Trace	0.75^{b}	0	0
NAM_{10}	Trace	1^{b}	0	0
Control	0	1.5^{a}	1.75	0

Data in colum followed by the same letter are not significantly different at $p<0.05$.
F35= *Fusarium oxysporum*
Hel= *Helminthosporium oryzae*
NAM= Eo from none mycorhizal plant,
AM= Eo from mycorrhizal plant.
CMC= carboxymethyl cellulose.

IV. Discussion

The mycelial growth inhibition pattern of the tested fungus by lemongrass Eos was shown to be origin and dose depend with large variation in regard of fungi species (table 2 and 3). In fact, at the concentration of 0.2 µl / ml, lemongrass Eo activities on fungi mycelial growth was significantly different in regard of the inhibition percentages. AM_{10} Eo was significantly more active on Cf fungus at the concentration of 0.2 µl/ml while AM_{14} Eo was significantly more active on Hel fungus, raising the importance of AM fungi on the modulation of bioactive compound in the plant. Such observation corroborate those of other studies who showed the influence of Am fungi on the modulation of the chemical composition of plant extracts, with potential extension to their biological activities (Kapoor et al., 2004; Kapoor et al., 2002; Chaudhary et al., 2008; Tajidin et al., 2012). Moreover, other researchers point out the influence of harvest periods, coupled or not, to the inoculation with AM fungi of plant on the modification of the chemical composition of Eos with extension to their antioxydative and probably antimicrobial properties (Fokom et al., 2019; Tsasi et al., 2017).

The mycelial growth inhibition of lemongrass Eo used in this study was shown to be concentration dependent. Mycelial growth was totally inhibited at the concentration of 0.8; 0.6 and 0.4 µl/ml respectively for the fungi F35, Hel and Cf with NAM_{10} and $_{14}$ Eo while it was totally inhibited at the concentration of 0.6 µl/ml for F35 and 0.4 µl/ml for Hel and Cf with AM_{10} and $_{14}$ Eo. These differences of action are certainly link to the chemical composition of the Eo which undergoing some modification within the growth of producing plant in symbiosis with AM fungi. In fact study on the impact of AM inoculation in regard of medicinal plant metabolism, not only point out the modification of their chemical composition but also the variation in the content of specific compound, most of the time credited with biological activities. Such observations are supported by results obtained from work on *Coriandrum sativum* (Kapoor et al., 2002), *Cymbopogum citratus* (Selim, 2011; Fokom et al., 2019), *Salvia officinalis* (Tarraf et al., 2017). It is known that key active constituents of lemongrass oil are α-citral, β-citral, limonene, citronellal, ß-myrcene and geraniol (Schaneberg and Khan, 2002; Teuscher et al., 2006; Tajidin, et al., 2012). Those compounds were shown to greatly vary following AM fungi inoculation and harvest period of plant (Fokom et al., 2019). This variation could explain the differences of activities of the lemongrass Eos observed on the tested fungi.

The susceptibility rate of the tested fungi to Eos was found to significantly vary according to fungi strain, with F35>Hel>Cf. This observation is probably linked to the proper physiology of each microorganism as they belong to different genera. A range of work did with Eos on mycelial growth inhibition showed great variation in their efficacy with respect to the strain of microorganism used (Thielmann et al., 2019; Tejeswini et al., 2014; Morcia et al., 2012; Campaniello et al., 2010; Browers and Locke, 2000).

Moreover, the susceptibility rate of the three fungi tested to lemongrass Eo significantly vary with the AM fungi status of the plant used for its production (Table 2 and 3). Fungi were more susceptible to Eo from AM fungi plant compared to those from none AM fungi plant. This observation may be as a result of variation in the compounds present in the Eos since studies on the chemical composition of lemongrass Eo following AM fungi inoculation of producer plant shows variation in individual compounds (Fokom et al., 2019), which may have great contribution to the differences in susceptibility rate recorded between AM and NAM Eo.

In regard of the growth inhibition properties of the studied Eos, their inhibition capacity on the activity of two key enzymes always produced by fungi within the colonization of host tissue was tested. They include pectinase and cellulase enzymes (table 4). In the absence of Eos, the fungus Hel developed no halo zone on the CMC media. This was observed after 5 days of growth and exposure to congo-red solution. However, a growing body of halo with low intensity was recorded in CMC media containing Eos after 5 days of growth and exposure to cong-red solution. This observation may explain a growing body of cellulase enzyme activity. Although

slowly active, this observation made us believed that this fungus produce cellulase enzyme during its growth but only in harsh conditions such as the presence of Eos in the media. In contrast, we recorded a halo zone arrow the colonies of Hel fungus after 5days of growth and exposure to iodine solution in pectin media. The diameters of the arrow in control and Eo treatments considerably varied, with the largest recorded in the culture media without Eo followed by those with NAM_{10} and AM_{10} Eo respectively. It seemed possible that Hel fungus naturally produced pectinase enzyme during its growth for metabolic purposes but the production capacity of this enzyme is inhibited in the media containing Eos. The inhibition rate recorded was high in the presence of AM_{10} Eo follow by NAM_{10} Eo compared to the control (table 4). While testing the fungus F35 (table 4), we recorded a halo zone around colonies after 5 days of growth and exposure to congo-red solution in CMC media without Eos. However, no halo zone was recorded around colonies in the CMC media containing Eos, whether it was AM_{10} or NAM_{10}. Such observation may explained, the aptitude of the fungus F35 to produced cellulase enzyme during its growth for metabolic purposes in normal condition. However they lost this capacity is in harsh environment such as the presence of lemongrass Eos independent of the oil origins. In contrast, no halo zone was recorded arrow the colonies of F35 fungus after 5 days of growth and exposure to iodine solution in pectin media for both the control and treatments, meaning no activity of pectinase enzyme. Probably these fungi did not produce pectinase enzyme within its growth. Previous study on Eos revealed that, some of their constituents cause a strong inhibitory effect on the activity of the enzymes pectinase and cellulase isolated from plant pathogenic fungi (Marei et al., 2012; Marei et al., 2018). Those compound may have considerably contribute to such observation.

V. Conclusion

Mycelial Growth inhibitory rate exhibited by lemongrass Eos was shown to be fungi species dependent. All the tested lemongrass Eos was active on the studied fungi, with significant more active pattern addressed to Eos from plant grown under AM symbiosis. The tested Eo was shown to inhibit the activity of pectinase and cellulase enzymes produced by Hel and F35 fungi for their metabolism, with significant activity record for cellulase compare to pectinase. Results obtained from this study are alternative way to safely fight against these pathogenic fungi. AM fungi are therefore a promotional way to improve the chemical content of plant with pharmacological properties for various usages.

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