Isolation of endophytic fungi from *Cola acuminata* Schott & Endl, and antifungal activity against *Candida Sp*

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ABSTRACT

The discovery of novel antifungal agents with low toxicity and high therapeutic efficacy is needed to overcome the limitation of the actually existing antifungal therapies. Endophytic fungi acknowledge for their outstanding ability to produce bioactive metabolites can be exploited for this purpose. Therefore, this study was designed to investigate the antifungal potential of endophytic fungi leaving in tissues of *Cola acuminata*. Endophytic fungi associated with *Cola acuminata* were isolated from healthy and matured plant tissues and characterized based on their morphological and microscopic characters. Each isolated fungus was cultured on potato dextrose broth for ten days and crude extract prepared from the resulting medium after filtration. The resulting extracts were tested for their antifungal potential using broth microdilution method. One hundred and six fungal isolates were obtained from leaves, fruit, stem, stem bark, root, and root bark of *C. acuminata*. They belong to six genera including *Aspergillus*, *Alternaria*, *Curvularia*, *Cunninghamella*, *Fusarium*, *Trichoderma*. Non-sporulating isolates were designated as *Mycelia*. Out of the 106 extracts screened for their antifungal activities, 21, 13 and 43 showed antifungal activities against *C.albicans* NR-29450, *C.parapsilosis* ATCC 22019 and *C.krusei* ATCC 6258 respectively. Five of these isolates including Cal14, Casb122, Cab259, Cab31 and Cab244 were the most potent, inhibiting the growth of the three tested *Candida* species more than 85% against. Isolate Casb122 appears to be one of the most potent antifungal against *Candida* species (MIC 500-1000µg/mL). The results suggest that endophytic fungi from *C. acuminata* can produce metabolites with antifungal activity.

Keywords: *Cola acuminata*, endophytic, fungi, antifungal, *Fusarium oxysporum*.

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I. INTRODUCTION

Mycoses still one of the major health issues in the world [1]. Due to the continue increase in number of immunocompromised patients and other factors such as the use of broad spectrum antibiotics aggressive anticancer chemotherapy and organ transplantation, the incidence of candidiasis have drastically increase over the past decades [2]. Antifungal agents available for the treatment of systemic or mucosal candidiasis are restricted to only a few classes of compounds with limited efficacy. For decades, the treatment has relied on fungicidal polyenes drugs such as amphotericin B, which binds to the fungal ergosterol and recently, fungistatic drugs such as the azoles (fluconazole) have become more widely used to treat fungal infection due to the comparative ease of their use [3]. However, adverse side effects, toxicity, and emergence of drug resistance limit the use of these drugs [4]. More distressing situation is the fact that various *Candida* species can acquire resistance to different antifungals or, even worse, to more than one drug [5]. Indeed, multidrug-resistant *Candida* spp. have been reported all over the world over the past decade [6] and recently with the discovery of a new worrisome and globally emerging life threatening and multidrug resistant *Candida auris*, posing a further threat to our ability to use antifungal drugs to treat candidiasis [7]. Therefore, new compounds that could constitute starting points for new drug antifungal discovery are clearly needed. The investigation of natural products for new lead discovery has proven to be a successful approach for centuries. Indeed, estimates suggests that more than 70% antimicrobial drug in clinical use are natural product or natural product derivatives [8]. In fact, natural products are adapted to a specific function in nature and constitute therefore an excellent source for novel secondary metabolites. The search for new agents should concentrate on organisms such as endophytic fungi inhabiting novel biotopes in nature [9]. Endophytes defined as “microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects” [10], are found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. In culture, outside of their host tissue, endophytic fungi are also known to produce a number of important secondary metabolites including anti-cancer, anti-fungal, anti-bacterial anti-diabetic and immunosuppressant compounds [11], [12]. Therefore, investigating this group of microorganisms can lead to the discovery of new antifungal agents. The current study was conducted to isolate, characterize and screen endophytic fungi from *C. acuminata* growing in Cameroon for their antifungal activity.

II. MATERIALS AND METHODS

A. Materials

1. Microorganisms

Fungal strains used to test endophytes antifungal activities included *Candida albicans* NR-29451 from Biodefense and Emerging Infections (BEI resources), *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 from the American Type Culture. These microorganisms were maintained on agar slope at 4°C and sub-cultured for 48 h

before use.

2. Plant material

Mature and healthy *Cola acuminata* were collected in west-Cameroon and stored in sterile plastic bags and transported to the laboratory within 48hours for isolation of endophytic fungi. The identification of plant species was done at the Cameroon National Herbarium by comparison to Voucher specimens under the identification number 5368/SRFK.

B. Methods

1. Isolation and identification of endophytic fungi from *Cola acuminata*

a) Isolation

For each plant tissue and organs (leaves, fruit, stem, stem bark, root or root bark), fragments of about 5 mm were thoroughly washed separately in running tap water, then surface sterilized by submerging them in 70% ethanol for 5 minutes, followed by 1% sodium hypochlorite (NaOCl) solution for 15 min, 70% ethanol for 2 min and rinse with sterile distilled water. The resulting disinfected samples were plated (20 pieces/organ/plate) onto potato dextrose agar (PDA; pH 5.6) supplemented with chloramphenicol (200 mg/L) and kept in dark at room temperature ($25 \pm 2^\circ\text{C}$). Five plates were prepared per sample. Mycelial tips emerging from plant fragments were transferred onto fresh PDA free of chloramphenicol to obtain pure cultures (Fig. 2). Continuous maintenance of endophytes was achieved by regular transfer on PDA slants under aseptic conditions to keep the culture freehand viable. Each colonization frequency of each isolated and identified endophyte was calculated using the following formula [13]:

$$\text{Colonisation frequency} = \frac{\text{Number of fragments colonized by the endophytic fungi}}{\text{Number of fragments deposit on the culture medium}} \times 100$$

b) Identification of fungi isolates

The isolated endophytes were identify based on the morpho-cultural characteristics. For this purpose, they were cultured on PDA plates at 28°C for 7 days. For identification macroscopic characteristics of colonies including shape, size, color, and surface texture, observed daily were used. These macroscopic characters were associated to microscopic features like the size and shape of hyphae, conidia and conidiophores of isolates grown on PDA plates for 7 days [14]-[17]. These identifications were limited at the genus level.

2. Screening of endophytes antifungal activities

a) Fermentation and preparation of endophytes extracts

The isolated endophytic fungi were cultured at 28°C in a 250 mL round bottom flask containing 20 ml of potato dextrose broth (PDB) medium for 3 incubation periods: 5, 10 and 15 days. The culture was shaken (with an electronic shaker IKA-VIBRAX-VXR) at 150 rpm all over the incubation periods. At the end of each incubation time, culture media was separated from mycelium by centrifugation at 4500 rpm for 20 min at 4°C . The resulting culture media was extracted thrice, using 20 mL of ethyl acetate. This mixture was then transferred to a separator funnel that permitted to separate the organic phase from aqueous phase. The crude extract was obtained after evaporation of the upper phase at 40°C in a rotary

evaporation system (BÜCHI 461). The obtained residue was subjected to evaporation to dryness to afford the crude ethyl acetate extract.

b) Antifungal screening of endophytes crude extracts

Preparation of stock solution and reference drug

The stock solution of crude extract was prepared at 160mg/mL using DMSO 20% in water. Indeed, 160mg of extract were introduced in 100 µL of 20% DMSO and the final volume completed to 1mL with sterile distilled water. The stock solutions were filter-sterilized with a 0.2 µm syringe and stored at 4°C. Fluconazole (Sigma Aldrich) was used as reference drug and prepared at 2mg/mL in DMSO 20%.

Antifungal activity

Each endophytic extract was tested for its antifungal activities using broth dilution method in 96-wells microtitre plates as described by the Clinical Laboratory Standards Institute M27-A3 [18]. In the first ligne of the 96-wells plate, 190µL of Sabouraud dextrose Broth were introduced and 10µL of stock extract solution added. From these wells, 100 µL of the solution were pipetted and introduced in the next well containing 100 µL of SDA and so on and so for. Extract solution (100 µL) and reference drugs (fluconazole) in Sabouraud dextrose broth (Hi Media) were added to the wells, followed by addition of 100 µL of candida inoculum standardized at 2.50×10^3 cells/mL to reach 4 mg/mL concentration for each extract. A blank column was included for sterility control. After 48 hours of incubation at 37 °C, absorbance of each plate was measured using a Magellan Infinite M200 multi-well plate reader (Tecan) 490 nm. The percentage inhibition was calculated as compared to negative control. Only extract with percentage inhibition higher than 80% was considered as active.

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of test well}}{\text{absorbance of the negativ control well}} \times 100$$

The minimum inhibitory concentration (MIC) of selected extracts against yeasts was determined as previously described [20] using 96-wells microtiter plates. The concentrations of extracts tested ranged from 125, 250, 500, 1000, 2000, and 4000 µg/mL. For a fluconazole, concentration was ranged from 4µg/mL to 128µg/mL according to the preliminary test on agar medium. After 48 hours of incubation at 37 °C, the turbidity was observed as an indication of growth. MIC was defined as the lowest concentration inhibiting the visible growth of yeasts.

III. RESULTS

A. Identities of isolated endophytic fungi from *C. acuminata*

One hundred and six (106) endophytic fungal isolates were obtained from 600 tissue segments of *C. acuminata*, including leaves, fruit, stem, stem bark, root, and root bark. Based on their morphocultural and microscopic features, they were identified as belong to six different genera: *Aspergillus*, *Alternaria*, *Curvularia*, *Cunninghamella*, *Fusarium* and *Trichoderma*. Fifty-six of these isolates were imperfect non-sporulating fungi and were categorized as *Mycelia sterilia*.

1. *Trichoderma* spp.

Trichoderma colonies showed variable aspects, sometimes slightly flaky and other times compacted in clumps. Between these two extremes, intermediate aspects have been observed. The conidium gives rise to a sterile, white, sterile first mycelium, and then takes on a visible green color on the aerial parts of the mycelium, corresponding to conidiogenesis (Fig. 1a and 1b). Under microscope, the mycelium appears composed of yellow, septate, branched, smooth-walled hyphae (Fig. 1c). Conidiophores have a conical or pyramidal shape. Very branched, they wear phialides in the form of flanges or bowling pins. In turn, phialides carry spores (Fig. 1d).

Fig. 1. Morphological characteristics of *Trichoderma* sp on Potatoes Dextrose Agar Medium. General mycelial aspect and color (both sides), c. hyphae d. Microconidia.

2. *Fusarium* Spp.

Colonies of *Fusarium* were characterized by a rapid growth on PDA medium. Colonies were flat, whitish to yellowish in color (Fig. 2a). These colors either remained with time or changed into purple or whitish on the reverse of the culture (Fig. 2b). Macroconidia present several lodges, with a little or no curved shape, form of the basal cell (Fig. 2c). The microconidia are dispersed among the mycelium, small in size compared to macroconidia and more often consist of one or two cells fusiform, pear-shaped, ellipsoid, ovoid or subglobose (Fig. 2d). Chlamydospores when they exist are terminal, or intercalated, isolated or in groups or chains (Fig. 2d).

Fig. 2. Morphological characteristics of *Fusarium* sp on Potatoes Dextrose Agar Medium. General mycelial aspect and color (both sides), c. macro and microconidia.

3. *Aspergillus Spp.*

Cultured on PDA medium, *Aspergillus* colonies showed variable aspects on the front: white then green, green-gray then dark green, fluffy to powdery, white then yellow to yellow-green, white then yellow then granular and blackish (Fig. 3a). The reverse of these colonies were colorless, yellow, green, red-brown, or pale yellow (Fig. 3b). Microscopic features include hyphae that develops into vegetative and reproductive hyphae with conical heads with large and black globes (Fig. 3c).

Fig. 3. Morphological characteristics of *Aspergillus* sp on Potatoes Dextrose Agar Medium. General mycelial aspect and color (both sides), c. conidiophore.

4. *Alternaria Spp.*

Cultured on PDA medium, *Alternaria* is fast-growing, with black or gray or greenish-gray and woolly colonies (Fig. 4a). The reverse of the culture is black (Fig. 4b). Conidiophores are erect and swollen at their ends in spherical or ovoid heads (Fig. 4c). The conidia are wall-shaped, isolated or grouped from the conidiophore. They are brown, pear-shaped or ovoid, with an enlarged base with transverse, longitudinal, oblique, variable number of septations.

Fig. 4. Morphological characteristics of *Alternaria* sp on Potatoes Dextrose Agar Medium General mycelial aspect and color (both sides), c. macroconidia d: conidiophore.

5. *Curvularia Spp.*

Grown on PDA, it produced effuse colonies with grey

cottony mycelia becoming blackish – brown when forming conidia and conidiophores (Fig. 5a). The reverse of the culture is black (Fig. 5b). The observed conidiophores were smooth-walled mononematous and macronematous. Conidia were solitary, often curved, rounded at the end with mostly 3 rarely 4- 5 transverse septates with dark bands (Fig. 5c).

Fig. 5. Morphological characteristics of *Curvularia* spp on Potatoes Dextrose Agar Medium. General mycelial aspect and color (both sides), c. macroconidia.

6. *Cunninghamella Spp.*

The colonies on PDA are fast growing and extensive, flaky, white in color becoming gray (Fig. 6a). The reverse is colorless to yellowish (Fig. 6b). The mycelia are broad, irregular and not septate. The sporocystophores are simple or branched with the dilated end into a spherical to oval vesicle, of variable size. On these vesicles are formed external sporangioles containing a spore (Fig. 6c).

Fig. 6. Morphological characteristics of *Cunninghamella* Spp on Potatoes Dextrose Agar Medium. General mycelial aspect and color (both sides), c. conidiophore.

B. Distribution of endophytes isolates in different tissues and organs of *C. acuminata*

The isolated endophytes were unequally, both in qualitative and quantitative point of view, distributed in the various screened organs of *C. acuminata*. *Alternaria spp*, *Cunninghamella spp* and *Trichoderma spp* were isolated only in stem. *Fusarium* was found in stem bark and leaves while *Aspergillus*, the most predominant genera in *C. acuminata* was present in almost all the organs except in fruits (Table 1). *Cola acuminata* stem and root were dominantly infected by *Aspergillus spp* followed by *Mycelia sterilia*, whereas stem bark, root bark and leaves were dominated by *Mycelia sterilia* followed by *Aspergillus spp*.

TABLE 1: FREQUENCY OF ENDOPHYTES FUNGI ISOLATED PER ORGANS OF *COLA ACUMINATA*

Fungi genera	Stem	Stembark	Rootbark	Leaves	Fruit	Root	Total number of isolates
<i>Mycelia sterilia</i>	11	9	15	11	4	6	56
<i>Alternaria</i>	2	-	-	-	-	-	2
<i>Aspergillus</i>	12	5	2	6	-	12	37
<i>Curvularia</i>	-	-	1	1	-	1	3
<i>Fusarium</i>	-	1	-	1	-	-	2
<i>Cunninghamella</i>	1	-	-	-	-	-	1
<i>Trichoderma</i>	4	-	-	-	-	-	4
Total number of isolates	30	15	18	19	4	20	106
Infection frequency (%)	30	15	18	19	4	20	-

Fig. 7. Variation of extraction yield of endophyte cultures as a function of incubation time.

Extracts of endophyte isolates that were prepared exhibited different antifungal activities vis-a-vis of the three *Candida* strains tested and depending on the organ where they were isolated (Table 2). The isolates obtained from fruits and roots were practically not active on the tested *Candida* species tested. On the other hand, stembark and rootbark gave more active isolates than stem. *C. krusei* was susceptible to 43 extracts while *C. albicans* and *C. parapsilosis* were sensitive to 21 and 13 endophytes extracts respectively. The number of active endophytes from root and fruits on the three *Candida* species was very low. However, based on the percent inhibition of the extracts from endophytes cultures, and having set the bar at 80%, only five isolates were actually

C. Quantities of endophytes extracts and their antifungal properties

The evolution extraction yields of culture media with ethyl acetate varied according to endophyte isolates. Then the production of secondary metabolites by endophytes in culture varied according to isolates. Thus, with isolate cab244, the extraction yield increases from days 5 to day 15, while with isolates Cab14 and Cab31j, yield remain constant between day 5 and 10, and then increased between day 10 and 15. A reverse situation was observed with Casb122. With Cab259, the extraction yield increase between day 5 and 10, then remain constant between day 10 and 15 (Fig. 7).

active (Table 3). Concerning MIC, only extract of isolate Casb122 identified as *Fusarium spp* was appears to be one of the most potent antifungal against *Candida* species (Table 4).

TABLE 2: DISTRIBUTION OF ACTIVE ISOLATES IN DIFFERENT ORGANS OF *COLA ACUMINATA*

Human pathogenic fungal strains			
Plant organs	<i>C. albicans</i> NR-29450	<i>C. parapsilosis</i> ATCC 22019	<i>C. krusei</i> ATCC 6258
Stem	3/30 (10.0%)	6/30 (20.0%)	9/30 (30.0%)
Stembark	9/15 (60.0%)	2/15 (13.33%)	10/15 (66.66%)
Rootbark	4/18 (22.22%)	2/18 (11.11%)	9/18 (50.0%)
Leaves	5/19 (26.31%)	3/19 (15.78%)	13/19 (68.42%)
Fruits	0/4 (0.0%)	0/4 (0.0%)	1/4 (25.0%)
Roots	0/20 (0.0%)	0/20 (0.0%)	1/20 (5.0%)
Total	21/106 (19.81%)	13/106 (12.26%)	43/106(40.56%)

TABLE 3: THE MOST ACTIVE ISOLATES AGAINST THE THREE *CANDIDA* SPP

Candida species			
Endophytic fungi	<i>C. albicans</i>	<i>C. parapsilosis</i>	<i>C. krusei</i>
Cal14	99,27 %	86,55 %	80,96 %
Casb122	90,41 %	98,59 %	81,83 %
Cab259	86,73 %	90,35 %	87,29 %
Cab31j	87,73 %	88,38 %	88,66 %
Cab244	99,01 %	89,65 %	79,49 %

TABLE 4: MIC OF ENDOPHYTE EXTRACT AGAINST SEVEN *C. ALBICANS* ISOLATES (X102 μ G/ML)

Human pathogenic <i>C. albicans</i> (C.a) isolates								
Endophytes extract	C.a Is1	C.a Is2	C.a Is3	C.a Is4	C.a Is5	C.a Is6	C.a Is7	C.a NR29451
Cal14	20	20	20	20	20	20	20	20
Cat31j	20	20	20	20	20	20	20	20
Cat259	20	20	20	20	20	20	20	20
CaEt122	10	10	5	10	10	10	10	10

Cat244	20	20	20	20	20	20	20	20
Fluconazole	1.28	0.64	0.3	1.28	1.28	1.28	0.64	1.28

IV. DISCUSSION

The role of endophytes in plants is comparable to that of the intestinal microbiota in animals. Several endophytes have properties that potentiate the growth of host plants or its resilience to certain stress factors [19], [20]. In this study, one hundred and six (106) endophytic fungal isolates were obtained from different organs of *C. acuminata*. These results demonstrate that all the organs investigated are host of one or more endophytic fungi as previously described [21]-[23]. In some cases, endophytes appear to be non-pathogenic and then benefit to host plant in a symbiotic association. But in some cases, in plant-endophyte interactions, endophytic fungi can potentially become pathogens or saprotrophs [24], [25].

These endophytic fungi were classified by morphological analysis into six (6) genera including *Aspergillus*, *Alternaria*, *Curvularia*, *Cunninghamella*, *Fusarium*, *Trichoderma* and *Mycelia sterilia* for the non sporulating fungi. All the genera found in the present study have been also reported in several other studies [26], [27]. The most dominant genus found in all *C.acuminata*'s parts was *Aspergillus* spp. which is the common endophytic fungi from several tropical plants investigated till date.

The 106 endophytic fungi isolated were cultured in liquid potatoes medium and afforded extracts were tested against *Candida* spp. Out of the extracts tested, 21, 13 and 43 extracts were active against *C.albicans* NR-29450 and *C.krusei* ATCC 6258. The results show that a great percentage of fungi are producing active metabolites against *candida* spp. These results are in accordance with previous studies with reported that a high proportion of endophytic fungi (80%) produce biologically active compounds in tests for antibacterial, fungicidal and herbicidal activities [28], [29]. Endophytic fungi are therefore an alternative source for the production of new antifungal agents [30], [31]. Among the active isolates, five including *Cal14*, *Casb122*, *Cab259*, *Cab31* and *Cab244* were the more potent with percentage inhibition of more than 80%. The most potent extract against all the tested microorganisms with MIC values ranged from 500-1000 µg/mL was from isolate *Casb122*. This may suggest that this isolate can produce potent antifungal compounds. Indeed, previous studies reported the antifungal metabolites produced by some endophytic fungi species from medicinal plants. In fact, ambuic acid, a highly functionalized antifungal compound was isolated from both endophytic fungi *Pestalotiopsis* sp and *Monochaetia* sp by [32]. Another compound named Fusidicactones with good antifungal activity was produced by endophytic *Fusidium species* [33]. In addition, the endophytic fungus *Cryptosporiopsis quercina* was found to produce Cryptocandin and Cryptocin. In fact, Cryptocandin and its related compounds named, echinocandins and pneumocandins demonstrated excellent antifungal activity against *Candida albicans* and *Trichophyton* spp, *Sclerotinia sclerotiorum* and *Botrytis cinerea* and are currently being used against a number of fungi causing diseases of the skin and nails [11], [34].

V. CONCLUSION

The present investigation gives an insight into the fungal endophytes associated with the Cameroonian medicinal plants, *Cola acuminata* and their antifungal potential. The results demonstrate that endophytes isolated have the potential to provide novel pharmacophores against the *Candida* spp. This also supports the fact that screening of endophytes from medicinal plants can lead to the identification of potent compounds. Further studies are ongoing to optimize the condition for antifungal production by the most potent fungus *F.oxysporum*.

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