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Antifungal activity of endophytic *Fusarium oxysporum* from *Cola acuminata* against *Candida albicans* isolates from HIV-infected patients

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Abstract

Background: The Human Immunodeficiency Virus (HIV) infection has exacerbated the occurrence of opportunistic diseases with *Candida* infection being the most prominent. Unfortunately, the growing ineffectiveness of the available antifungal drugs along with the emergence of new multi-resistant strains further worsen the matter. In the search for new active molecules, the present study aims at investigating the optimal growth conditions for the production of antifungal metabolites from the endophytic fungus *Fusarium oxysporum*.

Methods: *Fusarium* sp. isolated from *Cola acuminata* was identified using the ITS1-5.8S rRNA-ITS2 nucleotide sequence. For the production of various metabolites, the fungus was cultivated for ten days in various media supplemented with different sources of carbon, amino acids, nitrogen, ion and organic acids. All extracts were submitted to antifungal activity test against seven *Candida albicans* isolates using the broth microdilution method. The most active extract was evaluated for cytotoxic activity against the Vero cell line using the MTT assay.

Results: The endophytic fungal isolate Casb122 was identified as *Fusarium oxysporum* based on the ITS sequence. Extracellular extracts showed better activity against *Candida albicans* (MICs ranges 500-1000 µg/mL) compared to intracellular extracts (2000 µg/mL). The growth media supplemented with carbon, amino acids, nitrogen, ion and organic acids promoted mycelium growth and production of secondary metabolites. The MIC of extracts obtained from certain media supplemented with carbon, amino acids, nitrogen, ion and organic acids were 2 (500 µg/mL), 4 (250 µg/mL), 8 (125 µg/mL) and 16 (62.5 µg/mL) times lower than that of the control (extract obtained from unsupplemented media). Active extracts with a Cytotoxic concentration 50 (CC_{50}) >200 µg/mL were not cytotoxic to Vero cell line.

Conclusion: The results from this study demonstrate that the culture of endophytic *Fusarium oxysporum* in supplemented media improves biomass production and antifungal activity against *Candida albicans*. This isolate could be an excellent source for the discovery of new antifungal compounds. Current investigations are ongoing to isolate and characterize potential antifungal metabolites.

Keywords: *Fusarium oxysporum; Candida albicans*; antifungal activity; Supplemented media; Cytotoxicity.

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Background

Candida albicans is an important pathogen among yeasts associated with other chronic infections and noninfectious disease conditions [1]. *C. albicans* can be recovered as normal biota of the vagina and skin of humans. However, it can become pathogenic and invade the organ when the immune system is compromised. This is the case with HIV/AIDS women, in whom vulvo-vaginal candidiasis due to *C. albicans* is more common and recurrent. Although this candidiasis causes little or no systemic fungal infections or mortality [2], interventions to prevent and treat this disease are essential to maintain the life quality of patients. Looking at the limited number of antifungals drugs available and the appearance of resistance for some of them, it is important to find alternative solutions to improve the management of patients suffering from candidiasis. The endophytes could appear as a novel way for the search of new antifungal molecules [3].

Endophytes are microorganisms living in a symbiotic association with plants without causing harm [3]. Endophytes have a broad range of benefits among others: protection of the host plant against predators, pathogens, and insects, increase in the plant's resistance to stress, and production of components of technological interest such as enzymes and antibiotics [4]. Endophytes equally play a role in the environment by intervening in the nutrient cycle via the process of biodegradation and bioremediation. Omnipresent in all plants, endophytes have the capacity to produce bioactive molecules sometimes identical to those found in plants [5]. Over the last few years, attention has been focused on endophytes and their exact role in nature and particularly in the medical domain [6]. Many studies are now focused on the isolation of endophytes from plants and on the study of the properties of metabolites produced by endophytes [3,7]. Indeed, the interest in endophytes lies in their ability to produce secondary bioactive metabolites such as steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasins, which have been identified to possess biological activities such as antibiotic, antiviral, volatile antibiotic, anticancer, antioxidant, insecticidal, antidiabetic, and immunosuppressive properties [7,8]. In plants, the presence of endophytes would improve the ecological adaptability of hosts, protect against certain parasitic pathologies, and increase resistance against abiotic stress [3,9]. The production of the compounds responsible for these activities by the endophytes in the plant would depend to the plant's need for these compounds, the species of plant, the nature of nutrients, and the type of soil [3]. All these factors can create a quantitative and qualitative variation of the bioactive molecules produced within the plant. Consequently, the therapeutic potential of the plant can be altered. Furthermore, bulky quantities of plant material are needed to harvest considerable amounts of bioactive molecules. For these reasons, isolating the endophytes and cultivating them under well-defined conditions would make it possible to control the production of certain metabolites, increase the yield and also prevent the extinction of certain plant species.

Among the most widespread endophytes with the ability to colonize a large variety of plants, *Fusarium* species represents a large cosmopolitan genus comprising more than 70 species capable of producing a wide array of active metabolites [10]. Endophytes from *Fusarium* genus are described as a promising source of antimicrobial compounds active on a broad range of pathogenic bacteria and fungi. Several compounds isolated from *Fusarium* species have demonstrated antiparasitic, antiviral and antimicrobial activities [7]. Furthermore, they are potential sources of anticancer, antioxidant, antithrombotic, immunosuppressive and immunomodulatory agents [7]. Several species of *Fusarium spp.* have been exploited for their ability to produce bioactive compounds and for the biological activities of these compounds. Despite these advances, few data are available regarding the optimization of the production of bioactive compounds from endophytes and the evaluation of their activities. Some studies have shown that the optimization of *Fusarium spp.* production could induce the metabolism of new molecules and/or increase that of existing ones, thereby potentiating their bioactivities [11,12]. Therefore, the aim of this study was to optimize the production of bioactive compounds from *Fusarium oxysporum* using several organic molecules and ions and evaluate the activities of these bioactive compounds on *Candida albicans* isolated from HIV patients.

Methods

Source of the endophytic fungus used in this study

The endophytic fungus used in this study was isolated from healthy and mature stem bark of *Cola acuminata* (registration code: 601610HNC of Cameroon National Herbarium). The plant sample was collected in Badenkop, Cameroon, in December 2017. Small pieces of plant materials measuring about 2 mm were surface-disinfected through a 5-min rinse with 70% ethanol, followed by treatment with 1% active chlorine solution for 15 min, 2 min in 70% ethanol, and a final rinse three times in sterile distilled water. Disinfected material pieces were plated on potato dextrose agar (PDA; HiMEDIA, India) containing chloramphenicol (200 mg/L) and kept in the dark at room temperature (22-26°C). After the emergence of endophyte mycelium from plant tissues into the agar, mycelial fragments were transferred to fresh PDA plates and maintained under natural light at room temperature. All endophytes are kept at -80°C in a 50% glycerol solution at the Antimicrobial & Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Cameroon [13].

Molecular identification of the fungal endophyte

The endophytic fungal isolate was identified based on the sequencing of the ITS1-5.8S rRNA-ITS2 nucleotide sequence. Briefly, fungi genomic DNA was extracted from mycelium grown in potato dextrose broth (PDB, HIMEDIA) using a commercial kit (RedExtract-N-Amp Plant PCR, Sigma Aldrich, USA). The extracted DNA's concentration and purity (A260/ A280 ratio) were measured with a Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Scientific, Germany) using 1 µL of each sample. The ITS1-5.8S rRNA-ITS2 region was amplified by PCR using ITS4 and ITS5 primers (ITS5; 5'GGAAGTAAAAGTCGTAACAAGG3' and ITS4; 5' TCCTCCGCTTATTGATATGC3') (Sigma-Aldrich, Germany) and the protocol described by White et al [14]. The PCR amplification program containing denaturation step at 95°C for 2 min, followed by 35 cycles. Each cycle had a denaturation step at 94°C for 1 min, annealing step at 54°C for 1 min, and elongation step at 72°C for 1 min. This was followed by a final step of 72°C for 10 min. PCR product was analyzed by agarose (1% agarose) gel electrophoresis using 1×Tris-EDTA (TE) buffer containing 1 µg/mL of Ethidium Bromide (EtBr) and a constant voltage of 100 V for 20 min. DNA bands were visualized, and images were acquired using

Gel Doc XR+ imaging system (Bio-Rad Laboratories Inc., Germany). Amplicons were purified by filtration (MSB Spin PCRapace, Invitek, Germany), and only one strand of the PCR amplicon was sequenced. The sequence reaction was started at the 5′ end of the ITS1-5,8S rRNA-ITS2 region, using primer ITS4.

The BLAST algorithm was used to find sequences similar to those obtained from fungal isolates. The criteria for identifying isolates were based on the similarity of their sequences to those of reliable reference isolates included in open access nucleotide databases. A dendrogram was made with the nucleotide sequences of the isolates and those of reference strains deposited in Centraal bureau Voor Schimmel cultures (CBS) and American Type Culture Collection (ATCC) collections. Sequences were aligned using the following parameters: pairwise alignment parameters (gap opening = 10 and gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, and delay divergent sequences = 25%) and optimized manually in MEGA 7.0. For the phylogenetic analyses based on Maximum Likelihood (ML), the best-fit models of nucleotide substitution for each data partition was determined using the software MEGA 7.0 and incorporated into the analyses. Alignment gaps were treated as partial missing information, and one thousand replications estimated the robustness of the classifications. The initial trees for the heuristic ML search were obtained by applying the Neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach, allowing some sites to be evolutionarily invariable. Groups of sequences at proximity within the same branch of the dendrogram were individually aligned to determine their similarity percentage. Sequences with close similarity with reference sequences used for phylogenetic analysis were considered to belong to the same species as the reference sequence [15].

Preparation of crude fungal ethyl acetate extracts

Culture of Fusarium oxysporum in supplemented media

Fusarium oxysporum was grown at 28°C for ten days under constant agitation (150 rpm) in liquid potatoes dextrose medium (PDB) supplemented with 0.5 % (w/v) of various sources of carbon (starch, glycerol, maltose and cellulose), nitrogen (urea, thiourea and ammonium sulfate), amino acids (L -tryptophan, L-arginine and L- proline), mineral salts (sodium sulfate, potassium phosphate, zinc sulfate, potassium iodide, potassium chloride, sodium chloride, magnesium sulfate and sodium citrate) and organic acids (gallic acid, tannic acid, sulfanilic acid and 3-5 dinitrosalicylic acid) [16-18]. At the end of the incubation period, each culture was centrifuged at 4500 rpm for 20 min. The mycelium was separated from the liquid medium, and both were submitted to extraction using ethyl acetate.

Extraction of crude extracellular and intracellular metabolites

For the preparation of extracellular crude metabolites, the supernatant was extracted using 100 mL of ethyl acetate using liquid-liquid partition method. The mixture was transferred to a separator funnel, shaken vigorously and allowed to stand for about 30 min. The organic phase was then separated from the aqueous phase and the process repeated twice on the aqueous phase to maximize extraction. The mixture of the three organic phases was subjected to evaporation for dryness at 40°C in a rotary evaporation system to afford the crude extracellular ethyl acetate extract. For intracellular crude metabolites, pellets collected after centrifugation of cultures were dried in a ventilated oven at 45°C for two days, weighted and ground to fine powder. Powder for each culture was macerated for 72 hours with 50 ml of ethyl acetate after which the organic phase was filtered and evaporated to dryness at 40°C in a rotary evaporation system to afford the crude intracellular extract.

Source of Candida albicans used in this study

Isolation of Candida albicans from vaginal samples

Prior collection of fungal isolates, the ethical clearance was requested, and the study was approved by the Ethical Committee of the Ministry of Public Health of Cameroon with reference number N˚2021/03/84/CE/CNERSH/SP. Only infected HIV women whether or not developing AIDS, not under antifungal treatment were eligible for this study. Informed consent was obtained from all participants before samples were collected by the medical personnel. The low vaginal swabs were collected from each participant and cultured at 37°C for 48 hours on Sabouraud dextrose agar media supplemented 1% chloramphenicol. All *Candida* species obtained were subcultured on Chromagar medium and submitted to germ tube assay to differential *C. albicans* isolates from non albicans species [19].

Antifungal susceptibility profile of C. albicans isolates

Among *C. albicans* isolated from HIV/AIDS patients, seven were selected and submitted to antifungal susceptibility testing against six antifungal drugs including Econazole (10µg), Ketoconazole (10µg), Fluconazole (50µg), Miconazole (50µg), Amphotericin-B (20µg), and Nystatin (100µg) using the disk diffusion method as developed by the National Committee for Clinical Laboratory Standards [20]. Briefly, one milliliter of *C. albicans* cell solution at 2.5×10^5 CFU/ml was inoculated on Sabouraud dextrose agar medium in the Petry dish, allowed to rest for 15 minutes after which the supernatant was removed, and the various antifungal discs were deposited on the surface. After 48 hours of incubation, the diameters of inhibition zone were measured. The isolate *C. albicans* was considered sensitive when the diameters were equal to or greater than 15 mm for Amphotericin-B, 16 mm for Miconazole, 18 mm for Nystatin and 20 mm for Ketoconazole, Econazole and Fluconazole. It was considered resistant if the diameters were below the set standard.

Antifungal activity of Fusarium oxysporum crude extracts

The antifungal activity of extracts was determined using the broth microdilution method in 96-wells microtiter plates as described by the Clinical Laboratory Standards Institute M27-A3 microdilution method. The tested extracts were prepared in Sabouraud dextrose broth at an initial concentration to 4 mg/ml and final to 31.25 µg/ml. *Candida albicans* inoculum was standardized at 2.5×10^5 CFU/ml. Each test was performed in triplicate. For these tests, a blank column (sterility control) containing culture medium and a negative control column containing the culture medium, supplemented compounds, microorganism and 2% DMSO were added. After 48 hours of incubation at 37°C, MIC was defined as the lowest concentration of extract for which no visual growth was observed.

Evaluation of the cytotoxicity of the extracts

The extracts that showed best antifungal properties were tested for their cytotoxicity using a colorimetric method as described by

Mosmann [21,22]. The Vero cell line used was cultured under standard conditions (37°C and 5% CO_2) in DMEM complete medium. In a 96-well microplate, 100 µL of a cell suspension (5×10^3 cells) were introduced into each well and incubated for 24 hours to promote cell adhesion. After incubation, the medium contained in the wells was replaced with same medium containing extracts prepared at concentration range of 12.5-200 µg/mL. The plates were incubated for 24 hours under the same conditions mentioned above. The negative control consisted of wells having the Vero cells only. Twenty microliters solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5 mg/ml in phosphate saline buffer) was added to each well and the whole plate was incubated for 4 hours at 37°C. At the end of the incubation, the contents of the wells were emptied, and 100 µL of DMSO 100% was added to dissolve the formazan crystals. The optical densities of each well were measured using the Infinite M200 plate reader (TECAN) at 570 nm. The colour intensity was proportional to the amount of formazan formed. From the optical densities obtained, the percentages of viable cells were calculated compared to the values from control wells. The non-linear regression curve using GraphPad Prism.7 software led to the determination of CC_{50} values (cytotoxic concentration that reduce cell viability to 50%).

Statistical analyses

Data on Biomass of *Fusarium oxysporum*, Mass of intracellular bioactive compound and Mass of extracellular bioactive compound were analysed by the One-way analysis of variance (ANOVA) using the Statistical package for social science (SPSS) software version 20.0 and plotted in Sigma Plot 11.0 software. The differences between the means were compared by the Waller Duncan test at 95% confidence ($p<0.05$).

Results

Molecular identification of the isolate Fusarium sp (Casb122)

The ITS rDNA region of the fungus was sequenced, and the identification was performed by comparison with published sequences in GenBank. The results from the BLAST search revealed that the fungal endophyte sequence showed 100% similarity with sequences from previously identified *Fusarium oxysporum* in the NCBI database. The new sequence generated in this study was deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov) under the accession number ON797481. To infer the evolutionary history of endophytic fungus, the Maximum Likelihood (ML) method based on the Jukes-Cantor model was used (Fig 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The ML phylogenetic analysis show that the endophytic fungus forms a well-supported clade with bootstrap value of 98 with several strains of *Fusarium oxysporum*. Since these results confirm those from the BLAST search, our endophytic fungus was identified as *Fusarium oxysporum* Casb122 (Figure 1).

Susceptibility of Candida albicans clinical isolates to currently used antifungals

Among the 320 participants recruited in this study, *C. albicans* was isolated from 145 (45.31%) women. Seven isolates of *C. albicans* were chosen randomly from 145 positive samples and their susceptibilities to currently used antifungals were evaluated. All isolates were sensitive to at least one antifungal disk. The Azoles' group (Ketoconazole, Econazole, Fluconazole and Miconazole) were more active on the isolates tested than the polyenes' group (Amphotericin-B and Nystatin) (Table 1). From the 7 isolates tested, 6 were resistant to Nystatin and Amphotericin-B, 4 to Fluconazole, Ketoconazole and Miconazole. Except isolate *C.a*Is3, all isolates were sensitive to Econazole.

Antifungal properties of intracellular and extracellular extracts of Fusarium oxysporum against clinical isolates of Candida albicans

Minimal inhibitory concentration (MIC) values of extracellular and intracellular extracts had ranges of 500 - 1000 µg/mL and 2000 µg/mL respectively against the seven isolates of *Candida albicans* (Table 2). The activity of extracellular compounds was better than that of intracellular compounds.

Effects of various supplements on the biomass and antifungal activity of crude metabolites

Regardless of the supplement, the production of extracellular metabolites in SDB was significantly higher than that of intracellular metabolites. After ten days of culture, threonine and proline significantly promoted the development of the mycelium compared to the negative control. However, they had an inverse effect on the production of crude metabolites compared to arginine, which rather boosted the production of crude metabolites albeit weak effect on mycelial growth compared to the control (Figure 2A). Concerning organics acids, we observed a beneficial effect of gallic acid on the production of metabolites while reducing the growth of mycelium. On the other hand, tannic and dinitrosalicylic acids promoted mycelial growth while reducing the production of metabolites (Figure 2B). The various carbon sources significantly slowed down mycelial growth and production of secondary metabolites with the exception of starch (Figure 2C). Among nitrogen sources, ammonium sulphate promoted mycelium growth while thiourea promoted secondary metabolites production (Figure 2D). An increase in metabolites production was observed with the addition of the mineral salts like sodium citrate and sodium chloride. The addition of other salts rather inhibited the metabolites production with the lowest amounts obtained when potassium phosphate and magnesium sulphate were used. While several of these minerals increased mycelial production, with masses significantly greater than the that of control, zinc sulfate showed no effect on mycelial growth (Figure 2E).

Antifungal and cytotoxic properties of extracts from supplemented Fusarium oxysporum

All intracellular and extracellular extracts obtained from medium supplemented with different molecules were tested by the disk diffusion method to determine the most active crude extracts. Only extracellular extracts from media supplemented with sulfanilic acid, glycerol, sodium chloride, potassium chloride, potassium iodide, potassium chloride, and thiourea showed activity with inhibition diameter (ID ≥10 mm) on at least two *Candida albicans* isolates. They were progress to MIC determination.

The results showed that various organic supplements have different effect on the activity of crude extracts obtained. The antimicrobial activity of crude extract is considered significant if MIC is less than 100 µg/mL, moderate if MIC ranges between 100 and 625 µg/mL and weak if it's greater than 625 µg/mL [23]. The most sensitive isolate was found to be *C.a*Is2. Overall, extracts from SDB supplemented with sulfanilic acid, glycerol, sodium chloride, potassium chloride and potassium iodide, the exhibited MIC values 2, 4, 8 times lower than the MIC of the non-supplemented extract. On the *C.a*Is7 isolate, the extracts supplemented with potassium chloride and thiourea exhibited MICs of 8 (125µg/mL) and 16 (62.5 µg/mL) times lower than that of the control (Table 3). All the bioactive extracts were non cytotoxic to Vero cell at concentrations as high as 200µg/ml (Table 4).

Discussion

Endophytic fungi are widely distributed in soil and plants. They aid in the mineralization of the soil, play a role in the defense of plants against infections [24]. Their interactions with plants result in the production of secondary metabolites and/or some growth hormones. *F. oxysporum* is an endophytic specie identified and isolated from *Cola acuminata*. *F. oxysporum* akin to other endophytic fungi have the capacity to produce a wide range of secondary metabolites with varied biological activities according to compounds produced. Several compounds isolated from *Fusarium* species have demonstrated antiparasitic, antiviral, antibacterial and antifungal activities [7] thereby justifying the antifungal activities of *F. oxysporum* observed on *Candida albicans* isolates.

The resistance profile of clinical isolates against commonly used antifungals at the Yaoundé General Hospital to treat candidiasis in HIV-positive patients varied from one class of compound to another or from drug to the other. Of the 7 isolates studied, 6/7 were resistant to polyene family molecules (Amphotericin B and Nystatin). In the azole family, the resistance profile varied among the different isolates. Four over seven (4/7) isolates were resistant to Fluconazole, Miconazole and ketoconazole. Such results show a very high proportion of strains resistant to antifungals used against candidiasis in HIV patients in Cameroon. These results are comparable to those obtained by Njunda et al. [25] at the Douala Nylon District hospital where resistance to antifungal drugs was also observed. This resistance can be explained by the fact that in Cameroon, most immunocompromised HIV patients with symptoms of candidiasis very often receive drugs from the azole family without a prior antifungigram.

The culture conditions were modified by growing the endophytes in the presence of several compounds as supplements. Hence, depending on the nature of the compounds, the growth of mycelia was varied, and the production of secondary metabolites was modified. Indeed, several compounds promoted mycelium growth and/or secondary metabolites production compared to the unsupplemented negative control. Such results suggest that these compounds could be silent gene activators. This suggestion verifies with studies conducted by Bergmann et al. [26] wherein, several clusters of secondary metabolic genes are silent under certain standard laboratory conditions. In addition, using genetic engineering techniques, co-culture with certain chemical compounds [11,27-29] has been successfully applied in recent years to activate these silent gene clusters in filamentous fungi and to induce the production of several new active metabolites. The growth optimization of *F. oxysporum* showed variable effects on mycelial growth and secondary metabolites production. Among the molecules used for this optimization; sodium citrate, potassium phosphate, potassium chloride, ammonium sulfate, tryptophan, proline, tannic and dinitrosallicylic acids promoted mycelial development. This observation suggests that these molecules would facilitate either the assimilation of nutrients or stimulate the synthesis of compounds necessary for mycelial growth, or even the substrates for the endophytes. Depending on the compounds used for optimization, the effect on secondary metabolites production was shown to be both beneficial and detrimental. For example, nitrogen and carbon sources are the most important elements for mycelial growth and bioactive metabolites production by *F. oxysporum* as demonstrated by other authors [30-31]. In this study, starch showed to be a good source of carbon in the production of bioactive metabolites by *F. oxysporum* whereas their activity against *C. albicans* was less than that obtained with glycerol. These results are similar to those obtained by Verma et al. [31]. Regarding nitrogen source, thiourea increases metabolite synthesis while inhibiting mycelial growth. However, Peighami–Ashmaei et al. [32] described the importance of various nitrogen sources on the growth of *Pseudomonas fluorescens* and *Bacillus subtilis*. Thus, the negative effect of thiourea compared to that of urea and ammonium sulfate on mycelial growth suggest that thiourea could be an inhibitor of growth factors for endophytes. Upon this observation, the importance of ions in endophytic is more emphasized since the quantity and quality of metabolites produced changed. For instance, *Fusarium oxysporum* uses Na^+ ion to better elaborate its metabolites while Toropova et al. [33] have shown the importance of Mg^{2+}, Mn^{2+} and Fe^{2+} ions for the production of antibiotics by *Hypomyces rosellus* due to their participation as biochemical cofactors in the biosynthetic pathways of the different classes of secondary metabolites. These ions have equally been shown to be important for antibiotic and pigment formation by fungus, with Mg^{2+} and Mn^{2+} ions being interchangeable. Amino acid supplements may play a role by exchanging their carbon and nitrogen skeleton in the process of primary and secondary metabolism of microorganisms [31,34]. The use of small molecules as elicitors seems to be a powerful tool to stimulate metabolic pathways to produce new compounds or to increase the production of desired compounds.

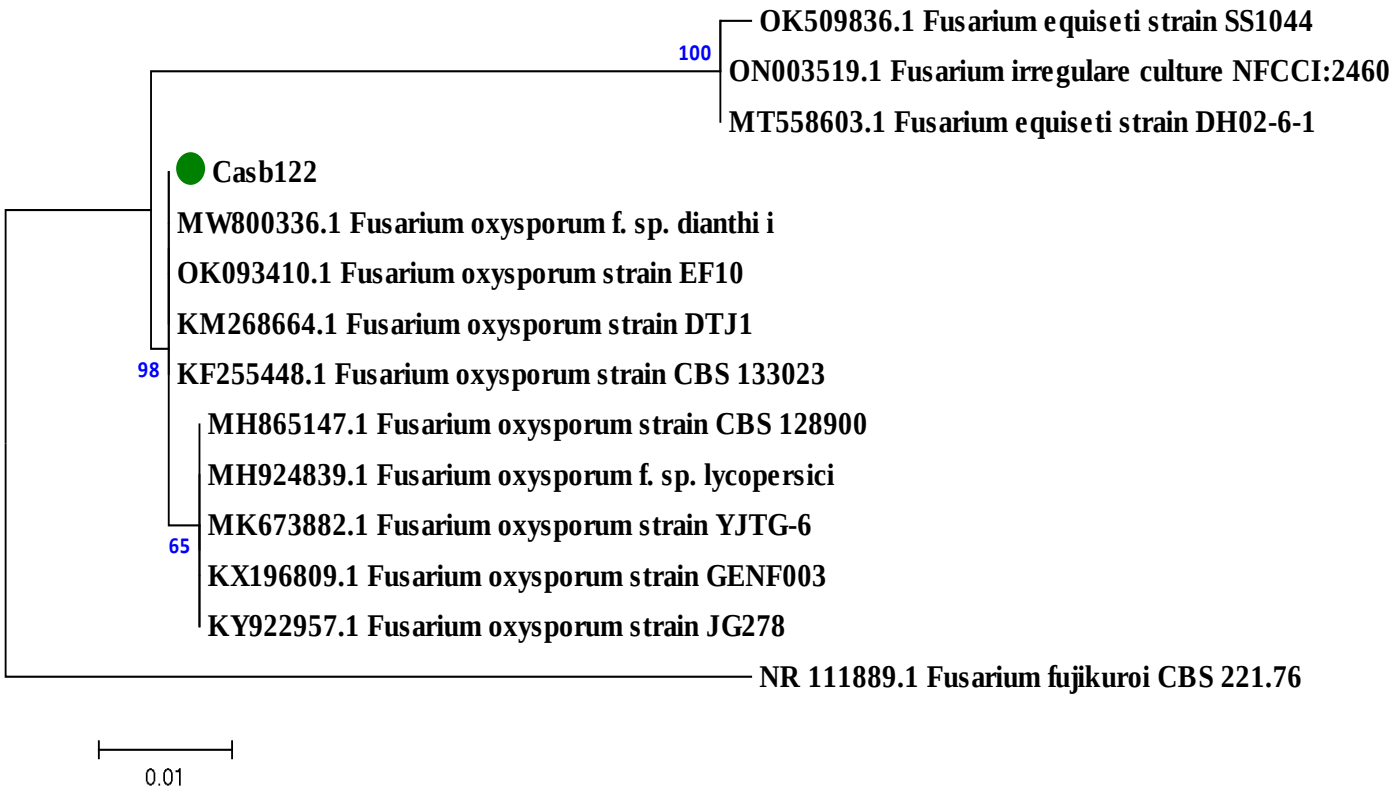

Figure 1. Molecular Phylogenetic analysis **of** isolate Casbl22 by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [1]. The tree with the highest log likelihood (-843.84) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.1654)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 435 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. *ITS4 of Casb122 sequence available under request to the corresponding author.*

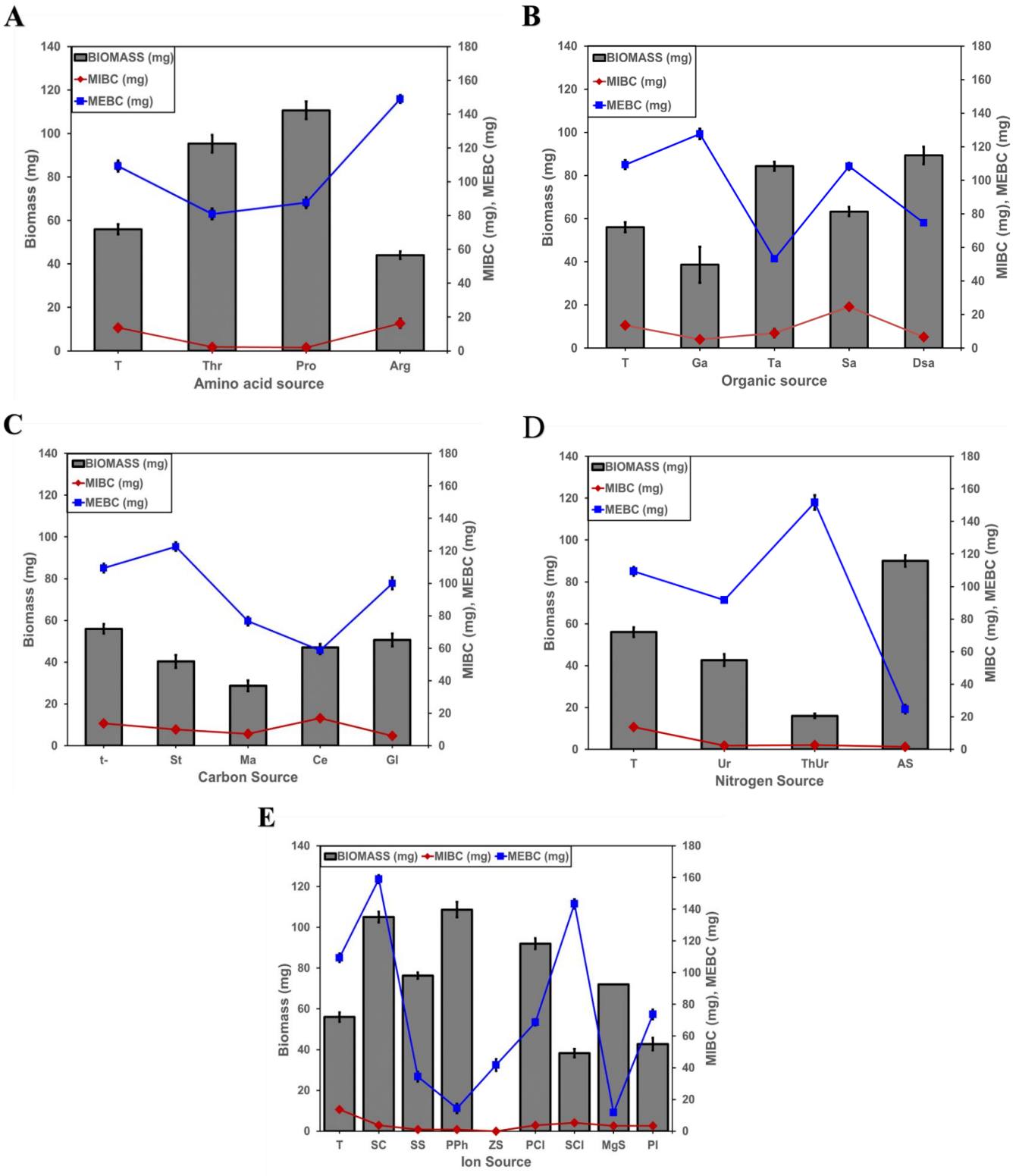

Figure 2. Effect of different molecules on growth optimization and bioactive metabolites production *of Fusarium oxysporum*.

MEBC; Mass of extracellular bioactive compound; MIBC: Mass of intracellular bioactive compound; T: negative control; Pr: proline; Thr: tryptophane; Arg: arginine; Ga: gallic acid; Ta: tannic acid; Sa: sulfanilic acid; Dsa: dinitrosalicylic acid; St: starch; Ma: maltose; Ce: cellulose; Gl: glycerol; Ur: Urea; ThUr: thiourea; AS: ammonium sulfate; SS: sodium sulfate; SC: sodium citrate; PPh: potassium phosphate; ZS: zinc sulfate; PI: potassium iodide; PCl: potassium chloride; SCl: sodium chloride; MgS: magnesium sulfate

Table 1. Susceptibility pattern of antifungals to *Candida albicans* isolates.

	***C.a*Is1**	***C.a*Is2**	***C.a*Is3**	***C.a*Is4**	***C.a*Is5**	***C.a*Is6**	***C.a*Is7**
Econazole	sensitive	sensitive	resistant	sensitive	sensitive	sensitive	sensitive
Miconazole	sensitive	sensitive	sensitive	resistant	resistant	resistant	resistant
Ketoconazole	sensitive	resistant	sensitive	resistant	resistant	sensitive	resistant
Fluconazole	resistant	sensitive	sensitive	resistant	resistant	resistant	sensitive
Amphotericin-B	resistant	resistant	resistant	resistant	sensitive	resistant	resistant
Nystatin	resistant	resistant	resistant	resistant	sensitive	resistant	resistant

*C.a*Is: *Candida albicans* isolates

Table 2. MIC (µg/mL) of *Fusarium. oxysporum* endophyte extracts against seven *Candida albicans* clinical isolates

	Human pathogenic *Candida albicans* (*C.a*) isolates						
	***C.a*Is1**	***C.a*Is2**	***C.a*Is3**	***C.a*Is4**	***C.a*Is5**	***C.a*Is6**	***C.a*Is7**
*Ca*Et122 Int	2000	2000	2000	2000	2000	2000	2000
*Ca*Et122 Ext	1000	1000	500	1000	1000	1000	1000
Fluconazole	128	32	64	128	128	128	32

*Ca*Et122 Int: bioactive intracellular compounds of *Fusarium oxysporum*; *Ca*Et122 Ext: bioactive extracellular compounds of *Fusarium oxysporum, C.a*: *Candida albicans; C.a*Is: *Candida albicans'* isolates

Table 3. MIC (µg/mL) of supplement *F. oxysporum* endophyte extracts against seven *Candida albicans* clinical isolates

Clinical isolates of *Candida albicans* (*C.a*)	**Supplements**	**MIC (µg/mL)**						
		*C.a*Is1	*C.a*Is2	*C.a*Is3	*C.a*Is4	*C.a*Is5	*C.a*Is6	*C.a*Is7
Bioactive extract from *F. oxysporum* supplemented with	NS	1000	1000	500	1000	1000	1000	1000
	Sulfanilic Acid	1000	**500***	1000	1000	1000	1000	1000
	Glycerol	**500***	**125***	1000	1000	**125***	1000	1000
	Soduim citrate	1000	1000	1000	**250***	1000	**250***	1000
	Sodium chloride	1000	**250***	500	1000	**250***	1000	1000
	Potassium chloride	1000	**125***	**250***	1000	1000	1000	**125***
	Thiourea	**500***	1000	1000	1000	1000	1000	**62.5***
	Potassium Iodide	1000	**250***	1000	1000	1000	1000	1000

*: concentration low than those of No Supplemented (NS) bioactive extract (first line value); MIC: Minimal Inhibitory Concentration; *C.a*: *Candida albicans; C.a*Is: *Candida albicans'* isolates

Table 4. Cytotoxic Concentration (CC_{50}) of different extracts

	Supplements	CC_{50} (µg/mL)	Interpretation
Bioactive extract from *F. oxysporum* supplement	NS	> 200	No Cytotoxic
	Potassium chloride	> 200	No Cytotoxic
	Glycerol	> 200	No Cytotoxic
	Sodium chloride	> 200	No Cytotoxic
	Thiourea	> 200	No Cytotoxic
	Sodium citrate	> 200	No Cytotoxic

NS : no supplemented.

Conclusion

Although some supplements have rather decreased the production of the bioactive extracellular compounds, the antifungal activity of glycerol and potassium chloride demonstrated an increase. This increase in activity could indicate that glycerol and potassium chloride favor either the production of new compounds or the production of one family of compounds to the detriment of other compounds or family of compounds. Optimization of metabolites production in endophytes would further promote their activities. Such results endow for the use of endophytes as an alternative to the use of plants, thereby justifying the up-growing interest in research on endophytes for the last 20 years.

Abbreviations

BLAST: Basic Local Alignment Search Tool
ML: Maximum Likelihood
$CC_{50:}$ Cytotoxic concentration that reduce cell viability to 50%
CFU: Colony forming unit
DMEM: Dulbecco's Modified Eagle *Medium*
DMSO: *Dimethyl sulfoxide*
DNA: Deoxyribonucleic acid
dNTP: deoxyribonucleotide triphosphate
HIV: Human Immunodeficiency Virus
MIC: Minimal inhibitory concentration
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCBI: National Center for Biotechnology Information
PCR: Polymerization chain reaction

Authors' Contribution

MMH, FSP, SV and TKRM carried out the study; FSP, TKRM and KJR analyzed the results; FSP wrote the manuscript; KJR designed the experiments, supervised the work; all authors read and approved the final manuscript

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Conflict of interest

The authors declare no conflict of interest

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