ENZYME AND PHYTOCHEMICAL SCREENING OF ISOLATED ENDOPHTIC FUNGI FROM SOUTHEAST PLANTS OF ALGERIA

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Abstract

Fungi that dwell internally in hosts that appear to be healthy and asymptomatic are known as endophytic fungi. For decades, fungi have demonstrated their value as sources of natural compounds for both industrial and biomedical development. This study aimedto identify some endophytic fungi, which were associated with the stems, leaves and roots of southeastAlgerianplants. Eleven endophytic fungi were evaluated for their ability of producing extracellular enzymes such as amylase, cellulase, laccase, lipase, protease, ligninase and protease on solid media, five endophytics were selected to determine the phytochemical composition such as alkaloids, flavonoids , phenols, tannins and saponins of their extracts. Cellulase activity was detected in 27.27% of the fungus that were tested for enzyme activity, 27.27% for amylase and 18.18% showed for ligninase. The phytochemical analysis revealed alkaloid as the only composition present in all selected endophytic's extracts. This study can be regarded as a preliminary work, endophytic fungi of high activity are proposed as possible resources for industrial applications.

Keywords: Endophytic Fungi, Extracellular Enzymes, phytochemicals, Sahara plants, Algeria

Introduction

The twenty-first century is marked by high population expansion, modernization and consumerism (Ayukekbong et al., 2017, Vasan et al. 2019). These generate a diverse range of problems, particularly, those induced by major industrial wastes and the overuse of pesticides (Ben Slama et al., 2021). establishing an eco-friendly lifestyle has been put into the spotlight recently by researchers(Khare et al.,2018), endophytes are microorganisms that live in the interior of host's tissues and perform ecological functions without affecting the host (Naik et al., 2021).These agents synthesize bioactive compounds that are comparable to those produced by the host plant (Yu et al., 2014). Additionally, secondary metabolite chemical of endophyticfungicontain antibacterial, antiparasitic, cytotoxic, anti-inflammatory, anticancer, antioxidant, and neuroprotective properties (Malhadas et al., 2017) among them: alkaloids, flavonoids, steroids, terpenoids, phenols, saponins tannins and cardiac glycosides (Desire et al., 2014).Furthermore, extracellular enzymes secreted by fungal endophytes include cellulase, lipase, amylase,laccase, protease and ligninase. Indeed, these enzymes play an

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essential role in biodegradation and hydrolysis, both of which are important in protecting against invading pathogens and in acquiring nourishment from the host plant(Sunitha et al.,2013). This study seeks to isolate endophytic fungi from four south east Algerian Saharan plants including:*ZygophyllumcornutumCoss.*, *MalcolmiaaegyptiacaSpr.*,*Phoenixdactylifera L.*,*Cyperusrotundus L*. to evaluate their biotechnological potential as extracellularcellulase, laccase, amylase, lipase, protease, ligninasenzyme producers and phytochemical screening (alkaloids, flavonoids, polyphenols, tannins and saponins) of endophytic fungal crude extracts.

Material and Methods

Source of endophytic fungi

Plant samples were collected from 4 regionsin southeast Algeria: leaves of *Phoenix dactylifera L*. from Touggourt (33° 06' 00"N 6° 04’ 00"E), leaves of *Malcolmiaaegyptiaca Spr*., stems and roots of *Cyperusrotundus L*. fromOuedSouf(33°22'4.12"N 6°51'5.91"E); leaves and stems of *ZygophyllumcornutumCoss*. Respectively from Sidi Mahdi (33° 4'18.27"N 6°5'43.14"E) and SidiSlimane (33°17'22.42"N 6° 5'33.16"E). These were cut at random with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The plant materials were transported to the laboratory within 12 h and stored at 4°C until isolation.

Isolation and identification of endophytic fungi

Isolation of endophytic fungi from 4 Algerian Saharan plants including:*ZygophyllumcornutumCoss*., *MalcolmiaaegyptiacaSpr.*,*Phoenix dactylifera L*. and*Cyperusrotundus L*. was carried out by using method of (Lakhdari et al., 2021).Leaves, stems and roots were cut into 1 cm long pieces and sterilized in series with 70% ethanol for 1 min, 1.0% sodium hypochlorite (NaClO) (v/v) for 1 min, and then cleaned with two sets of sterile distilled water.The sterile samples were put on plates, five fragments per dish with five dishes for each plant, each containing water agar (WA), potato-carrot (PCA), and oatmeal agar (OMA). The parafilm-wrapped petri dishes were incubated at 25±2° C until the fungal hyphae from the samples began to form.

Extracellular enzyme screening of pure cultures

Using qualitative techniques, pure cultures of endophytic fungi were screened for the production of extracellular enzyme activities (Amylases, Cellulase,Laccasse,Ligninase,Lipases, and Protease). All tests were performed in triplicate.

Amylase activity

Glucose yeast extract-peptone (GYP) agar medium (glucose 1g, yeast extract 0.1g, agar 15 g, distilled water 1L, and pH 6) containing 1% soluble starch was used to test the amylase production. The plates were flooded with 1% and 2% potassium iodide after incubation. The colony's surrounding clean zone indicated the production of amylase (Agrawal et al., 2016).

Cellulase activity

Cellulase-producing fungi testing each fungal isolate (5 mm agar disc) was grown for 4 days at 30°C on cellulose Congo red medium. This was achieved by filling all Petri dishes with Congo red solution (1% w/v), allowing them to sit at room temperature for 15 minutes, then draining the solution before

adding 1 mol/lNaCl for a while (Sopalun and Iamtham,2020).

Laccase activity

Activity of Laccasewas formed by growing the selected isolates in Czapek-Dox medium (3g $NaNO_3$, 1g K_2HPO_4, 0.5g MgSO4.H2O, 0.5g KCl, 30g Sucrose, 0.01g FeSO4, 15g Agar). After 3-5 days of incubation, the fully formed cultures were flooded with 0.2g of Bromophenol blue. The laccase activity was determinated by the formation of the halo around the colony (Amirita et al., 2012).

Lignocellulolytic activity

Enzymatic lignin hydrolysis was examined using the technique of with few necessary adjustments. Tannic acid media (TAM) [5.0 g tannic acid, 15.0 g malt extract agar, 20.0 g agar, and 1000 ml of distilled water] was autoclaved at 121°C for 15 min and poured into petri dishes at 20 ml per plate. A 5 mm diameter agar disc that was taken out of an old pure culture and put in the center of each TAM plate was then incubated for 4 days at 28 2°C and maintained in total darkness for four days. The degradation of lignine was reported by formation of dark brown pigments as which was a result of polyphenol oxidase (PPO) activity in the inoculation area (Saili et al., 2014).

Lipolytic activity

For this activity, the culture medium is Peptone Agar (peptone 10g, NaCl 5g, CaCl 2H 0.1g, agar- 16g, distilled water-1L; pH 6.0) supplemented with Tween 20 separately sterilized and added 1% to the medium.A visible precipitate that appeared around the colony by the end of the incubation time as a result of the synthesis of calcium salts of the lauric acid produced by the enzyme, indicated positive lipase activity (Sunitha et *al*.,2013).

Protease activity

Proteolytic activity is established by cultivating the strains on sterilized GYP culture media agar that contains 0.4% gelatin separately sterilized (8g of gelatin in 100ml distilled water). After incubation, the saturated aqueous ammonium sulphatewas poured onto the culture. The clear zone surrounding the colony denotes the hydrolysis of gelatin in the media, and ammonium sulphate precipitates the unhydrolyzed gelatin (Amirita et *al*., 2012).

Relative Enzyme Activity (RA)

Enzyme activity was estimated by measuring the diameters of the clear zones (activity zone) that formed around the fungus colonies in each replicate. The measurement was repeated in two orthogonal dimensions, and the mean value was reported. The RA was calculated using the formula:

Relative enzyme activity = (Clear zone diameter- Colony diameter)/Colony diameter

Isolates with a RA were considered to have ‘significant activity’(Bradner et *al*., 1999, Duncan et al., 2008).

Phytochemical screening of secondary metabolites

The crude extract obtained was used to screen five metabolites (Laib et al., 2022). Five isolates were tested for phytochemical screening. all test were done in duplicates(Devi et al., 2012).

Alkaloids

The fungal extract was dissolved in diluted HCL, filtered and then treated with nesslerreagent, the presence of alkaloids was detected by the production of a yellow colored precipitate. In a boiling water bath, the fungal crude extract was evaporated to dryness. 2N HClwas used to dissolve the residue. The mixture was divided into 3 equally parts. One part was treated with a few drops of Mayer reagent, another with an equal

quantity of dragondroff reagent and the last portion with an equal amount of Wagner's reagent. The presence of alkaloids is indicatedby the appearance of creamy, orange and brown precipitate.

Flavonoids

5-10 drops of diluted HCl and a little piece of zinc were added to a test tube holding 0.5 mL of crude extract, and the solution was heated for a few minutes. Flavonoids produced a reddish pink or dirty brown color.

Phenols

The extract is dissolved in 5 mL of distilled water. A few drops of neutral 5% ferric chloride solution are added to this. A dark green color revealed the presence of phenolic compounds.

Tannins

The alcoholic $FeCl_3$ reagent was used to treat the fungal crude extract. A bluish black color, which vanishes on addition of a little dilute H_2SO_4 was followed by the formation of yellowish brown precipitate.

Saponins

The Frothing test was used to assess the presence of saponins. The fungal extract crude dry powder was forcefully mixed with distilled water and left to stand for 10 minutes. No froth shows the lack of saponins, while more than 1.5 cm of stable froth indicates the presence of saponins.

Results and discussion

In our study, 11 species were isolated from stems, leaves and roots of plants samples the results were presented in Table 1.

Table 1: Endophytic fungi isolated from plants samples

Endophyte isolates	**Plant**	**Area**
ASPERGILLUS FLAVUS	Stems of*ZygophyllumcornutumCoss.*	Sidi Mahdi
*ASPERGILLUSNIGER*SP$_1$	Leaves of *Malcolmiaaegyptiaca Spr.*	OuadSouf
*ASPERGILLUSNIGER*SP$_2$	Stems of *ZygophyllumcornutumCoss.*	Sidi Mahdi
ASPERGILLUS TERREUS	Leaves of *Phoenixdactylifera L.*	Touggourt
*BIPOLARIS*SP	Stems of *ZygophyllumcornutumCoss.*	Sidi Mahdi
*CHAETOMIUM*SP	Leaves of*Cyperusrotundus L*.	OuadSouf
*FUSARIUM*SP$_1$	Leaves of *ZygophyllumcornutumCoss.*	SidiSlimane
*FUSARIUM*SP$_2$	Roots of*Cyperusrotundus L*.	OuadSouf
*FUSARIUM*SP$_3$	Leaves of *Malcolmiaaegyptiaca Spr.*	OuadSouf
PENICILLIUM SP	Leaves of *Phoenix dactylifera L*.	Touggourt

*TRICHODERMA*SP	Leaves of *Phoenix dactylifera* L.	Touggourt

Extracellular enzyme assay was carried out to investigate the potentialendophytic organisms to produce extracellular enzymes (amylase, cellulase, laccase, lignase, protease and lipase). Fungal digestion of substrates suspended or dissolved in agar was used to measure the activities (Table 2).

Three endophytics were able to produce cellulase: *Aspergillusflavus*, *Aspergillusterreus*and *Chaetomium*sp indicated by the appearance of the clear zone around the colony (Fig.01). *Aspergillusflavus*,*Aspergillusterreus*and*Fusarium*sp_3 produced amylase (Fig.02), for ligninase activity *Fusarium*sp_1 and *Fusarium*sp_3were reported to form a dark brown pigments (Fig.03).

Positive enzymatic activity was determined by the measuring the clear zone around the colony the resultants are reported in Table 3.

The results of phytochemicale analysis of the fungus crude extract are presented in Table 4.

Table 2: Endophytic fungi with potential for producing industrial enzymes

ENZYME ACTIVITY	CELLULASE	LACCASE	AMYLASE	LIPASE	PROTEASE	LIGNIN
ASPERGILLUS FLAVUS	+	-	+	-	-	-
ASPERGILLUSNIGER SP_1	-	-	-	-	-	-
ASPERGILLUSNIGER SP_2	-	-	-	-	-	-
ASPERGILLUS TERREUS	+	-	+	-	-	-
*BIPOLARIS*SP	-	-	-	-	-	-
*CHAETOMIUM*SP	+	-	-	-	-	-
FUSARIUMSP_1	-	-	-	-	-	+
FUSARIUMSP_2	-	-	-	-	-	-
FUSARIUMSP_3	-	-	+	-	-	+
*PENICILLIUM*SP	-	-	-	-	-	-
*TRICHODERMA*SP	-	-	-	-	-	-

+: presence;-: absence

Table 3: Relative enzyme activity in selected fungi.

	CELLULASE	LACCASE	AMYLASE	LIPASE	PROTEASE	LIGNIN
ASPERGILLUS TERREUS	1.16	-	0.33	-	-	-
ASPERGILLUS FLAVUS	0.27	-	0.26	-	-	-
*CHAETOMIUM*SP.	0.03	-	-	-	-	-
*FUSARIUM*SP$_1$	-	-	-	-	-	0.12
FUSARIUMSP3	-	-	0.17	-	-	0.14

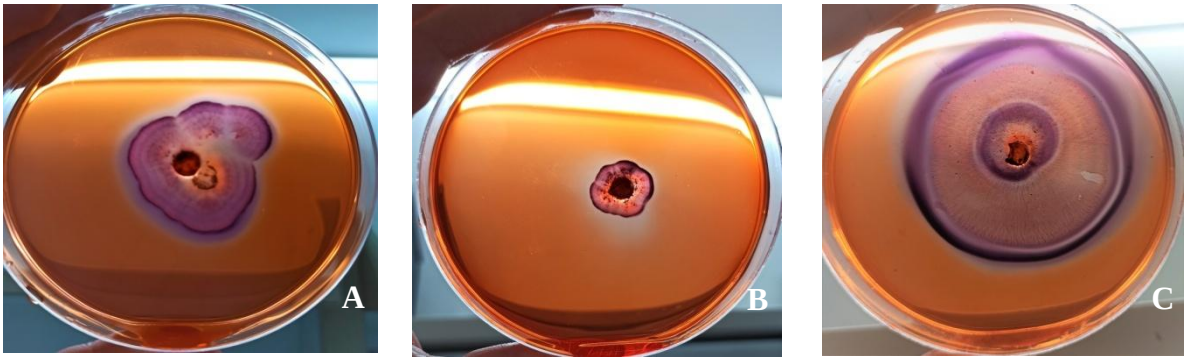

Fig.01- Cellulolytic activity by endophytic fungi: A)*Aspergillusflavus* B)*Chaetomium*sp C)*Aspergillusterreus*

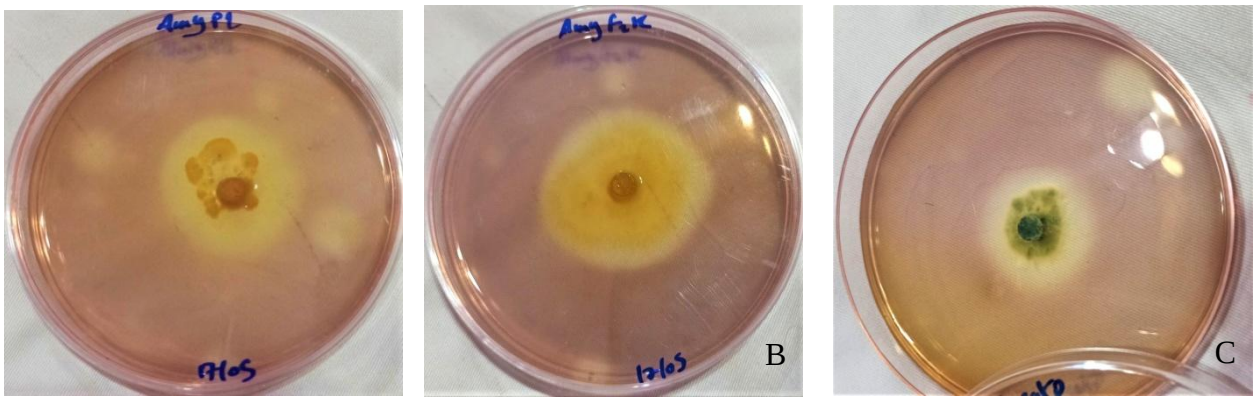

Fig.02- Amyloytic activity by endophyticfungi.A) *Aspergillusterreus* B)*Fusarium*sp3 C)*Aspergillusflavus*

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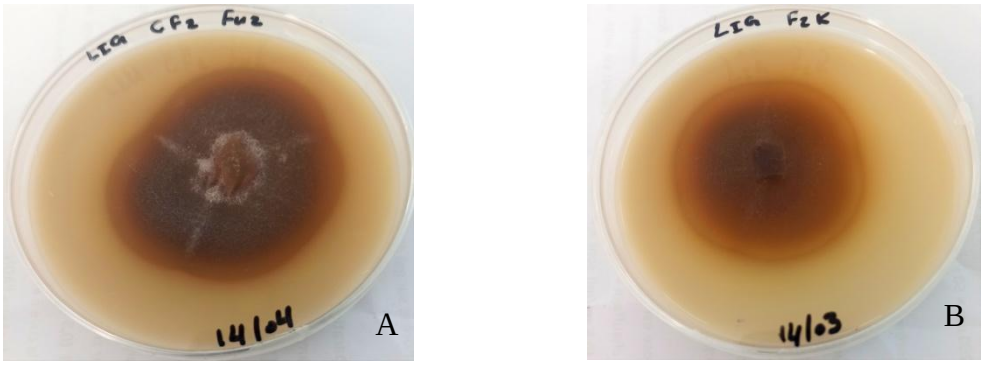

Fig.03- Lignocellulolytic activity by endophytic fungi. A)*Fusarium*sp1 B)*Fusarium*sp3

Table 4: Phytochemical Screening of isolated fungal extracts.

	ALCALOID 1	ALCALOID 2	FLAVONOID	POLYPHENOL	TANIN	SAPONIN
ASPERGILLUSNIGER SP2	+	-	-	-	-	-
*BIPOLARIS*SP	+	+	-	-	-	-
FUSARIUM SP_1	-	-	-	-	-	-
FUSARIUM SP_3	+	+	-	-	-	-
*PENICILLIUM*SP	+	-	-	-	-	-

+: presence **;**-: absence

Endophytes' functional role can be established by determining their patterns of substrate use and the enzymes they produce. This method is a useful and efficient tool for evaluating and characterizing the genetic capacity of endophytic fungi, which proposes its utility in determining various fungal applications. Table 1 lists the fungal isolates that were investigated across the six different enzyme types. five isolates *(Aspergillusflavus, Aspergillusterrus, Cheatomiumsp, Fusarium* sp_1 and *Fusarium* sp_3)showed considerable activity for cellulose 27.27%, amylase 27.27% and ligninase 18.18%.

Concerning the enzymatic activity, the production of cellulase was significant for *Cheatomiumsp, Aspergillusterrus* followed by *Aspergillusflavus*. *Cheatomium*sp displayed moderated cellulolytic activity, supporting the lignocelluloses degrading(Abdel-Azeem et *al*., 2016), These results are consistent with those previously observed for several endophytic fungi (Sharma and Shukla, 2008). Where, *Cheatomium*sp was reported not significant (Sunitha et al., 2013). *Aspergillusflavus* has a high cellulolytic activity (Onofre et *al*., 2013). It was reported the activity of this fungus is not significant (Sunitha et *al*., 2013).*Aspergillus* species produce a wide range of extracellular enzymes, of which amylases and proteases are of substantial economic value(Pandey et *al*., 2000) . In contrast ,no amylolytic activity for *Aspergillussp*and positive one for *Fusarium*sp(Maria et al., 2005),These result are in line with previous study for various endophytic fungi (Hegde, 2011).

Most endophytes are believed to begin to develop when the host plant falls sick or dies and suggested that endophytes may be better able to degrade simple substrates like starch, cellulose, and hemicelluloses than more

complicated ones like lignin(Carroll and Petrini, 1983). In our study only two fungus were able to degrade lignine*Fusariumsp*$_1$ and *Fuzariumsp*$_3$, this result are in conformity with earlier research on several endophytic fungi (Gajera et *al*., 2015,Martinho et *al*., 2019).

The phytochemical study was carried out to ascertain the existence of chemical components as a potential source for medical and industrial usage.Table 4 presents some secondary metabolites that are present in the selected endophytes and highlights the outcomes of the preliminary phytochemical analysis.

Alkaloids were present in an extract from *Aspergillusniger*sp$_2$. Our results are in accordance with earlier reports(Lai et *al*., 2010). Alkaloid and flavonoid were noted absent for *Aspergllus*sp(Hema et *al*., 2015), similarly, reported the presence of alkaloide and absence of flavonoid (AlurappaandChowdappa, 2018). the presence of all selected qualitative metabolites for *Penicillium*frequentans except saponin(Bhardwaj et *al*., 2015), this result is comparable to previous research on a diversity of endophytic fungi (Sharma et *al*., 2016). The chemical composition of endophytic fungi varies depending on the species, however, *Fusarium*sp has only shown presence of Tannin according to our selected bioactive metabolites (Ladoh-Yemeda et al.,2015), Also, the presence of alkaloid, flavonoid and saponin(Flora et *al*., 2022). In deeded, *Bipolaris*sp shows presence of all metabolites except tannin(JagadishandChowdappa, 2021).

Conclusion

Endophytic fungus have a high level of biodiversity and are a good source of new active compounds and may also produce several compounds of pharmaceutical significance, which is currently attracting scientific investigations worldwide. Endophytic fungal isolates was found to be associated with leaves, stem and root of the southeast plants of Algeria. Few isolated endophytesexhibited some kind of enzymatic activity, this indicates that the enzyme production differs between fungus and often corresponds to the requirements of its habitats. Phytochemical analysis was carried out for endophytic fungal extracts to determine the presence of chemical compositions as a potential source for medical and industrial usage. Their existence provides a signal that can be used to enhance the development of synthetic medications by serving as precursors. However, phytochemical analysis has been carried out in several plant species but very few reports are available on endophytes.Further research at advanced molecular level may offer better insights into endophyte biodiversity and the regulation of fungal secondary metabolism.

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