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Original Research Article

Identification and Phytochemical Screening of Endophytic Fungi from Haustoria of *Phragmanthera capitata* (Sprengel) S. Balle (Loranthaceae)

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Abstract

Loranthaceae are epiphytic hemiparasitic plants causing a lot of damage to cultivated or spontaneous woody species, but these parasitic plants are also used to treat a wide spectrum of diseases traditionally. It is with a view to searching for new sources of bioactive compounds that the present study was conducted with the aim of exploring the endophytic mycoflora of the haustoria of *Phragmanthera capitata*. The isolation of the endophytic fungi of *P. capitata* was done on a PDA (Potato Dextrose Agar) medium. The identification was on the basis of macroscopic and microscopic observations using identification keys. The mass culture of the isolated fungi was made on a solid medium based on rice and the extraction with ethyl acetate. 20 endophytic fungi belonging to 7 genera and 2 unidentified species were isolated from *P. capitata: Aspergillus, Trichoderma, Penicillium, Cladosporium, Aureobasidium, Mucor* and *Fusarium*. Flavonoids, tannins, anthocyanins and coumarins were detected in all extracts of these endophytic fungi. The endophytic fungi associated with this plant would also be a potential source of bioactive compounds and the exploration of medicinal plants, and habitats of endophytes would be an advantageous path for the discovery of new species of endophytes and new bioactive secondary metabolites.

Keywords: *Phragmanthera capitata*, Phytochemical screening, Loranthaceae, Endophytic fungi.

INTRODUCTION

Loranthaceae, also called African Mistletoe, are epiphytic hemiparasitic plants that cause a lot of damage to woody plants. These parasitic plants, however, have medicinal properties that several studies have shown. These plants would have many potentials such as antibacterial, antioxidant, analgesic and antipyretic [1, 2]. The Loranthaceae are therefore a source of bioactive compounds to be explored. It turns out that all plants are in continuous interaction with different microorganisms, both pathogenic and beneficial, living on the surface of the plant (epiphytes), in interactions with these roots (mycorrhizae) and even inside (endophytes) [3]. Endophytes are organisms that live in symbiosis with plants and play an important role in protecting them against pests, viruses, fungi and phytopathogenic bacteria. Endophytic fungi have received enormous interest and proved to be a good source of natural bioactive molecules of various structures possessing various therapeutic potential, and which can be used in different industries in agriculture, medicine, food, cosmetics, etc. [4]. The objective of this study is to explore the endophytic mycoflora of the

haustoria of *Phragmanthera capitata* harvested from *Psidium guajava*.

MATERIALS AND METHODS

Isolation and characterization of endophytic fungi isolated from *Phragmanthera capitata*

Collection of plant material

The haustoria of *Phragmanthera capitata* was harvested from *Psidium guajava* in the orchard of the chiefdom of Ndogbong. Harvesting targeted plants showing no visible pathological signs. The sample was put in a sterile plastic bag to be transported to the laboratory and kept at 4°C, for 24 [5, 6].

Isolation and purification of endophytic fungal strains

The haustoria is washed with water to remove epiphytes and debris. In a sterile hood, the sample is cut into fragments, the latter are disinfected in ethanol at 70° for 1 min, followed by immersion in a 2.4% sodium hypochlorite (NaOCl) solution for 30 s. The plant fragments or pieces sterilized in this way are rinsed with sterile distilled water for 1 min, then dried on sterilized filter paper [7]. The fragments are then transferred in Petri dishes containing PDA medium supplemented with Chloramphenicol (0.05 mg/ml) to inhibit any bacterial growth. The boxes are incubated at room temperature, after 48 h daily observations are made. Several subcultures are carried out on PDA medium. The choice of species to sample is made with the naked eye, taking into account the resemblance of the thalli or mycelium and the spores. Once these have been isolated in pure culture, they are subcultured in PDA medium to measure their apical growth rate and observe the macroscopic and microscopic characters after 5 to 7 days of incubation [7]. They are stored at +4°C in screw tubes on inclined PDA agar. All microbiological manipulations are carried out under aseptic conditions in a fume hood using a Bensen burner.

Morphological identification of isolated endophytic fungi

The identification of the fungal strains isolated was done by observation of the cultural characters and by the morphology of the strains isolated on PDA oriented by identification keys [8-10].

Microscopic identification

The microscopic examination of a fungal colony consists of taking a sample from a Petri dish after 2 to 3 days of incubation, using the sterilized platinum loop and performing a spread between slide and coverslip with cotton blue as mounting liquid. Observation is done under an optical microscope with x 10 and x 40 objectives. The structures observed are the mycelium (septate or septate), the nature of the conidiophore (brush or aspergillus head), the conidiogenous cells (biseriate or uniseriate), the presence or absence of zygospore and the spores. Macroscopic photos showing the morphological appearance of the thalli and microscopic photos of the mycelium, conidia and conidiogenesis were also taken.

Mass culture (fermentation) and extraction of secondary metabolites from endophytic strains

Ten fungal isolates were chosen for mass culture, the medium used for the fermentation of endophytic fungi is the solid medium, prepared from 100g rice + 100ml distilled water per 500ml capacity glass jar. This medium thus prepared is closed using a cloth and its lid and autoclaved at 121°C for about 20 min. A few fragments of a young culture of each mushroom from 3 to 5 days old are taken and each introduced into a test tube containing sterile distilled water with the addition of tween 40 then vortexed for a few minutes to form the inoculum. After the medium has cooled, each inoculum is introduced into a jar which is incubated at rest at room temperature. After 4 weeks, 2 ml of chloroform is introduced into each fermentation box 24 hours before extraction to kill fungal spores, thus avoiding any contamination. 400 ml of ethyl acetate are introduced into each jar, maceration lasts 48 hours. the content of each jar is filtered and concentrated in a Heidolph brand rotary evaporator [7]. Each extract was then chemically screened for the presence of secondary metabolites.

RESULTS

Diversity and characterization of endophytic fungi of *Phragmanthera capitata*

Macroscopic and microscopic identification of endophytic fungi

In total, 40 segments or fragments of the haustoria of *Phragmanthera capitata* were examined for isolation of some endophytic fungi, in view of the results obtained previously. After isolation and based on morphological characteristics, 20 fungal endophyte strains were isolated from *P. capitata*. These strains belong to 7 genera: *Aspergillus*, *Trichoderma*, *Penicillium*, *Cladosporium*, *Aureobasidium*, *Mucor* and *Fusarium*. Six genera belong to the class of Hypomycetes divided into hyaline hypomycetes (order *Moniliaceae*): *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp. and *Fusarium* sp. and in dark Hypomycetes (order *Dematiaceae*): *Cladosporium* sp. and *Aureobasidium* sp., only *Mucor* sp. belongs to the class Zygomycetes (order *Mucorales*). However, 2 strains could not be identified due to lack of spore production. The isolation rate (%I) of these endophytic strains in this study is 50%. Shannon-wiever and Simpson indices were determined to assess the diversity and specific dominance of endophytic fungi that colonize the haustorium of *P. capitata*. These indices were 1.82 and 0.15 respectively. The strains of *Aspergillus* sp. and *Penicillium* sp. are the most abundant with the highest frequency and isolation rate (Table 1).

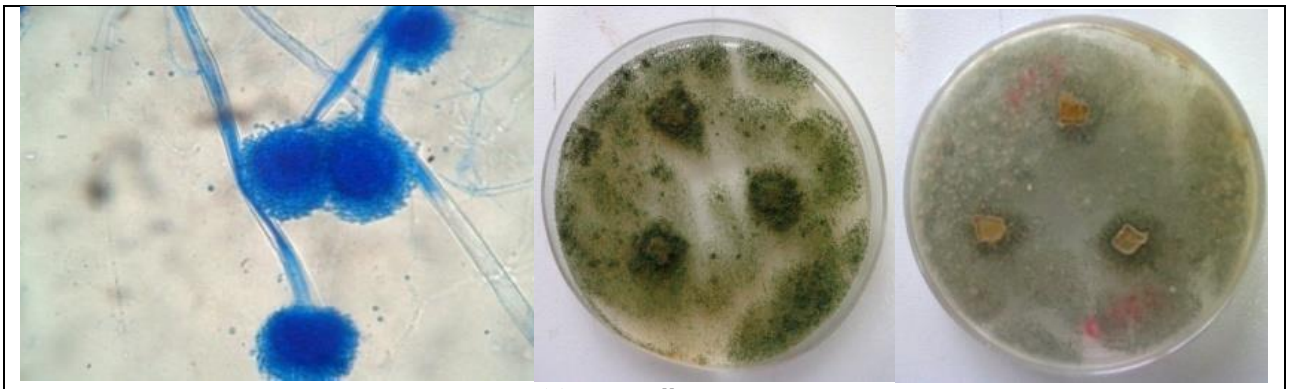
Table-1: Frequency and isolation rate of endophyte strains of *Phragmanthera capitata.*

Strains	Classification	Number of isolates	Frequency (%)	(% I)
***Aspergillus* sp.**	Class: Hypomycetes hyalines Order: *Moniliaceae*	6	30	15
***Penicillium* sp.**		5	25	12.5
***Trichoderma* sp.**		1	5	2.5
***Fusarium* sp.**		3	15	7.5
***Mucor* sp.**	Class: Zygomycetes Order: *Mucorales*	1	5	2.5
***Aurobasidium* sp.**	Class: Dark hypomycetes Order: *Dematiaceae*	1	5	2.5
***Cladosporium* sp.**		1	5	2.5
Not identified		2	10	5
Total	-	20	100	50

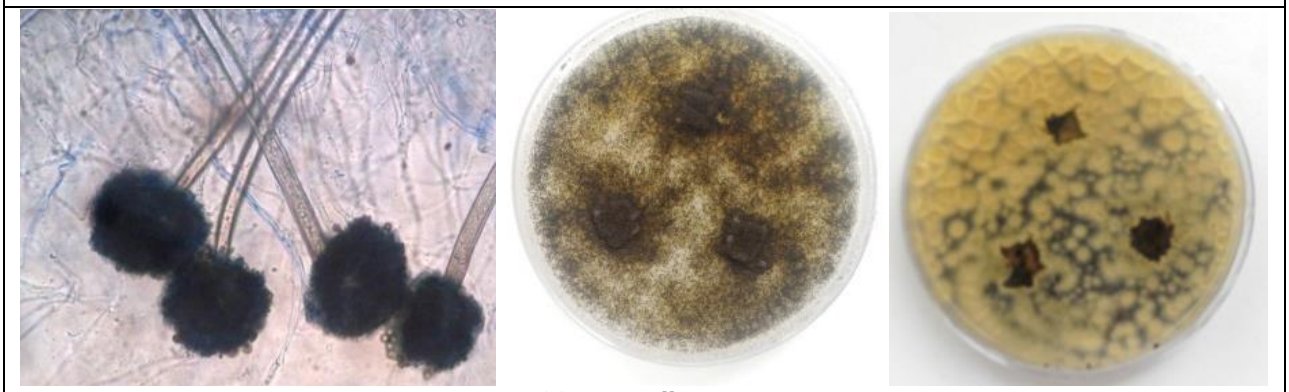
% I: Isolation rate

Identification of the genus *Aspergillus*

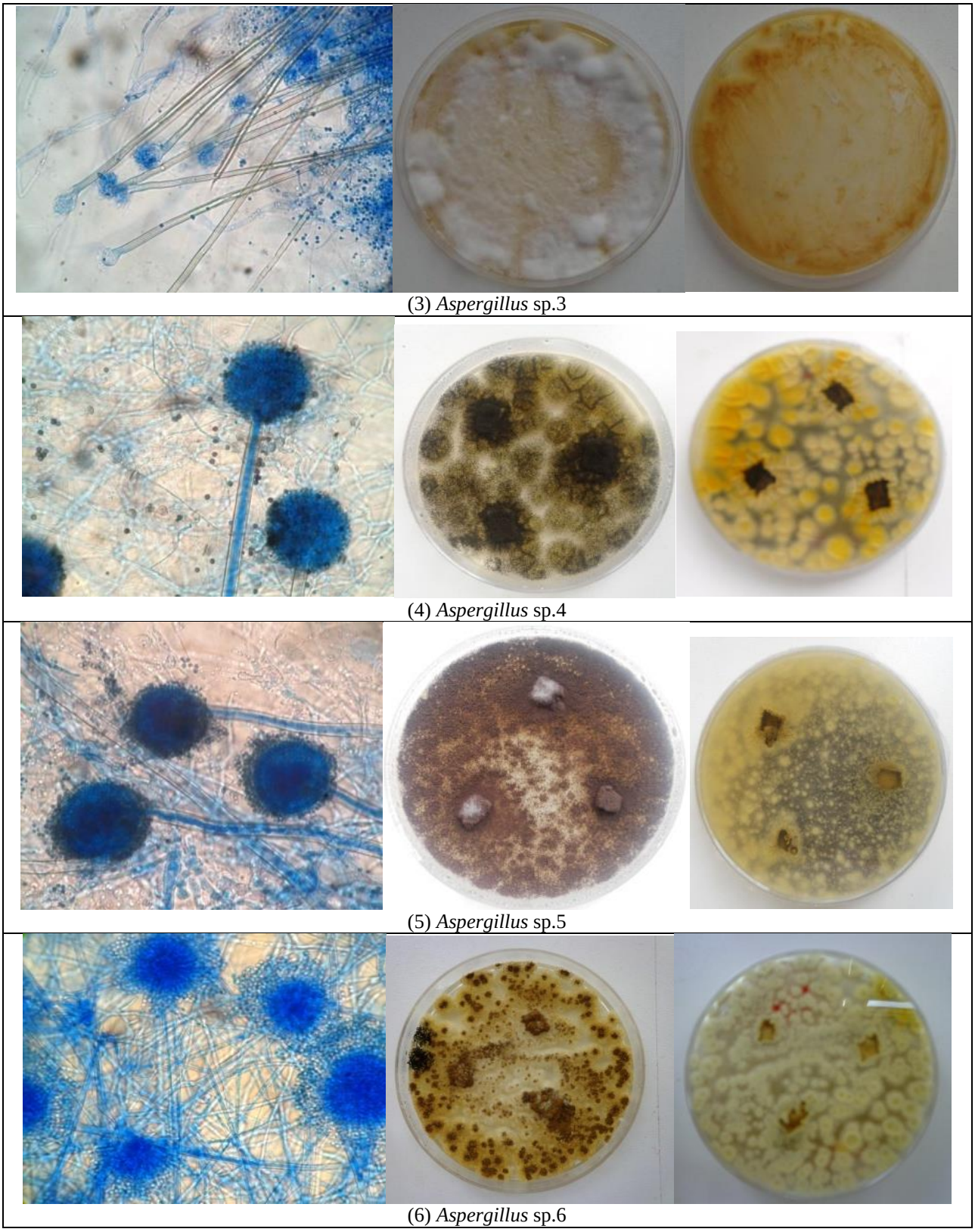
Strains of the *Aspergillus* genus have a characteristic shape and bright colour which makes them quite easily identifiable. Six species of this genus were identified in this study (Figure 1). The mycelial colonies of the isolated species are either fluffy, black and green in colour (*Aspergillus* sp.1, *Aspergillus* sp.2, *Aspergillus* sp.4 and *Aspergillus* sp.5), or cottony, white in colour (*Aspergillus* sp.3), or brown in colour (*Aspergillus* sp.6). The presence of a diffusible yellow pigment was observed for the strain *Aspergillus* sp.4. These strains are also characterized by a septate mycelium and an unbranched conidiophore of variable length and shape, ending in a characteristic bulge or vesicle of the genus called an *Aspergillus* head.



(1) *Aspergillus* sp.1



(2) *Aspergillus* sp.2



(3) *Aspergillus* sp.3

(4) *Aspergillus* sp.4

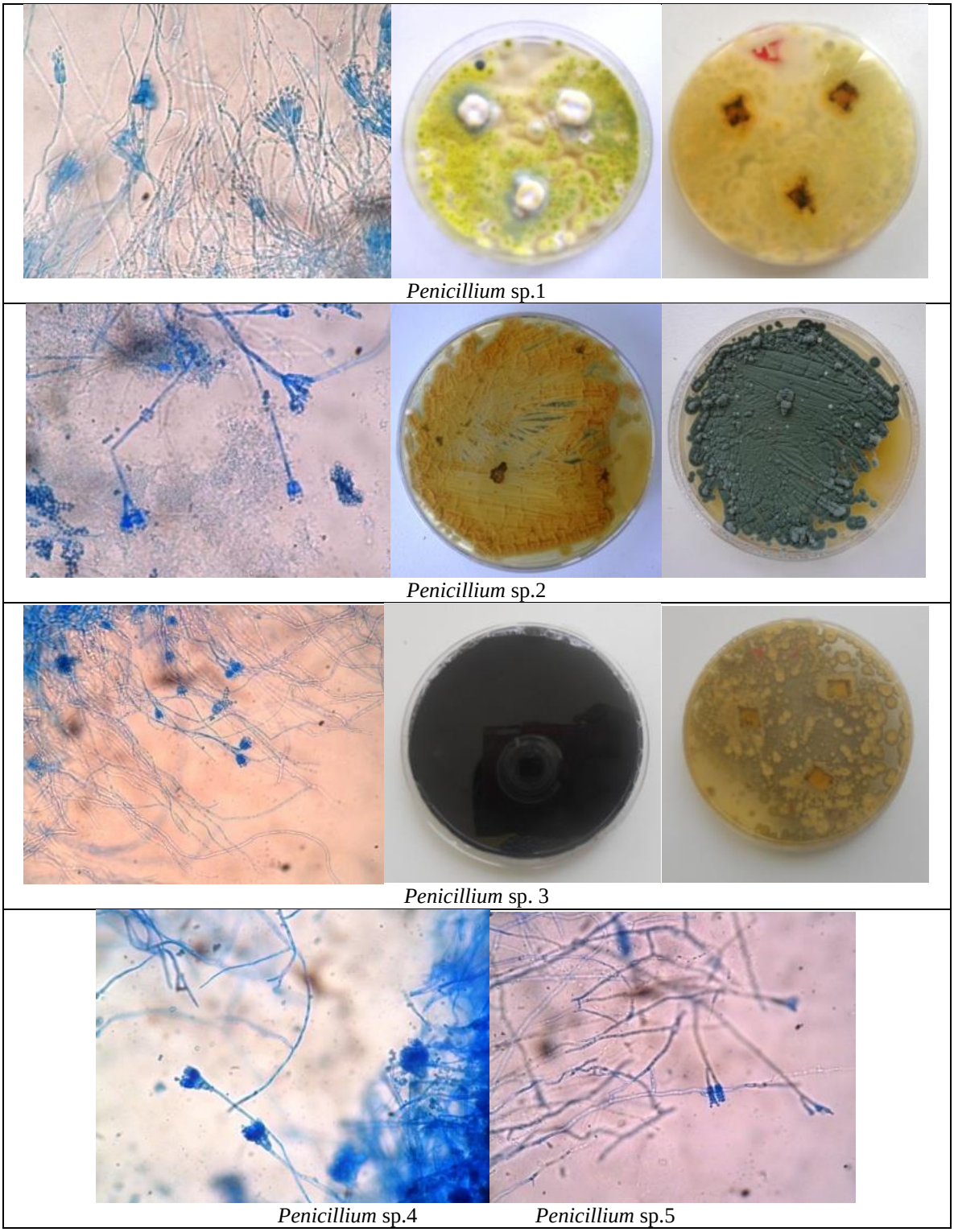
(5) *Aspergillus* sp.5

(6) *Aspergillus* sp.6

Fig-1: Microscopic and macroscopic appearance of species of the genus *Aspergillus* isolated.

Identification of the genus *Penicillium*

The strains of the *Penicillium* genus isolated show powdery colonies of variable green colour (green, lime green, dark green), with the presence of diffusible yellow pigment. They are also characterized by a septate mycelium and a branched conidiophore of variable length and shape ending in a brush. Five species of this genus were identified in this study (Figure 2).



Penicillium sp.1

Penicillium sp.2

Penicillium sp. 3

Penicillium sp.4 *Penicillium* sp.5

Fig-2: Microscopic and macroscopic appearance of isolated species of the genus *Penicillium*.

Identification of the genus *Trichoderma*

The strain of the genus *Trichoderma* presents powdery colonies of white colour initially, then green as it ages. It has a very rapid and extensive growth, with the presence of a diffusible yellow pigment. The mycelium is septate and branched and the conidiophores are branched and have a conical shape. The conidia are gathered in clusters at the top of the phialides and thus form "false heads" (Figure 3).

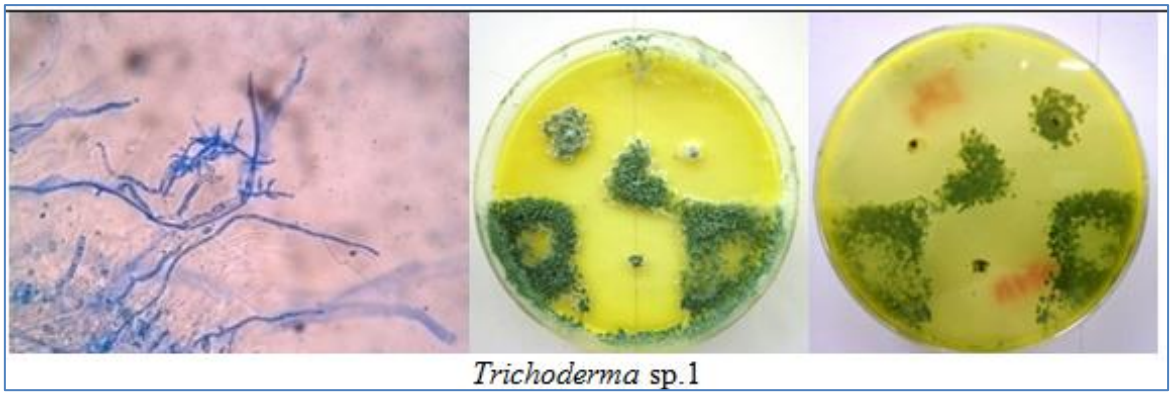


Fig-31: Microscopic and macroscopic appearance of species of the genus *Trichoderma* isolated

Identification of the genus *Fusarium*

Fusarium colonies are woolly pink and/or white in colour. The conidia are spindle-shaped, hence the name *Fusarium* (Figure 4).

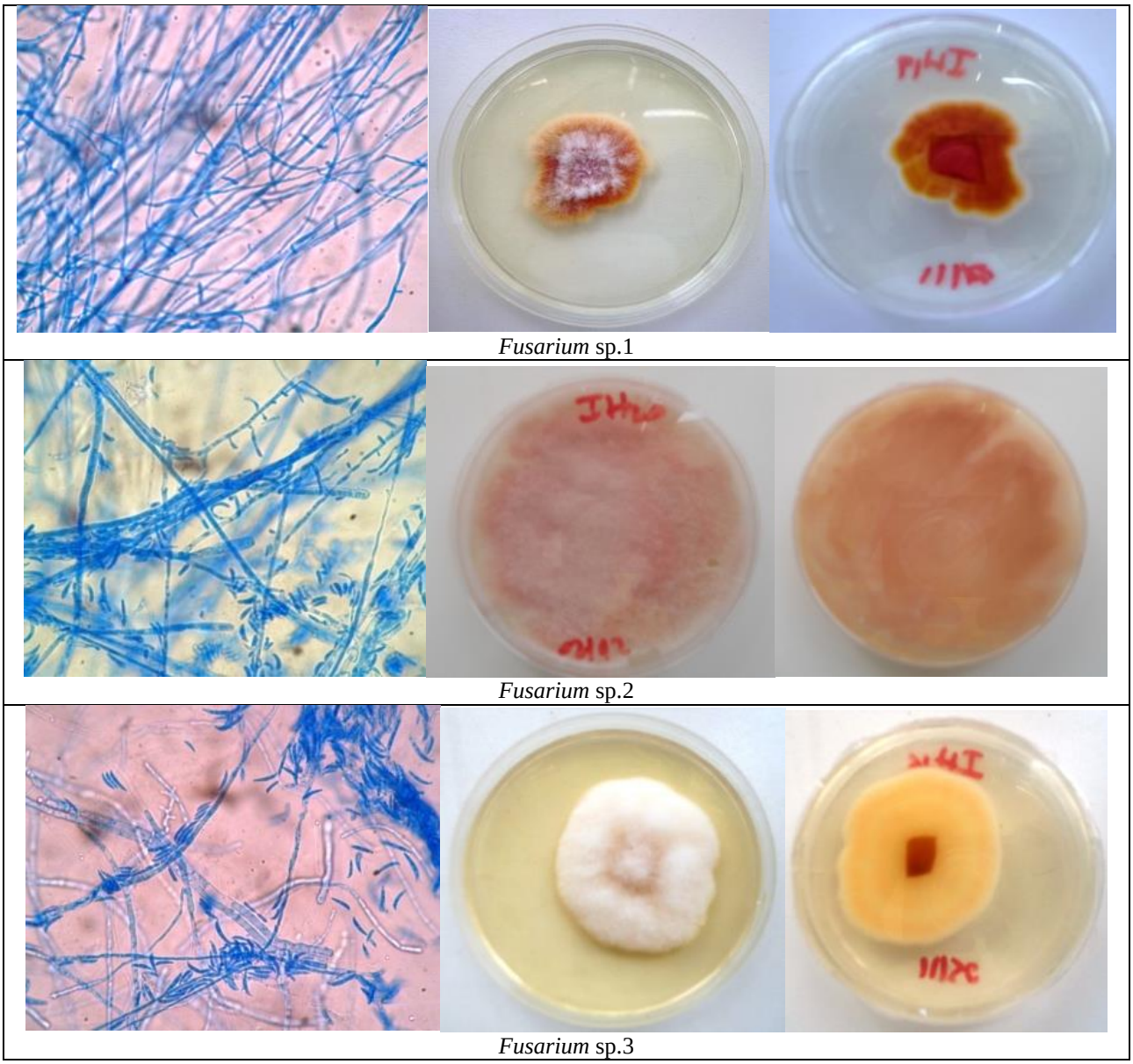
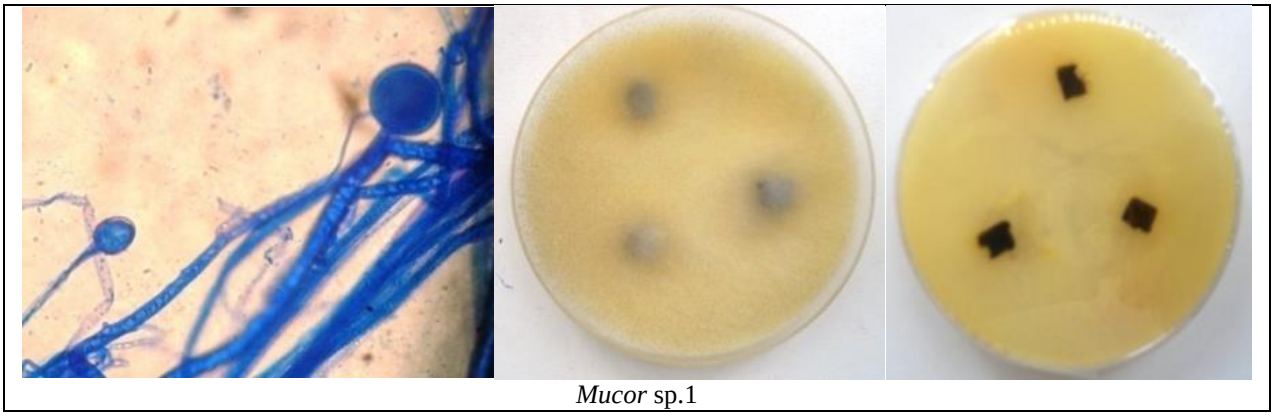


Fig-4: Microscopic and macroscopic appearance of isolated species of the genus *Fusarium*.

Identification of the genus *Mucor*

The colonies are woolly with rapid and extensive growth, grey on the surface and colourless on the back. Filaments or sporangiospores broad and slightly septate terminating in an ovoid columella without apophysis, with the absence of rhizoids (Figure 5).



Mucor sp.1

Fig-5: Microscopic and macroscopic appearance of the isolated species of the genus *Mucor*.

Identification of the genus *Aureobasidium*

Presence of arthroconidia, conidia formed from conidiogenous cells embedded in the filaments (Figure 6).

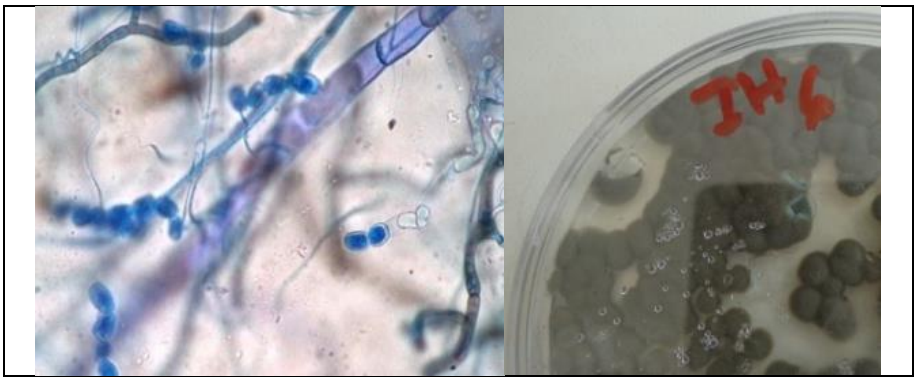


Fig-6: Microscopic and macroscopic appearance of the isolated species of the genus *Fusarium*.

Identification of the genus *Cladosporium*

Slow growing and velvety and powdery texture of olive-green colour and black underside. Septate mycelium and conidiospores of variable length (Figure 7).

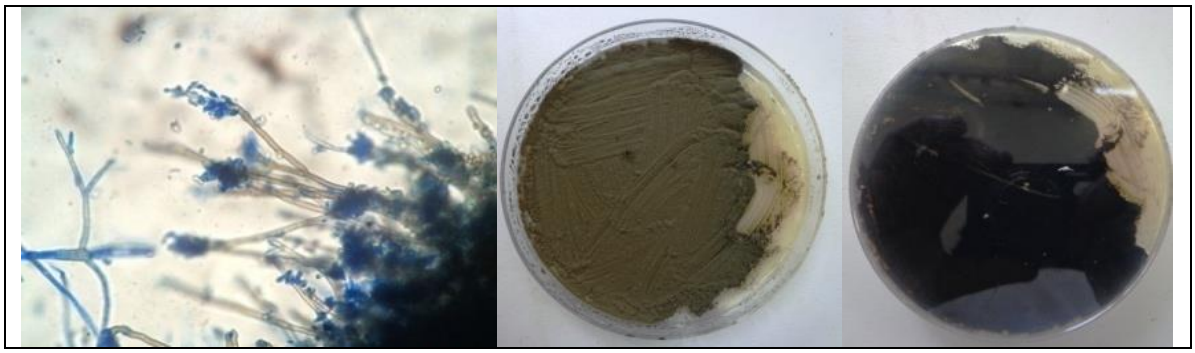


Fig-7: Microscopic and macroscopic appearance of the isolated species of the genus *Cladosporium*

Gender identification

Two isolated strains did not sporulate on PDA medium, hence the difficulty in identifying them (Figure 8).

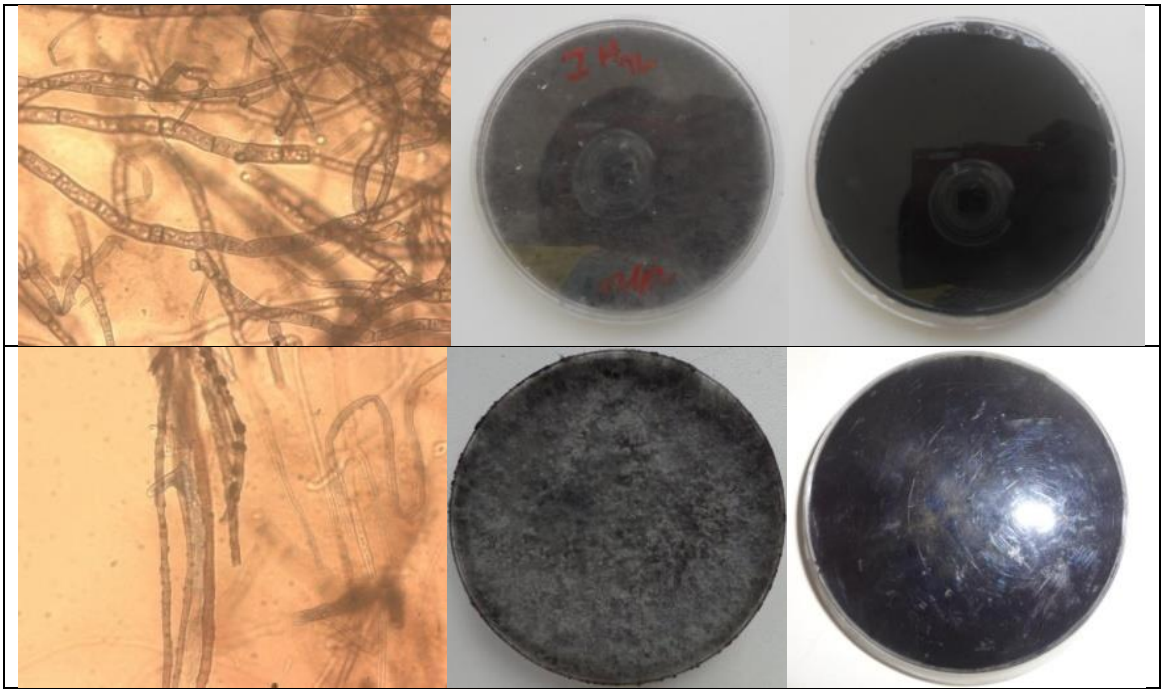


Fig-8: Microscopic and macroscopic appearance of unidentified isolated species.

Chemical screening of endophyte extracts

Ten strains were chosen for extraction and chemical screening. Flavonoids, tannins, anthocyanins and coumarins are present in all endophyte extracts except alkaloids and saponins. Phenols are present only in extracts of *Trichoderma* sp., *Aspergillus* sp.3 and *Penicillium* sp.2. Limonoids are present in all extracts except *Aspergillus* strains and *Penicillium* sp.1 (Table 2).

Table-2: Chemical screening of endophyte extracts.

Fungi extracts	Tri	Asp1	Asp2	Asp3	Pen1	Pen2	Muc	Fus	NI 1	NI 2
Alkaloids	-	-	-	-	-	-	-	-	-	-
Phenols	+	-	-	+	-	+	-	-	-	-
Polyphenols	+	+	+	+	+	-	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+
Anthocyanins	+	+	+	+	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+	+	+	+	+
Limonoids	+	-	-	-	-	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-	-
Sterols	+	-	+	+	-	+	+	-	+	+

Tri: *Trichoderma* sp., Asp1: *Aspergillus* sp.1, Asp2: *Aspergillus* sp.2, Asp3: *Aspergillus* sp.3, Pen 1: *Penicillium* sp. 1, Pen 2: *Penicillium* sp. 2, Muc: *Mucor* sp., Fus: *Fusarium* sp., NI 1 et NI 2: not identified 1 et 2.

DISCUSSION

Mycoflora endophyte of *Phragmanthera capitata*

The haustorium of *Phragmanthera capitata* was selected for the isolation of the fungi in this study and also to compare their secondary metabolites with those of the plant. Numerous studies have shown that endophytic fungi are potential sources of bioactive compounds and these have captured the attention of researchers in recent decades. These endophytes would produce special active metabolites [11, 12].

A total of 20 endophytic fungi were isolated and identified from the haustorium of *Phragmanthera capitata* with an isolation rate of 50%. These fungi belong to 7 genera (*Aspergillus*, *Trichoderma*, *Penicillium*, *Cladosporium*, *Aureobasidium*, *Mucor* and *Fusarium*). Various endophytic fungi have been isolated from medicinal plants and particularly from Loranthaceae species. Ladoh *et al*. [13] reported the presence of 11 endophytic fungal species belonging to 4 genera (*Apergillus*, *Penicillium*, *Trichoderma* and *Fusarium*) in the stems of *P. capitata*. Similar work carried out in Brazil on a Loranthaceae species also revealed the presence of the *Aspergillus* and *Trichoderma* genera as endophyte fungi of *Cladocolea micrantha* [14]. These endophytes have also been isolated from many medicinal plants [15-17]. Generally, species of the genus *Aspergillus* such as *A. fumigatus*,

and *A. niger*, as well as species of the genus *Penicillium* and *Fusarium* adapt to different plant tissues [18]. Several studies have reported Ascomycotina, Deuteromycotina and Basidiomycotina as endophytic fungi [19, 20]. A large number of genera and species of fungi belonging to the first two are able to live endophytically in plant tissues [21]. In this study, the strains isolated belong to the division of Deuteromycotina (*Aspergillus*, *Trichoderma*, *Penicillium*, *Cladosporium*, *Aureobasidium* and *Fusarium*) and Zygomycotina (*Mucor*). The Deuteromycotina were the most abundant. A recent study showed that these endophytes are not specific to a single host [22]. No study had clearly demonstrated the association of endophyte-haustorium fungus in Loranthaceae.

Phytochemical screening of endophyte strains revealed the presence of secondary metabolites such as flavonoids, tannins, anthocyanins, phenols, limonoids and coumarins. The chemical composition of endophytic fungi is variable depending on the species. In this study, the absence of alkaloids and saponins was noted in all mushroom extracts. The ability of an endophyte to produce certain metabolites and not others has been described by Selim et *al.* [23]. Different endophyte strains in a plant can produce different secondary metabolites, each having different functions in the plant. Endophytic fungi play an important role in the physiology of their host plants. They receive nutrition, protection and opportunity for propagation from their hosts and in return offer their protection against insects, and herbivores and also help their hosts to adapt to different stressful conditions in the environment [24-26]. However, these endophytes under certain conditions can become opportunistic microorganisms [27, 28].

The production and quality of bioactive compounds from endophytic fungi depend on the natural conditions of the association and the nature of the synthetic medium used [29]. Gunatilaka [30] in his review on natural products of endophytes, lists the biological activities of metabolites of endophyte fungi such as antibacterial, antiviral, antifungal and anticancer. Strategies should be developed to utilize these fungi in the production of bioactive compounds. In addition, the use of endophytes as a potential product for the production of secondary metabolites could revolutionize pharmaceutical, agricultural and biotechnological research [31].

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