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Isolation and identification of *Carpobrotus edulis* endophytic fungi growing in Morocco

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ABSTRACT

Fungal endophytes are microfungi that live in plants without causing apparent symptoms of infection. The purpose of this work was to identify the endophytic fungi isolated from the medicinal plant *Carpobrotus edulis* collected in the Atlantic coast region of Rabat city in Morocco. The isolates were obtained from different parts of the plant (flowers, stems and leaves). A total 12 isolates were characterized and identified using molecular biology analysis based on the internal transcribed spacer (ITS) region sequencing. 5 fungal species of endophytic fungi were successfully isolated from Carpobrotus edulis including *Trichoderma asperellum*, *Fusarium culmorum*, *Penicillium radicum*, *Aspergillus flavus* and 8 isolates represented the same fungal species *Aspergillus fumigatus*. The 5 fungal species isolated in the current study are reported for the first time as *Carpobrotus edulis* endophytes.

Key words: Fungal endophytes, *Carpobrotus edulis,* ITS region Sequencing

INTRODUCTION

Fungal endophytes are microfungi that colonize and live within healthy plant tissues without inducing symptoms of disease (Petrini, 1991). The endophytic fungi play important physiological and ecological roles in their host life. They protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll and Carroll, 1978; Strobel, 2002a).

Endophytes have proved to be the promising sources of biologically active products, which are of interest for specific medicinal applications such as antibiotics and antimicrobial metabolites (Strobel, 2002b). A compound polyketide citrinin produced by the endophytic fungus *Penicillium janthinellum* from fruits of *Melia azedarach*, presented 100% antibacterial activity against *Leishmania* sp. (Marinho et al. 2005). Moreover, endophytes comprise a rich and reliable source of genetic diversity and biological novelty, which have been applied in pharmacology and agriculture (Strobel and Daisy, 2003).

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The present study was carried out to identify endophytic fungi, which are isolated from *Carpobrotus edulis*. It is a medicinal plant belonging to the family *Aizoaceae*, represented mainly in the dry climates of southern Africa. In indigenous medicine, *C. edulis* and *C. acinaciformis* are the most commonly used herbs from the genus *Carpobrotus*, of which a preparation is gargled to treat infections of the mouth and throat (Springfield et al. 2003). Solvent extracts of *C. edulis* have been tested against various micro-organisms and found to exhibit antimicrobial activity (Van der Watt and Pretorius, 2001). In fact, Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products (Zhang et al. 2006). Therefore, researchers are paying more attention to the drug development from the endophytic fungi living in symbiosis with medicinal plants (Tan and Zou, 2001).

The aim of the study is to isolate and identify *Carpobrotus edulis* endophytic fungi by using the analysis of the ribosome spacer sequence (ITS) gene sequencing. The originality of the current research work is that few studies about *Carpobrotus edulis* endophytic fungi isolation and identification were carried out.

MATERIAL AND METHODS

The Plant material collection

The whole plant was collected from fully matured *Carpobrotus edulis* in March 2012 in the Atlantic coast region of Rabat city in Morocco. The plant species identification was carried out by Prof. S. Ouchbani, Scientific Institute, Mohammed V-University Rabat, Morocco.

The endophytic fungi isolation

The endophytes were isolated using a modified method described by Arnold et al. (2000). The material was thoroughly washed in sterile water, surface-disinfected by soaking in 70% ethanol for 2 min then rinsed in sterile demineralized water. The plant material was subsequently rinsed in sterile demineralized water. Small pieces of healthy tissues were dried under laminar flux hood. The external tissues were removed and several lesions were applied into inner tissues to allow endophytes to disperse in petri plates filled with Malt Agar culture medium supplemented with antibiotic streptomycin (3mg/100 mL). Petri plates were incubated in 28°C for several days until fungi growth. After purifying the isolates several times as described above, the purity of each fungal culture was assessed by examination of colony morphology.

Each pure isolate was removed from Malt Agar than placed on a sterile nylon membranes fixed on PDA petri plates as support for fungi solid culture (Utermark and Karlovsky, 2008). After 7 days of incubation at 28°C the mycelium is scratched then lyophilized and stocked in Eppendorf tubes ready for DNA extraction.

DNA Preparation, and ITS region Sequencing

DNA was extracted using ISOLATE II Plant Kit according to the manufacturer's protocols (Bioline). PCR were conducted in a thermal cycler Gene AmpR PCR System 2700 (Applied Biosystems, Foster City, USA) to amplify the internal transcribed spacer (ITS) region of the extracted DNA, which includes the 5.8s rDNA. The Amplification was carried out, by using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), under the following conditions: 94°C for 1 min (initial denaturation) followed by 35 cycles of 95°C for 15 sec (denaturation), 57°C for 20 sec (annealing), 72°C for 15 sec (extension), and final extension at 72°C for 3 min.

The polymerase chain reaction mixtures consisted of 13.8 µl sterile distilled water, 5µl of 5x buffer MyTaq DNA polymerase (Bioline), 1µl (10µM) of each primer (sigma), 5 µl (150 ng) of DNA and 0.2 µl (5 U/ µl) of Taq DNA polymerase (Bioline). The amplified products (5 µl) were visualized, separated by electrophoresis on 1% (w/v) agarose gel, and purified using the Eppendorf PerfectPrep Gel Clean-up Kit (Hamburg, Germany) following the manufacturer's instructions.

DNA sequencing was performed on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) using the POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software.

Phylogenetic analysis and Identification of Fungi

Preliminary identification was performed by FASTA search of the NCBI database. However, a more precise identification was performed by phylogenetic analysis with type strains of the nearest neighbours. Phylogenetic trees were constructed using the Neighbor joining methods implemented in the program MEGA version 6 (Tamura et al. 2013). In each case, bootstrap values were calculated based on 1000 replicates.

Nucleotide sequence accession numbers

The ITS region sequence determined in this study, have been deposited in the Genbank database under the accession number KX066052 to KX066063.

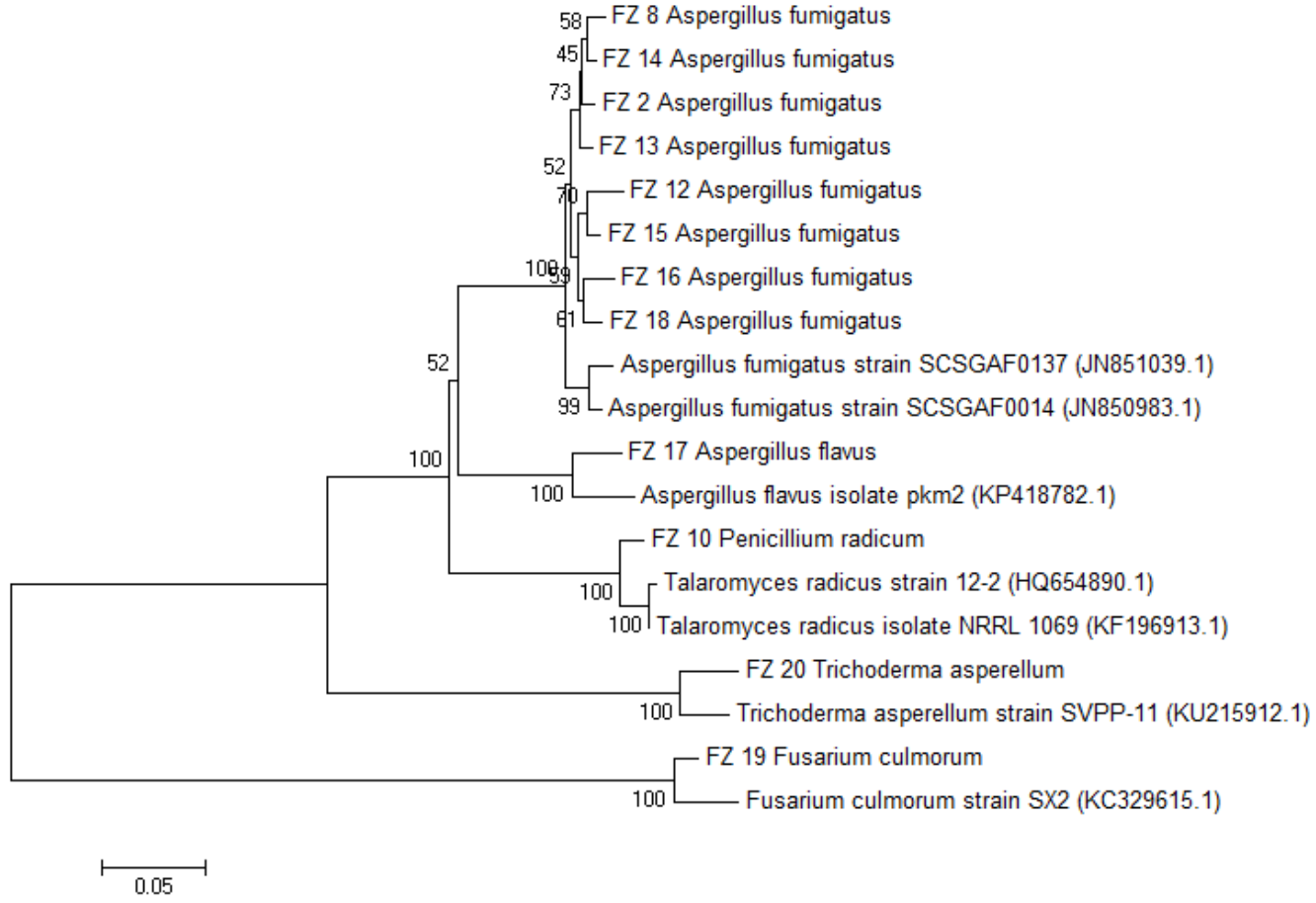
RESULTS AND DISCUSSION

A total of 12 endophytic fungi strains were isolated from *Carpobrotus edulis.* The ITS region of the 12 isolates were sequenced and compared with those in GenBank database. The ITS sequences of strains shared 98–99 % similarity with their closest NCBI relatives.

Table-1: ITS region sequencing of endophytic isolates from *Carpobrotus edulis*

Strain number (FZ number)	N° of bp sequenced	Similarity with nearest type strain (%)	Tentative identification based on nearest neighbor	Accession number
FZ 20	540	99	*Trichoderma asperellum*	KX066063
FZ 19	485	98	*Fusarium culmorum*	KX066061
FZ 10	535	98	*Penicillium radicum*	KX066062
FZ 17	535	98	*Aspergillus flavus*	KX066052
FZ 18	532	98	*Aspergillus fumigatus*	KX066060
FZ 16	532	99	*Aspergillus fumigatus*	KX066059
FZ 15	527	99	*Aspergillus fumigatus*	KX066058
FZ 14	534	98	*Aspergillus fumigatus*	KX066057
FZ 13	535	98	*Aspergillus fumigatus*	KX066056
FZ 12	546	99	*Aspergillus fumigatus*	KX066055
FZ 8	539	99	*Aspergillus fumigatus*	KX066054
FZ 2	532	99	*Aspergillus fumigatus*	KX066053

Figure-1 Phylogenetic dendrogram of fungal endophytes and its related species based on ITS gene sequence similarities. The tree was constructed using the neighbour-joining method implemented in the program MEGA version 6. Bar, 0.05 nt substitutions per site. The numbers at each branch point represent percentage bootstrap support calculated from 1000 replicate.



BLAST analysis revealed that the 12 fungal isolates belonged to 4 fungal genera (*Aspergillus, Fusarium, Trichoderma* and *Penicillium*) (Table 1). *Aspergillus* was the most common genus with isolates. The identity of these sequences was further confirmed using phylogenetic analysis. As shown in Figure 1, FZ 2, FZ 8, FZ 12, FZ 13, FZ 14, FZ 15, FZ 16 and FZ 18 strains are placed into clade with *Aspergillus fumigatus* strain SCSGAF0137 and *Aspergillus fumigatus* strain SCSGAF0014. Moreover, the neighbour-joining phylogenetic tree further confirmed that the strains FZ 17, FZ 19 and FZ 20 form clades with *Aspergillus flavus* isolate pkm2, *Fusarium culmorum* strain SX 2 and *Trichoderma asperellum* strain SVPP-11 respectively.

In addition, the strain FZ 10 forms clade with *Talaromyces radicus* strain 12-2 and *Talaromyces radicus* strain 12-2 (Synonymy: *Penicillium radicum*) (Samson et al., 2011).

In conclusion, the profile neighbour-joining tree construct based on our sequence-structure alignment resulted in well-separated clades. All the investigated isolates belonged to 2 orders (Eurotiales and Hypocreales) and 4 genera (*Aspergillus*, *Trichoderma*, *Penicillium,* and Fusarium) of Ascomycota (Wang et al., 2009).

Rodriguez et al. (2009) have previously reported that two endophytic groups: clavicipitaceous (C) (which infect some grasses) and nonclavicipitaceous (NC) (which can be recovered from tissues of nonvascular plants, ferns, conifers and angiosperms), have been discriminated based on phylogeny and life history traits.

Earlier, Kock et al. (2007) and Zhang et al. (2008) reported the presence of fungal endophytes, from different parts of *Carpobrotus edulis.* However, as far as we know, this is the first report of isolation and identification of endophytic fungus, *Aspergillus fumigatus,*

Aspergillus flavus, *Trichoderma asperellum, Fusarium culmorum*, and *Penicillium radicum* from medicinal plant *Carpobrotus edulis.* Also, it has been reported that endophytic fungi belonged to genera *Aspergillus, Trichoderma, Fusarium*, and *Penicillium* were isolated from many medicinal plants such as *Bauhinia forficate, Artimisia nilagirica, Tylophora asthmatica,* and *Phyllanthus amarus* (Bezerra et al., 2015; Myrchiang et al., 2014; Nalini et al., 2014).

Several studies showed that secondary metabolites from fungal endophytes have broad spectrum of biological activities, such as antibiotics, antipathogens, anticancer, and antioxidant. It has been also reported that a single endophytic fungi strain may produce multiple bioactive compounds (Gaikwad, 2011). Indeed, Kock et al., 2007 have previously reported that endophytic fungus *Coniothyrium* sp. from *Carpobrotus edulis* was able to produce several bioactive secondary metabolites such as Massarilactones, Massarigenin E, and Coniothyrenol. In addition, Zhang et al. (2008) showed that *Blennoria* sp., a *Carpobrotus edulis* endophyte, produced antifungal and antibacterial compounds against *Microbotryum violaceum*, *Bacillus megaterium* and *Escherichia coli*.

Most recently, Patil et al. (2015) showed that *Aspergillus flavus* obtained from *Aegle marmelos* produced bioactive flavonoids with pharmaceutical potentials.

Moreover, many studies described the relationships between endophytic fungi and their host plants. In fact, it was reported that endophytic fungi were able to protect their hosts by secreting specific bioactive secondary metabolites (Zhao et al., 2011). Furthermore, Myrchiang et al. (2014) reported that the endophytic fungi *Trichoderma viride, Aspergillus fumigatus* and *Penicillium atrovenetum,* associated with *Artimisia nilagirica* may be recommended as good biocontrol agents of the potato pathogen fungus: *Phytophthora infestans.*

CONCLUSION

In this study, we investigated the culturable endophytic fungi from *Carpobrotus edulis* via traditional cultivation techniques coupled with the analysis of the ribosome spacer sequence (ITS) gene sequencing. Species characterization and identification revealed that the dominant specie was *Aspergillus fumigatus.* Our results provide useful information about the endophytic fungi but more studies are needed to explore their full potentiality. Moreover, the 5 fungal species isolated in the current study: *Trichoderma asperellum*, *Fusarium culmorum*, *Penicillium radicum*, *Aspergillus flavus* and *Aspergillus fumigatus* are reported for the first time as *Carpobrotus edulis* endophytes.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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