

# Endophytic Fungi Isolated from *Crocus sativus* L. (Saffron) as a Source of Bioactive Secondary Metabolites

Imane Chamkhi\*, Laila Sbabou, Jamal Aurag

Imane Chamkhi\*, Laila Sbabou, Jamal Aurag

Microbiology and Molecular Biology Team, Center of Plant and Microbial Biotechnology, Biodiversity and Environment. Faculty of Sciences, Mohammed V University in Rabat, MOROCCO.

## Correspondence

Imane Chamkhi

Center of Plant and Microbial Biotechnology, Biodiversity and Environment. Faculty of Sciences, Mohammed V University in Rabat, Avenue IbnBattouta, BP 1014, Rabat 10000, MOROCCO.

Phone no : 21 26318 09913

E-mail: chamkhi.imane@gmail.com

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## ABSTRACT

**Introduction:** Endophytic fungi are becoming an important source of new natural bioactive products. Many interesting endophytic fungi have been isolated from traditional medicinal plants. *Crocus sativus* L. (saffron) is one of the most expensive and rarest spices in the world, used as a dye, aroma and for medicinal purposes. This study reports on the molecular characterization of endophytic fungi isolated from roots of saffron plants growing in Taliouine-Morocco, and the examination of the antibacterial and antioxidant activities of secondary metabolites extracted from these endophytes. **Methods and Material:** The fungi were isolated from sterilized saffron root fragments and identified using internal transcribed spacer (ITS) rRNA gene sequence analysis. Three endophytes were fermented and extracted with ethyl acetate (EtOAc) and chloroform (CHCl<sub>3</sub>). The antibacterial activity of fungi extracts was evaluated using the Agar Diffusion Method against six strains, *Bacillus* sp., *Stenotrophomonas* sp., *Pseudomonas putida*, *Pantoea* sp., *Luteibacter* sp. and *Escherichia coli*. The antioxidant activity was tested by using the  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) analysis and  $\beta$ -Carotene bleaching test (BCBT) methods. **Results:** Sixty fungal isolates were recovered and purified from saffron roots. ITS rDNA sequences were 99-100% identical to three different species: *Rhizopus oryzae*, *Aspergillus fumigati*affinis and *Aspergillus niger*. Both *Rhizopus oryzae* extracts had high antibacterial activity against most of the bacteria tested, while *Aspergillus niger* and *Rhizopus oryzae* extracts showed an antioxidant capacity using DPPH and BCBT methods, respectively. **Conclusion:** The fungal endophytes inhabiting saffron roots could be a potential source of natural plant bioactive secondary metabolites especially for antibacterial and antioxidant purposes.

**Key words:** *Crocus sativus* L., Endophytic fungi, ITS rDNA, Secondary metabolites, Antibacterial activity, Antioxidant activity.

## INTRODUCTION

Endophytic fungi are microorganisms that live in the intercellular spaces of stems, petioles, roots, and leaves of plants.<sup>1</sup> These endophytes colonize plants internally without apparent adverse effects and they are not considered as saprophytes since they are associated with living tissues without causing any noticeable symptoms of a disease. However, some endophytic fungi may become pathogenic under stressful conditions.<sup>2</sup> Endophytic fungi have been detected in hundreds of plants including many important agricultural commodities.<sup>3</sup> Different studies demonstrated that they produce a large number of interesting secondary metabolites including anti-cancer, antifungal, antidiabetic and immune-suppressant compounds comparable to those produced by host plants. The production of these interesting compounds makes endophytic fungi an alternative and natural source of important plant secondary metabolites.<sup>4</sup> In addition, fungal endophytes could be beneficial to host plants by increasing their tolerance to biotic and abiotic stress factors.<sup>5</sup> Many endophytes have the potential to synthesize various

bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases, such as paclitaxel, podophyllotoxin, hypericin, vinblastine, camptothecine and diosgenin which are also produced by their host.<sup>6</sup> The production of bioactive compounds by endophytes, especially those exclusive to their host plants, are highly important from ecological, biochemical and molecular perspective.

Investigations concerning 29 medicinal plant species and 1.160 fungal endophytes showed the existence of high biodiversity, host recurrence, tissue specificity, and spatial heterogeneity.<sup>7</sup> Some phenolic compounds were found to more likely coexist with certain endophytic fungi in the same plants, and their systematic investigation revealed that traditional medicinal plants are a rich and reliable source of novel endophytic fungi. This information has led us to assume that saffron could constitute another source of endophytic fungi with biological activity.

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Saffron is a perennial crop culture that belongs to the large family of Iridaceae and to the genus *Crocus* which includes about 162 species (National Center for Biotechnology Information). *Crocus sativus* represents the most interesting species because of the natural coloring, bitterness and aromatic power of its dried stigmas.<sup>8</sup> The saffron have been used as a dye in cosmetics and perfumes, as spice in food and also as medicinal plant, with a potent chemotherapeutic effect for the treatment of various diseases.<sup>9</sup> Saffron is cultivated under different climates in different countries of Asia and the Mediterranean basin. Iran is the main producer of saffron, accounting for more than 90% of world production, followed by India (5%) and countries of the Mediterranean basin (Greece, Morocco, Spain, Italy and Turkey).<sup>10</sup> However, the Mediterranean region is recognized worldwide as producing the best quality of saffron, which is attributed to many factors.<sup>10-11</sup>

However, up to date no studies have been carried out on the endophytic fungi of Moroccan saffron. At the international level studies focused on endophytic fungi recovered from corms and stems of saffron, but to our knowledge no study has looked at the roots that are the entry point for endophytic fungi.<sup>12-13</sup> In this context our main objective was the isolation of endophytic fungi from roots of saffron growing in Morocco, their molecular identification and assessment of their possible antibacterial and antioxidant activities through the extraction of secondary metabolites.

## MATERIAL AND METHODS

### Study area, plant sampling and endophytic fungi isolation

Plant samples of *C. sativus* were collected randomly during April 2016 from 1.2.3. SAFRAN farm (N30°28'12.99"/W7°46'22.479") located in Talakhat-Taliouine zone at Taroudant province, Souss-Massa region (Morocco). Root samples were separated from individual healthy plants. Each root sample was washed thoroughly under running tap water to remove soil particles, dried and cut into 1 cm length segments. The root segments were surface sterilized following the protocol of Fernández Di Pardo,<sup>13</sup> then placed in Petri dish plates containing potato dextrose agar (PDA) and incubated in the dark at 25±2°C until fungal growth appeared.

### Molecular identification of endophytic fungi

A lyophilized sample of fungal hyphae (0.2 g) was powdered in a Mixer Mill for 10 min. DNA isolation was performed using the Promega™ Wizard™ Genomic DNA Purification Kit according to the manufacturer's protocol. The isolated DNA was dissolved in 50 µl of the elution buffer. PCR reaction was performed using my Taq TMHS mix bioline kit and ITS1 (5'-TCCGATGGTGAACCTGCGG-3') as the forward primer and ITS4 (5'-TCCTCCGCTTATGATATGC-3') as the reverse primer. The PCR program was carried as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of a denaturation at 95°C for 1 min, annealing at 56°C for 30s and an extension at 72°C for 1 min then a final extension of 10 min at 72°C.<sup>14</sup> Purified PCR products were sequenced using the universal primer ITS1 at Genoscreen (Lille, France). The sequences were corrected using BioEdit software and compared to the available databases deposited in the NCBI.

### Extraction of endophytic fungi secondary metabolites

The fermentation broth of the different fungus species was prepared as explained by Barik.<sup>15</sup> Metabolites were extracted using two organic solvents: EtOAc and CHCl<sub>3</sub>. Each filtrate was mixed with an equivalent volume of solvent and placed under stirring for 2 h. The organic layer (EtOAc) was separated from the aqueous layer in the separating funnel, this is the EtOAc fraction. The CHCl<sub>3</sub> fraction was also obtained using

the same procedure. The collected organic phase was recovered and concentrated under pressure by evaporating the solvent using a rotary evaporator.<sup>16</sup> Finally, the resultant compound was dissolved in methanol.

### Antimicrobial activity

The endophytic fungi extracts were evaluated against six bacterial strains from the collection of the Microbiology and Molecular Biology team (Faculty of sciences, Rabat, Morocco). One Gram-positive *Bacillus sp.* strain and five Gram-negative strains belonging to different genera: *Stenotrophomonas sp.*, *Pseudomonas putida*, *Pantoea sp.* and *Luteibacter sp.* and the reference strain *Escherichia coli* (DH5α). All bacterial suspensions were prepared in liquid medium, *Escherichia coli* was grown in Luria-Bertani (LB) medium for 20 h at 37°C while the other strains were grown in Tryptone Yeast (TY) medium for 20 h at 28°C until the stationary growth phase. Microbial suspensions were diluted with either LB or TY media to a final concentration of 10<sup>6</sup> CFU/ml. The antimicrobial test was carried out using the Agar Diffusion Method.<sup>17</sup> Each microbial suspension was spread on the surface of the appropriate agar media. Sterilized Whatman paper discs of 7 mm diameter was deposited on the agar surface and 15 µl of each extract was added on the top of the disc, then incubated at 37°C/28°C for 24 h. The antimicrobial activity was measured by the diameter of the inhibition zone.<sup>18</sup>

### Antioxidant activity

The antioxidant activity of the extracts was measured by two methods;

#### The DPPH method

DPPH is a stable, nitrogen-centered free radical of a purplish color which fades to shades of yellow color in the presence of scavenging compounds (antioxidants). The absorbance measurement used to calculate the percentage of DPPH radical inhibition is proportional to the anti-radical power of the sample. To test the antioxidant activity of the extracts studied, 50 µl of each endophytic fungus extract was mixed with 1.95 ml DPPH (0.025 g/l) and incubated in darkness at 37°C for 30 min. The DPPH scavenging capacity of the sample was calculated using the equation adopted by Wang.<sup>19</sup> Ascorbic acid (Asc A) was used as a positive control and all the tests were carried out in triplicate.

#### The BCBT method

The mixture of β-carotene linoleic acid was prepared as follows: 2 mg β-carotene was dissolved in 1 ml chloroform and 25 µl of linoleic acid and 200 mg of Tween 40 were added, then chloroform was completely evaporated using a vacuum evaporator at 40°C for 5 min. Finally, 100 ml of H<sub>2</sub>O<sub>2</sub> 50 mM was added while shaking vigorously. Five milliliters of this reaction mixture was dispensed into test tubes and 100 µl of a prepared methanol extract of endophytic fungi were added and vortexed. The absorbance of the mixtures was measured at 470 nm immediately (T=0 h), then the emulsion system was incubated at 50°C during 3 h to calculate the percentage (A%) of antioxidant activity by using the equation mentioned in Liu.<sup>20</sup> Butylhydroxytoluene (BHT) and methanol were used as positive and negative controls, respectively. The discoloration kinetics of the emulsion of each sample was measured using a spectrophotometer at 470 nm immediately after sample preparation (T=0 h) and at 1 h, 2 h, 3 h, 24 h, and 48 h.

### Statistical analysis

All parameters were statistically analyzed using XLSTAT program (Version 2014.5.03), with \ one-way ANOVA and compared using Fisher's Least Significant Difference test with a 5% probability threshold.

**Table 1: Accession numbers of endophytic fungi isolated from the roots of *C. sativus***

Fungal isolate	Fungus species	Accession number
LMR568	<i>Rhizopus oryzae</i>	KY042103
LMR569	<i>Aspergillus fumigatiaffinis</i>	KY042104
LMR570	<i>Aspergillus niger</i>	KY042105

**Table 2: Antibacterial activity of EtOAc and CHCl<sub>3</sub> extracts of endophytic fungi isolated from the roots of *C. sativus***

Extracts	Inhibition zone (mm)						
	Chloroform extracts			Ethyl acetate extracts			
	Control	<i>Aspergillus fumigatiaffinis</i>	<i>Aspergillus niger</i>	<i>Rhizopus oryzae</i>	<i>Aspergillus fumigatiaffinis</i>	<i>Aspergillus niger</i>	<i>Rhizopus oryzae</i>
Endophytic fungi							
<i>Stenotrophomonas</i> sp	0±0 <sup>f</sup>	12±0 <sup>d</sup>	0±0 <sup>e</sup>	20±0 <sup>c</sup>	11.5±1.15 <sup>d,e</sup>	11±0 <sup>d,e</sup>	15.03±0.05 <sup>c</sup>
<i>Escherichia coli</i>	0±0 <sup>f</sup>	0±0 <sup>f</sup>	0±0 <sup>f</sup>	11±0 <sup>d,e</sup>	26±0 <sup>a</sup>	10±0 <sup>e</sup>	11±0 <sup>d,e</sup>
<i>Pseudomonas putida</i>	0±0 <sup>f</sup>	12±0 <sup>d</sup>	10±0 <sup>e</sup>	23±0 <sup>a</sup>	0±0 <sup>f</sup>	10±0 <sup>e</sup>	12±0 <sup>d</sup>
<i>Bacillus</i> sp	0±0 <sup>f</sup>	12±0 <sup>d</sup>	0±0 <sup>f</sup>	0±0 <sup>f</sup>	12±0 <sup>d</sup>	10±0 <sup>e</sup>	9±0 <sup>e</sup>
<i>Pantoea</i> sp	0±0 <sup>f</sup>	12±0 <sup>d</sup>	10±0 <sup>e</sup>	10±0 <sup>e</sup>	15±0 <sup>c</sup>	10±0 <sup>e</sup>	14±0 <sup>c</sup>
<i>Luteibacter</i> sp	0±0 <sup>f</sup>	10±0 <sup>e</sup>	0±0 <sup>f</sup>	22±0 <sup>b</sup>	11±0 <sup>d,e</sup>	10±0 <sup>e</sup>	20±0 <sup>b</sup>

Values with different letters differ significantly (P < 0.05)

## RESULTS

### Isolation and molecular identification of isolated endophytic fungi

Sixty pure fungal isolates were obtained from roots of saffron plants and their DNA was extracted, amplified and sequenced. Their ITS rDNA sequences were 99–100% identical to three fungal species. *Rhizopus oryzae* was the most abundant (93.54%) followed by *Aspergillus fumigatiaffinis* (4.83%) and *Aspergillus niger* (1.61%). All sequences obtained in this study were submitted to NCBI data bank. Accession numbers of the three isolates used in this study are available in Table 1.

### Antimicrobial activity

The effect of three endophytic fungi extracts against six different studied bacteria by the Agar Diffusion Method showed an interesting antibacterial activity (Table 2). Antibacterial effects of EtOAc solvent fraction extracts were superior to those of CHCl<sub>3</sub> extracts, particularly the EtOAc extract of *Rhizopus oryzae* inhibited all bacteria tested. However, different levels of bacterial sensitivity were recorded from high to low, with *Luteibacter* sp. being the most sensitive followed by *Stenotrophomonas* sp., *Pantoea* sp., *Pseudomonas putida*, *Escherichia coli* and finally *Bacillus* sp. The EtOAc extract of *Aspergillus niger* also inhibited all tested strains with a similar zone of inhibition while the EtOAc extract of *Aspergillus fumigatiaffinis* inhibited almost all the strains tested except *Pseudomonas putida* which was more resistant, while the *Escherichia coli* strain was the most sensitive.

On the other hand, CHCl<sub>3</sub> extract of *Rhizopus oryzae* inhibited all the Gram-negative strains tested while the Gram-positive strain *Bacillus* sp. was resistant. The CHCl<sub>3</sub> extract of *Aspergillus fumigatiaffinis* inhibited all the bacteria except *Escherichia coli*, while the CHCl<sub>3</sub> extract of *Aspergillus niger* was only efficient on *Pseudomonas putida* and *Pantoea* sp. strains.

### Antioxidant activity

#### The DPPH tests

The calculated percentage of DPPH decolorization showed that the performance of EtOAc extract of *Aspergillus niger* was high but still

lower as compared to the standard substance: ascorbic acid (5µg/ml) (Asc A) (Figure 1). According to the inhibition percentage of DPPH, extracts of the endophytic fungi can be classified in a decreasing order: *Aspergillus niger* (EtOAc) > *Rhizopus oryzae* (EtOAc) > *Rhizopus oryzae* (CHCl<sub>3</sub>) > *Aspergillus fumigatiaffinis* (EtOAc) > *Aspergillus fumigatiaffinis* (CHCl<sub>3</sub>) > *Aspergillus niger* (CHCl<sub>3</sub>).

These results indicate that EtOAc fraction extracts has higher radical scavenging activities than CHCl<sub>3</sub> fraction extracts.

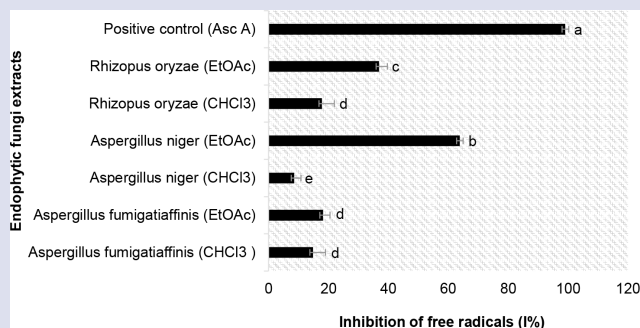
#### The BCBT test

The bleaching kinetics of β-carotene of the studied extracts were measured spectrophotometrically following the fading of the β-carotene at 470 nm during 48 h (Figure 2).

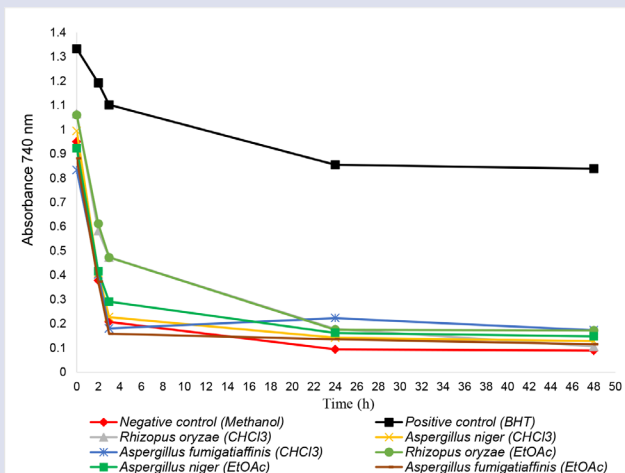
The β-carotene undergoes rapid discoloration in the absence of an antioxidant, the negative control, followed by *Aspergillus fumigatiaffinis* (EtOAc) and *Aspergillus fumigatiaffinis* (CHCl<sub>3</sub>). At the opposite both *Rhizopus oryzae* extracts appear to be the best inhibitor of linoleic acid oxidation. The results of β-carotene bleaching measurements presented in Figure 3 confirmed that both extracts of *Rhizopus oryzae* have good antioxidant potentials, with 43.17%±7.54b and 42.82%±4.04b respectively, compared to the other endophytic fungi extracts.

## DISCUSSION

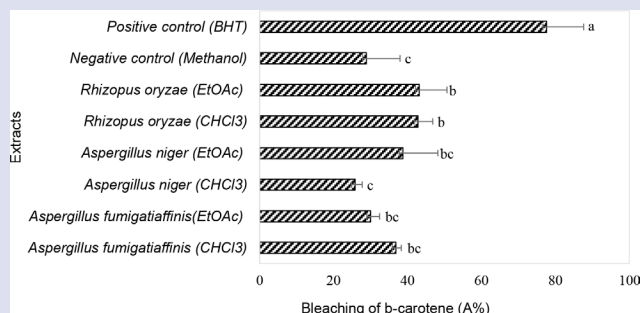
In this study, 60 fungal isolates were purified from segments of *C. sativus* roots. The most abundant fungi species was *Rhizopus oryzae* followed by *Aspergillus fumigatiaffinis* and *Aspergillus niger*. Two recent studies reported the results about fungal endophytes associated with saffron corms and/or stems collected from different sites in Jammu and Kashmir State (India).<sup>21-22</sup> The dominant endophytes in the first study were *Rhizoctonia* sp followed by *Fusarium* sp. and *Phytophthora* sp.,<sup>21</sup> while *Phialophora mustea* and *Cadophora malorum*. dominated in the second one.<sup>22</sup> In a third study conducted in China only *Penicillium vinaceum* was isolated from corms of *C. sativus* growing in the Changxing Island.<sup>12</sup> In our study the dominant endophytic fungi associated with saffron roots belonged to different genera and species than those reported in the previous studies. The striking differences in community composition



**Figure 1:** Antioxidant activity of EtOAc and CHCl<sub>3</sub> extracts of endophytic fungi isolated from the roots of *C. sativus* evaluated by the DPPH method. Data expressed as means  $\pm$  standard deviation. Values with different letters differ significantly ( $P < 0.05$ ).



**Figure 2:** Kinetics of the inhibition of  $\beta$ -carotene oxidation by EtOAc and CHCl<sub>3</sub> extracts of saffron endophytic fungi during the 48 h interval.



**Figure 3:** Antioxidant activity of EtOAc and CHCl<sub>3</sub> extracts of endophytic fungi isolated from saffron roots evaluated by the BCBT method. Data expressed as means  $\pm$  standard deviation. Values with different letters differ significantly ( $P < 0.05$ ).

can be explained by the different organs examined in the different studies and/or by differences in the environment of studied areas that might harbor different fungi-saffron plants associations. These facts may also suggest that the degree of endophytic colonization reflect the degree of adaptation of the host to the biotic (potential mycobiota) and the abiotic (climate, etc) conditions of the site.<sup>23</sup>

Endophytic fungi from medicinal plants are important regarding their ability to produce a variety of bioactive compounds possibly those produced by their host plant.<sup>24</sup> In this study, we investigated the antioxidant and antibacterial capacity of saffron endophytic fungi extracts. The five Gram-negative and one Gram-positive bacteria tested were highly sensitive to the extracts of the three endophytic fungi tested. The extract of *Rhizopus oryzae* was the most bactericidal followed by those of *Aspergillus fumigatiaffinis* and *Aspergillus niger*. Our results were consistent with those reported for *Rhizopus* sp. isolated from the medicinal plants *Dendrobium devonianum* and *Dendrobium thyrsiflorum*.<sup>25</sup> Other studies indicated that secondary metabolite compounds of *Aspergillus fumigatiaffinis* and *Aspergillus niger* possess antimicrobial activities.<sup>26-27</sup> The bioactive compounds of fungal endophytes tested showed an antibacterial effect against Gram-negative species despite the complex structure and arrangement of their cell wall compared to the Gram-positive bacteria.<sup>28</sup> This effect is probably due to the large number of bioactive compounds that can be found in endophytic fungi extracts exhibiting antibacterial activity such as chaetoglobosins A and C, pyrrocidines A and B, ergosterol and 5 $\alpha$ ,8 $\alpha$ -epidioxyergosterol, pestalachloride A and B, 3-O-methylalaternin and altersolanol A.<sup>29</sup>

Overall, the results showed that the antioxidant activity varies depending on the type of the test used. In the DPPH method, *Aspergillus niger* extract showed the highest antioxidant activity while it was *Rhizopus oryzae* extract in the BCBT method. The absence of significant correlation between the results obtained with both methods can be explained by the fact that the antioxidant activity can involve various mechanisms and different parameters.<sup>30</sup> Noting that DPPH method is the most known for the evaluation of free radical-scavenging activity because it is sample polarity-independent, very rapid, simple and reproducible.<sup>31</sup> Discrepancies between the two methods cannot affect the recognized efficacy of the bioactive antioxidant components of the endophytic fungi especially those of *Aspergillus niger*<sup>26</sup> and *Rhizopus oryzae*.<sup>32</sup>

On the other hand, the nature of the solvent used in the extraction process is crucial. In our study ethyl-acetate extracts exhibited the highest antioxidant activity. This result may be explained by the fact that the active components are not well dissolved out of the extracts when using chloroform as solvent, or and if so, a very small quantity is being extracted out which gives little or no effectivity. Another possibility is that the bioactive components have a polarity which is best extracted out by ethyl acetate among the solvent fractions.<sup>33</sup>

## CONCLUSION

In summary, roots of *C. sativus* collected from Taliouine in the south of Morocco harbor at least three species of endophytic fungi that possess not only an antibacterial activity against different bacteria tested but also an antioxidant activity. Our results suggest that the fungal endophytes of *C. sativus* could be an appropriate source of bioactive secondary metabolites.

## CONFLICT OF INTEREST

There are no conflicts of interest.

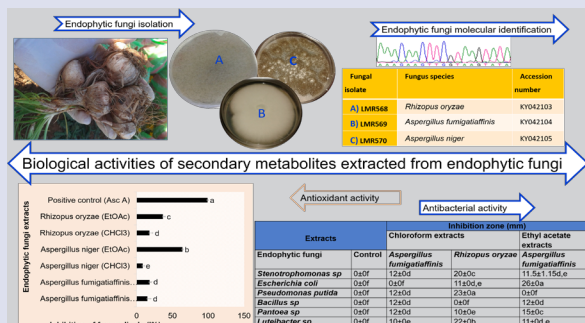
## ABBREVIATIONS

**PDA:** Potato dextrose agar; **PDB:** Potato dextrose broth; **LB:** Luria-Bertani; **TY:** Tryptone Yeast; **CFU:** colony forming unit; **EtOAc:** Ethyl acetate; **CHCl<sub>3</sub>:** Chloroform; **ITS:** Internal transcribed spacer; **DPPH:**  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl; **BCBT:**  $\beta$ -Carotene bleaching test; **Asc A:** Ascorbic acid; **BHT:** butylhydroxytoluene; **NCBI:** National Center for Biotechnology Information.

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## GRAPHICAL ABSTRACT



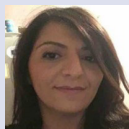
## SUMMARY

- Three species of fungal endophytes were isolated and identified from the roots of *Crocus sativus*: *Rhizopus oryzae*, *Aspergillus fumigatiifinis* and *Aspergillus niger*.
- The ethyl acetate (EtOAc) and the chloroform (CHCl<sub>3</sub>) extracts of *Rhizopus oryzae* showed growth inhibitory activity against the bacteria: *Bacillus* sp., *Stenotrophomonas* sp., *Pseudomonas putida*, *Pantoea* sp., *Luteibacter* sp. and *Escherichia coli*.
- The EtOAc extracts of *Aspergillus niger* and *Rhizopus oryzae* showed an antioxidant capacity using DPPH and BCBT methods, respectively.

#### ABOUT AUTHORS



**Dr. Jamal Aurag** is a professor of microbiology since 1984 and director of the Center of Plant and Microbial Biotechnology, Biodiversity and Environment at the Mohammed V University in Rabat. He obtained his PhD "Doctorat d'État" degree in Microbiology in 1996 and a "diplôme d'études supérieures" degree in 1984 in Microbial Ecology from Mohammed V University in Rabat.



**Dr. Laila Sbabou** is a Research professor of Genomics and Biotechnology at Mohammed V University of Rabat. She defended her national doctoral thesis in 2009 in Genomics and Biotechnology at the Faculty of Sciences of Rabat. She is a member of the Bureau of the Moroccan Association of Microbiology (AMM), the Moroccan Association of Biosafety and Biosecurity (AMBS) and the Moroccan Academy of Young Scientists (AMJS).



**Imane Chamkhi** is a Ph.D. student in the Microbiology and Molecular Biology Team, Center of Plant and Microbial Biotechnologies, Biodiversity and Environment, Faculty of Sciences, Mohammed V University in Rabat, Morocco. She obtained her master's degree in 'Plant Production'. A former head of the analytical laboratory of saffron quality at "1.2.3 SAFRAN" society, Taliouine, Morocco.

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