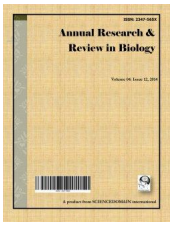

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Isolation and Biochemical Characterisation of Endophytic *Bacillus spp* from *Urtica dioica* and Study of Their Antagonistic Effect against Phytopathogens

Dahaieh Naoufal[1*], Hacham Amine[1], Bourakkad Ilham[1] and Ounine Khadija[1]

[1]Laboratory of Biology and Health, Applied Microbiology Team, Faculty of Sciences, Ibn Tofail University, B.P: 133 14000, Kénitra, Morocco.

Authors' contributions

This work was carried out in collaboration between all authors. Author DN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HA and BI managed the analyses of the study. Author OK managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Endophytic bacteria have been isolated from the roots of *Urtica dioica.* A total of 54 endophytic bacteria were isolated from the underground parts using suitable surface sterilisation protocol. Three isolates R45a; R45b; R21a were tested for antagonism effect against *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Phytophthora parasitica* in dual culture method. Significant inhibitory effects on mecylial radial growth have been revealed with a percentage superior or equal to 75%. These strains were Gram-positive rods. Cultures on nutrient agar showed irregular, entirely cream coloured colonies that are strictly aerobic and capable of forming endospore. They belong probably to the genus of *Bacillus spp*.

**Corresponding author: E-mail: n.profdoc@gmail.com;*

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1. INTRODUCTION

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products annually [1]. In order to combat these diseases, synthetic chemical fungicides have long been used to reduce their incidence. However, they are costly, can have negative effects on the environment, and may induce pathogen resistance. Consequently, biological control, including the use of microorganisms or their antibiotics, offers an attractive alternative or supplement to pesticides for the management of plant diseases without the negative impact of chemical control [2]. Endophytic bacteria reside within plant hosts without causing disease symptoms, these latter are relatively unexplored as potential sources of novel species and novel natural products for medical and commercial exploitation. In general, endophytes are more likely to show plant growth-promoting effects than bacteria exclusively colonising the rhizosphere [3,4].

Some endophytes have the capacity to control plant pathogens [5,6,7] and nematodes [8,9]. Gram-positive bacteria offer a biological solution through formulations as they form heat- and desiccation-resistant spores [10]. The use of *Bacillus spp*. is recognised as a powerful tool. They are able to synthesise more than 60 types of antibiotics which also act as plant growth promoters [11,12,13]. The present study is carried out with the intention of isolating and characterising endophytic *Bacillus spp* bacteria from *Urtica dioca* as well as screening out valuable antifungal activity.

2. MATERIALS AND METHODS

2.1 Isolation and Purification of Endophytic Bacteria

Three plants of genus *Urtica dioica* were carefully removed from field, roots (0.5 cm in diameter) were washed in running water to remove soil particles and sterilised by sequential immersion in 70% (v/v) ethanol for 2 min, and sodium hypochlorite solution (0.9%, w/v, available chlorine) for 2 min, followed by repeated rinsing in sterile water to remove residual sodium hypochlorite. The tissues were aseptically cut into small pieces and macerated with 10 ml of sterile NaCl solution (0.85%) using a mortar and pestle and further homogenised by vortexing for 60 s at high speed. The endophytic bacteria were isolated through enrichment method, using the standard dilution plating technique [14]. Plates were incubated at an appropriate temperature for 24-48.

To validate the sterilisation procedure, the nutrient agar plate was spread with water from the last washing of plant samples and incubated at the same temperature for endophytic isolation, then checked for microbial growth.

2.2 Antagonistic Effect of Endophytic *Bacillus spp.* Strains: Dual Culture Technique

The antagonistic activity of the endophytic strain *Bacillus sp* against phytopathogen fungus was tested via dual culture technique [15]. Bacterial isolates were streaked at one side of Petri dish (one cm away from the edge) containing Potato Dextrose Agar (PDA): Potato (peeled) 200 g, dextrose 20 g, agar 18 g, distilled water (DW) 1000 ml. 9 mm mycelial disc from seven days old PDA culture of phytopathogen fungus was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±2°C for 5-7 days. Petri dishes inoculated with fungal discs alone served as control. Three replications were maintained for each isolate. Observation on inhibition zone width and mycelia growth of test pathogen was recorded and inhibition percentage of pathogen growth was calculated using the formula proposed by Vincent [16].

Inhibition (percentage) (I) = C-T/C ×100

Where,

C- mycelial growth of pathogen in control
T- mycelial growth of pathogen in dual plate.

2.3 Identification and Biochemical Characterisation of Endophytic Strains

2.3.1 Biochemical identification

The isolated bacterial strains are subject to morphological and biochemical identification

tests according to the Manual of Bergey's Determinative Bacteriology, 2009.

2.3.2 Production of ammonia

Bacterial isolates were tested for ammonia production in peptone water using Nessler's reagent [17]. Development of brown yellow color indicated a positive test for ammonia production.

2.3.3 Phosphate solubilisation

The ability of the isolates to solubilise tri-calcium phosphate was observed as per Pikovskaya's [18]. Agar was inoculated with the isolates and incubated at 36±2°C for five days. Formation of halo indicated phosphate solubilisation.

2.3.4 Production of hydrolytic enzymes

The isolates of *Bacillus spp*. possessing the antagonistic effect and growth promoting characteristics were further characterised biochemically through tests for the production of oxidase, amylase, catalase, protease, urease, lipase, cellulase. The isolates were also tested for their ability to carry out nitrate reduction and carbohydrate fermentation.

2.3.5 Nitrate reduction test

Nitrate agar: Peptic digest of animal tissue 5 g; Beef extract 3 g; Potassium nitrate 1 g; Agar 12 g), are used to test for nitrate reduction.

Following incubation at 37°C, two reagents are added: Nitrate Reagent A (sulfanilic acid) and Nitrate Reagent B (alpha-naphthylamine). These reagents test for the presence of nitrites and the media will turn red if nitrites are present.

2.3.6 Sugar fermentation

Each sugar solution was prepared as 10% stock solution (10 g sugar was dissolved in 100 mL distilled water) and sterilised by tyndallisation. Peptone water (peptone 1 g, NaCl 0,5 g, distilled water 100 mL; pH 7,2) was also prepared. From this peptone water, 5 mL was dispensed in separate test tubes along with phenol red indicator (0, 01%) and placed in inverted Durham's tubes and sterilised.

To each test tube of peptone water, 0.5 mL of 10% sugar solution (separate for each type of sugar) was added and inoculated with tow drops of individual isolate suspension). The tubes were inoculated at 37°C for 7 days

Positive or negative results were observed by change in the color of media or air bubble formation in the Durham's tube indicating acid and gas production, respectively.

2.4 Statistical Study

Collected data were transferred to SAS software and subjected to analysis using a two-way ANOVA. Means were separated by Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

3.1 Isolation and Antagonistic Assay

Water washing from 10 surface-sterilised root samples showed no microbial growth on nutrient agar after 2 –day incubation at 20°C, 26°C, 30°C or 37°C. This indicates that epiphytic bacteria could not grow after surface sterilisation and that any subsequent bacteria isolates were in fact endophytic.

We isolated and purified 54 endophytic bacterial strains from the root interior of *Urtica dioca* plant with approximately 10^6 CFU g^{-1}. Similar result with various studies have shown that the population density in the rhizoplane range from 10^5-10^7 CFU g^{-1} of fresh weight [19,20]. Results of serial dilution showed various colonies of different nature. Colonies with gram positive bacilli, showed several characteristics among them were the endospore formation, catalase production, Mannitol fermentation, negative for VP, having the best inhibition percentage in addition to a broad spectrum of action, that why they were chosen for this study. Three isolates were selected for further studies and named R45a; R45b; R21a.

The antifungal activity of endophytic *Bacillus spp* were shown in Table 1.

Statistical analysis revealed that the average diameter size of the mycelium growth indicates the antagonistic activity of endophytic *Bacillus spp* isolates against *Fusarium oxysporum, Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Phytophthora parasitica* by dual culture method on PDA medium in-vitro (Fig. 1).

Control plates not treated with the bacterial isolates were completely covered by the

phytopathogens showing no inhibition. Except the effect of the strain R45a against *Phytophthora parasitica* which generated an inhibition of 59.52%; the other fungi are inhibited at a percentage higher than 73, 21% by the 3 strains, with a maximum of 85.45% driven by the strain 45a against *Colletotrichum gloeosporioides.*

This result was in accordance with the studies realised by Bargabus and al which showed that several bacterial strains of *Bacillus* genius isolates from various habitats have significant efficacy in biological control against pathogenic fungi [21]. Furthermore, other authors reported that the *Bacillus spp* have showed significant activity against many plant pathogens including *F. oxysporum*, *P. cinnamomi*; *Colletotrichum gloeosporoides*, *Botrytis cinerea*, *Monilinia laxa*, *Sclerotiumr olfsii* [22,23,24].

In the present sudy, the endophytic *Bacillus* isolates R45b, R45a and R21a inhibited the mycelium growth of *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Phytophthora parasitica* with a different percentage of inhibition. This suggests that this isolates produced a variation of secondary metabolite [25]. Concurrently, different investigation showed the ability of the *Bacillus* species to synthesise more than 60 different types of antibiotics which also act as plant growth promoters [11,12,13].

3.2 Biochemical Characterisation and Plant Growth Promoting Characteristics of *Bacillus* Strains

3.2.1 Biochemical characterisation and production of enzyme

The bacterial isolates were Gram-positive rods. Cultures on NA showed irregular, entirely cream colored colonies. The isolates were positive for oxidase, cellulase, production of catalase and Methyl red. They are motile, capable of forming endospore and they also utilise carbon sources namely, mannitol, glucose, lactose and mannose. They are negative for voges-proskauer test, production of indole, and utilisation of lipids. According to Bergy's manual of determinative bacteriology 2009, these strains probably belong to the *Bacillus spp* genus.

3.2.2 Phosphate solubilisation and production of ammonia

It was observed that Pikovaskaya's agar, when inoculated with the isolates of *Bacillus*, did not show any clear zone of hydrolysis around the bacterial growth, which pointed out their inability to solubilise phosphates. Various studies indicate that *Bacillus* isolates were not able to solubilise phosphate. Wahydi et al. [18] showed that one out of twelve isolate of *Bacillus* from soybean rhizosphere were unable to solubilise the phosphorus and Ashrafuzzamam et al. [26] demonstrated that phosphate were not able to be solubilise by all the seven *Bacillus* species isolated from rice rhizosphere. At the same time, Shobha and Kumudini [27] showed that 7 *Bacillus* isolates from the rhizosphere of rice, chili, ragi and beans were incapable to solubilisate phosphate.

The R45b, R45a, R21a *Bacillus* strains developed yellow-brown color in pepton water culture after addition of Nessler's reagent indicating a positive test for ammonia production. Mishra et al. [28] showed the aptitude of the ammnia-producing strains *B. subtilis* strain MA-2 to develop the biomass of plant medicinal aromatic Geranium. Moreover, ammonia production was detected in 95% of the isolates from the rhizosphere of rice, mangrove and effluent contaminated soil influencing plant growth promotion [17,29].

Table 1. The antagonism of the isolated *Bacillus* spp against *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Phytophthora parasitica* by dual culture method

	Fusaruim oxysporum		*Colletotrichum gloeosporioides*		*Rhizoctonia solani*		*Phytophthora parasitica*	
	Myceluim growth (cm)	**Growth inhibition (%)**	**Myceluim growth (cm)**	**Growth inhibition (%)**	**Myceluim growth (cm)**	**Growth inhibition (%)**	**Myceluim growth (cm)**	**Growth inhibition (%)**
R45b	1.2^{c}	78.57	1.3^{b}	76.63	1.8^{c}	79.06	0.8^{d}	75.55
R 45 a	1.1^{c}	80.35	0.8^{d}	85.45	2.2^{b}	74.41	1.7^{b}	59.52
R21a	1.5^{b}	73.21	1.0^{c}	81.81	2.2^{b}	74.41	1.0^{c}	76.19
Control	5.6^{a}	0	5.5^{a}	0	8.6^{a}	0	4.2^{a}	0

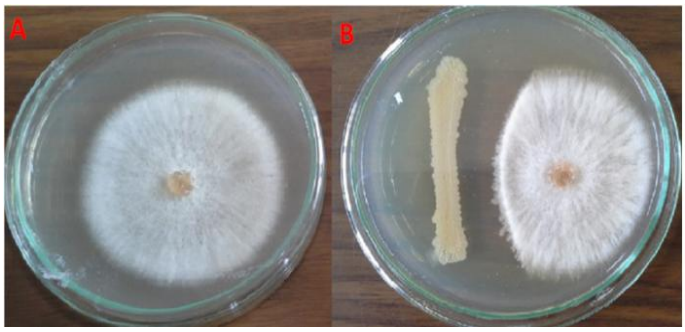

Fig. 1. Effect of the endophytique bacterial strain R45a against the fungus *Colletotrichum gloeosporioides* by dual culture method A: Control; B: Endophytic bacteria against fungus

Table 2. Biochemical and physiologic characteristics of endophytic *Bacillus sp.* strains

Test	Strain 21a	Strain 45a	Strain 45 b
Gram reaction	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
endospore	+	+	+
VogesPorsker	-	-	-
Nitrate reduction	-	-	-
Indole	-	-	-
Amonia production			
Phosphate solubilasation	-	-	-
Hypersonsitivity reaction	-	-	-
Urease	+	+	+
Lipase	-	-	-
esterase	-	-	-
cellulase	+	+	+
Amylase	+	+	+
Carbohydrates and polyalcohols			
mannitol	+	+	+
sucrose	+	+	+
sorbose	-	-	-
lactose	-	-	-
arabinose	+	-	+
fructose	+	+	+
dextrose	-	-	-
Maltose	-	-	-
Xylose	+	-	-
Raffinose	-	-	-
galactose	-	-	-
Mannose	+	+	+
Citrate	-	-	-
Glucose	+	+	+

4. CONCLUSION

The present study has illustrated that the endophytic strains R21a,R45a and R45b may be useful for biological control of diseases and plant growth promotion, on top of for the production of new metabolites and enzymes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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