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Screening of actinomycete bacteria producing antifungal metabolites which could be used in biological control against a phytopathogenic fungus (*Rhizopus stolonifer*)

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Abstract

A total of 77 actinomycetes strains were isolated from samples of water, soil and bark of plants. The isolation was performed on three culture media (Olson, Bennett and Glucose- Yeast extract-Malt extract: GLM). The antifungal activity against *Rhizopus stolonifer* is tested on two culture media (Bennett and GLM) using two different techniques (double layer technique and agar cylinder technique). Thus, 36 isolates (46.75%) were showed moderate to high inhibitory activity against the phytopathogenic fungus studied. Among the active strains, 8 isolates (SP6, Sfr1', SPO3, BEU1, SR1', SEU1, EC3 and EUS1) have showed strong antifungal activity which may provide a potent source for antifungal metabolites. They were selected to susceptibility testing and to determine the minimum inhibitory concentration (MIC). Results indicated that the extracts of 8 actinomycetes studied exert an interesting antifungal activity, especially the isolate BEU1 which has proved most efficient and exhibits an intense antifungal activity by inhibiting all phases of development cycle of *Rhizopus stolonifer*. It therefore appears necessary to use the Actinomycetales bacteria in biological control against plant diseases caused by this phytopathogenic fungus.

Keywords

Actinomycetes, Antifungal, *Rhizopus Stolonifer*, Phytopathogenic Fungi, Leak, Biological Control

1. Introduction

The phytopathogenic fungal poses serious problems worldwide and causes several plants and animal diseases. Plant diseases caused by fungi include rusts, smuts, rots, and may cause severe damage to crops [1], [2].

Rhizopus stolonifer is a phytopathogenic fungus causes a disease known as leak of some plants [3], especially strawberry, carrot, apple, plum, peach and pear [4]. It is a mold that grows at temperatures between 30°C and 5°C with an optimum around 25°C [5]. In a humid environment, this plant pathogen grows rapidly and produces a black and loose mycelium with aerial fruiting white then becoming black. The establishment of the infection takes 2 to 6 hours [6]. *R. stolonifer* is a cosmopolitan fungus located mainly in warehouses on the conditioning material and storage of fruits. The plant moisissement appears after harvest.

In post-harvest, how best to control this disease is the

rapid cooling of fruits and their conservation between 0 and 5°C [7]. The information about the use of fungicides in pre-and post-harvest is almost nonexistent [8]. The majority of fungicides are mostly chemical origin. Irrational and excessive use of chemical fungicides in fight against phytopathogenic diseases has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide. On account of these problems in fungal disease control, a several search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmentally friendly.

Microbial antagonists are widely used for the biocontrol of fungal diseases. Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists [9], [10], [11]. Antibiotics have been used in many fields including agriculture, veterinary and pharmaceutical industry. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes. They are the main source of antifungal. [12], [13], [14], [15].

This report describes the isolation of actinomycete strains producing antifungal secondary metabolites from samples collected from different Moroccan ecosystems. Our ongoing search for new antifungal metabolites led to the isolation of 36 actinomycete strains showing antifungal activity against *R. stolonifer*. The antifungal activity of the culture extract of 8 representative isolates have showed that the antifungal potential of extracellular metabolites produced by Actinomycetes could be exploited in biological control as an natural antifungal compounds against *R. stolonifer*.

2. Materials and Methods

2.1. Sampling Procedure

Several habitats were selected for isolation of Actinomycetes:

- Twelve soil samples were collected using the Pochon Tardieux technique [16]: Discharge Soils of Settat (SD), pottery discharge soil of Boujad (SP), soil irrigated by wastewater of Settat (SEU), mud of waste water of Boujad (BEU), Errachidia soil (SE), substrates attached to ratchet in middle Atlas of Beni-mellal (SR), forest soil of Settat (SFr), phosphatic soil of Oued zem (SPO) and rhizosphere soil of coniferous (Rhc, Rhp, Rhc-ac).
- Eight water samples (250 ml) were collected according to Rodier technique [17]: Dams (EBB, EBM), Water sources (ESB, ESS), Wadis (EOO, EOB), Wastewater (EUS, EUB).
- Three bark samples were collected according to the recommended technique by Kitouni [18] from Conifers (*Casuarina* sp: EC), from Eucalyptus (*Eucalyptus camaldulensis*: EE) and from Atriplex (*Atriplex nummularia*: (EA)

In order to reduce the incidence of bacteria and molds, the soil samples and bark samples were pretreated with calcium carbonate; the water samples were pretreated with phenol 7 $mg.mL^{-1}$ [19].

Soil dilution plate technique was employed to isolate the actinomycete strains on different media such as Olson, Bennett and Glucose- yeast extract-malt extract (GLM) with pH adjusted to 7.3 and the plates were incubated at 28°C for 14 days. The actinomycete strains predominant on different media were picked, purified and preserved on Bennett medium at 4°C [20].

2.2. In Vitro Screening of Isolates for Antifungal Activity

Determination of antifungal activities of pure actinomycete isolates was assessed using the double-layer method [1], [19]. Antagonism was measured by the determination of the size of the inhibition zone [21].

2.3. Production of Antifungal Metabolites

The secondary metabolites produced by 8 representative isolates (SP6, Sfr1', SPO3, BEU1, SR1', SEU1, EC3 and EUS1) were extracted by the method of Ellaiah *et al*. [22] Pure culture of the strains was transferred aseptically and individually into the 8 flasks (500 mL) containing 150 mL of synthetic medium [19]. After 24 h of incubation, the seed culture at a rate of 10% was inoculated into the production medium of the same composition. After incubation at 28 ° C under agitation at 250 rpm for eight days, 50 mL of each culture were centrifuged at 5000 rev / min for 20 min. Then the supernatants (crude extracts) were used directly for performing sensibility testing.

2.4. Sensitivity Testing of *R. Stolonifer* to Bioactive Metabolites Produced by Actinomycete Isolates

A Potato dextrose agar (PDA) medium, freshly prepared, containing 5%, 10%, 15%, 20% and 25% of the crude extracts (supernatants) to be tested was covered with a 0.6% agar layer of nutrient agar medium, previously seeded with spores of *R. stolonifer*. After 24, 48 and 72 hours incubation at 28°C, spore germination, fungus growth and sporulation were noted [8].

3. Results and Discussion

3.1. Isolation of Actinomycete Stains

This study aimed to isolate actinomycete strains presenting an antifungal activity using the selective isolation media. Thus, seventy seven different actinomycete strains were isolated from twenty three samples collected from different Moroccan ecosystems (Table 1).

Table 1. *Total number of actinomycetes strains isolated from various Moroccan ecosystems*

Sampling site		Code	Number of isolates	Total
Soil	Pottery discharge	SP	19	66
	Young discharge	SDJ	4	
	Swamped soil by leaching	SDL	1	
	Soil irrigated by wastewater	SEU	6	
	Errachidia soil	SE	0	
	Wastewater mud	BEU	4	
	Phosphatic soil of Oued-Zem	SPO	5	
	Forest	SFr	5	
	Substrates attached to ratchet	SR	2	
	Coniferous rhizosphere	Rhc	20	
Plant barks	*Eucalyptus camaldulensis*	EE	0	5
	Atriplex nummularia	EA	2	
	Casuarina sp.	EC	3	
Water	Wastewater	EUB	3	6
		EUS	2	
	Dam water	EBB	1	
		EBM	0	
	Source water	ESB	0	
		ESS	0	
	Wadis water	EOB	0	
		EOO	0	
Total				77

The results analysis presented in Table 1 showed that in all isolates 66 (85.71%) were isolated from telluric samples, 6 (7.79%) from water samples and 5 (6.49%) from bark of plants. The greatest number of isolates was obtained from telluric samples; this result is consistent with literature data [23], [24], [25].

Maximum number of actinomycetes was obtained in the soil collected from coniferous rhizosphere (30.30%). A similar result was reported by Miller *et al.* [26] and Crawford *et al.* [27], which show that rhizosphere soils are richer in actinomycetes.

The existence of 19 actinomycetes (28.79%) in soil sample of pottery discharge could be explained that this ecosystem is rich at mineral and organic matter coming from the pottery ashes which may be the reason for highest count.

3.2. Antifungal Activities of Isolates

The results of the antifungal activity of all isolates are given in Table 2.

Table 2. *Antifungal activity of actinomycete isolates*

Sampling site	Isolates	IZ	Sampling site	Isolates	IZ
Soil of Pottery discharge	SP1	10	Substrates attached to ratchet	SR1	0
	SP2	10		SR1’	16
	SP3	7	Sol of Coniferous rhizosphere	Rhp1	0
	SP4	0		Rhp2	0
	SP6	15		Rhp1’	0
	SP8	12		Rhc1	0
	SP9	10		Rhc2	0
	SP1’	13		Rhc1”	4
	SP2’	0		Rhc-ac1	0
	SP3’	9		Rhc-ac2	0
	SP4’	3		Rhc-ac1’	7
	SP6’	8		Rhc-ac2’	0
	SP7’	NT		Rhc-ac3’	0
Forest soil	SP8’	0	Soil irrigated by wastewater	Rhc-ac4’	0
	SP10’	4		Rhc-ac5’	0
	SP11’	6		Rhc-ac6’	0
	SP12’	0		Rhc-ac7’	0
	SP13’	14		Rhc-ac8’	0
	SP14’	0		Rhc-ac9’	8
	SFr1’	16		Rhc-ac10’	0
	SFr2’	7		Rhc-ac11’	7
	SFr3’	11		Rhc-ac12’	0
	SFr4’	0		SEU1	20
	SFr5’	0		SEU2	5
Phosphatic soil	SPO1	7		SEU1’	15
	SPO2	12		SEU2’	2
	SPO3	15		SEU3’	0
	SPO4	0		SEU4’	14
	SPO6	NT	Bark of *A. nummularia*	EA1	0
Discharge soil	SDJ1’	0		EA2	0
	SDJ2’	0	Bark of *Casuarina* sp.	EC1	0
	SDJ3’	3		EC2	0
	SDJ1”	0		EC3	17
Wastewater mud	SDL	0	Dam water	EBB	0
	BEU1	17	Wastewater	EUB1	0
	BEU2	0		EUB2	0
	BEU1’	14		EUB3	5
	BEU2’	0		EUS1	15
				EUS2	13

IZ: Inhibition zone in mm
These values represent the average of inhibition zones of three repeats.
NT: Not tested

The antifungal activity of the test isolates was varied. 36 of 75 actinomycetes isolates were shown to have interesting antifungal activity against the phytopathogenic fungus studied (Figure 1).

The diameters of inhibition zones ranged from 0 to 20 mm. The highest values of these zones are recorded with strains coming from polluted or arid environments; a similar result was reported by Boussaber *et al* [19]. Among the active

isolates, 8 (SP6, Sfr1', SPO3, BEU1, SR1', SEU1, EC3 and EUS1) were showed a strong antifungal activity against *R. stolonifer*.

3.3. Susceptibility Testing of *R. Stolonifer* Spores to Actinomycete Extracts

The crude supernatant extracted from Actinomycetes was used to test the antifungal activity against *R. stolonifer*. Different concentrations such as 5, 10, 15, 20 and 25% of supernatant were used to check antifungal activity and to determine the minimum inhibitory concentration. Table 3 summarizes result of spore germination after 24, 48 and 72 hours at 28°C in PDA medium containing different concentrations of supernatants from actinomycete cultures.

Table 3. *Spores germination of R. stolonifer after 24, 48 and 72 hours incubation on PDA medium containing different concentrations of actinomycete supernatants*

Composition of culture medium	24 hours					48 hours					72 hours				
	5%	10%	15%	20%	25%	5%	10%	15%	20%	25%	5%	10%	15%	20%	25%
PDA + Supernatant of SP6	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
PDA + Supernatant of SFr1'	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
PDA + Supernatant of SPO3	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-
PDA + Supernatant of BEU1	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
PDA + Supernatant of SR1'	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-
PDA + Supernatant of SEU1	+	+	-	-	-	+	+	x	-	-	+	+	+	-	-
PDA + Supernatant of EC3	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-
PDA + Supernatant of EUS1	+	+	+	x	-	+	+	+	+	-	+	+	+	+	-
PDA without supernatant	+					+					+				

+: Spores germination, -: No spores germination, x: Reduced and localized germination

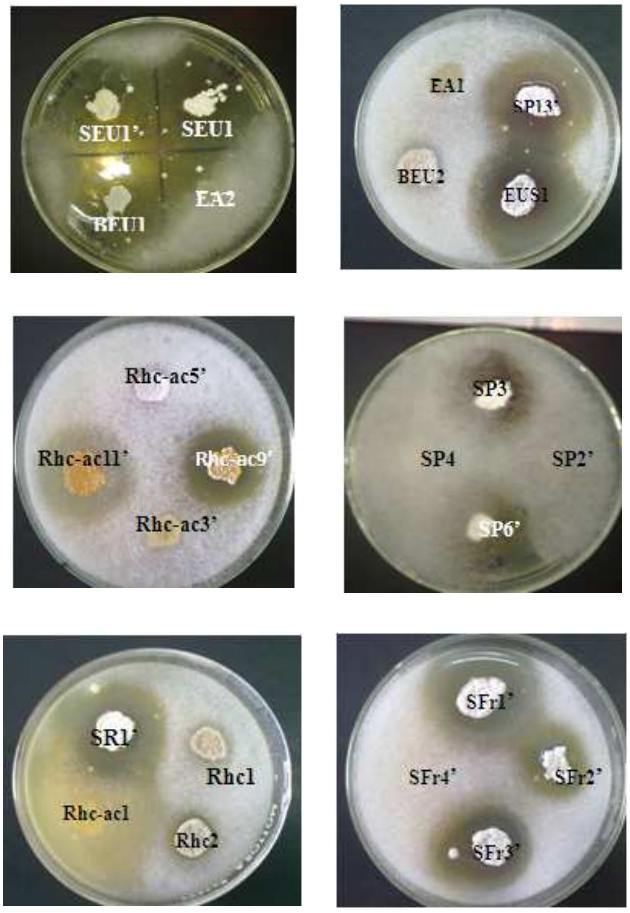

Fig. 1. *Highlighting of antifungal activity by double layer technique of some isolated actinomycete strains*

The resistance of fungus to antifungal contained in supernatant was evaluated according germinated spores (germ tube length is at least twice the diameter of spores). The resistance is much higher than the percentage of germinated spores is high. The absence of germination indicates good effectiveness of antifungal metabolite.

Spores germination begins after 8 hours incubation. The effect of actinomycete extracts on spore germination depends on concentration of supernatant. All extracts studied are not effective at 5% concentration to inhibit the spores germination.

The supernatant of SP6 (20%), SFr1' (25%) and EC3 (15%) seem to act as germination inhibitors and not like fungicides. All supernatants except that of SFr1' inhibited germination at concentrations of 25%.

Spores germination, fungal growth and sporulation on PDA medium containing the supernatant of SFr1', SR1' and EC3 after 72 hours (Table4) could be explained by metabolism or disabling antifungal substances (hydrolysis, oxidation, ...) by spores with time, which confirms the results obtained by Thibodeau *et al* in 2002 [8].

The supernatant of SR1' (10% and 15%) seemed to stimulate spores germination and inhibits it at 20% and at 25%. The contrary, after 72 hours, the supernatant at all used concentration of SR1', SFr1' and EC3 stimulates fungal growth and sporulation.

The contained bioactive metabolites in supernatants of BEU1 and SEU1 appear to be most effective in preventing spores germination, fungal growth and inhibition of sporulation.

The case of EUS1 is particular: at 25%, it completely inhibits the spore germination in the first 72 hours of incubation, after, it permits mycelial growth at all concentrations, but sporulation is subsequently completely inhibited even after 15 days (Table 4).

Table 5 shows the minimum inhibitory concentrations (MIC) in percentage of supernatant in culture medium. It varies according to incubation time and to supernatant origin. The lowest MIC is recorded with the supernatant of BEU1,

10% successively was sufficient to inhibit the germination, mycelial growth and sporulation. The supernatant of this strain thus contains bioactive metabolites able of preventing the development of *R. stolonifer* and consequently fight against leak caused by this fungus.

Table 4. *Actinomycete supernatant effects on fungal growth and sporulation*

Composition of culture medium	Growth				Sporulation			
	10%	15%	20%	25%	10%	15%	20%	25%
PDA + Supernatant of SP6	+	+	+	-	++	++	++	0
PDA + Supernatant of SFr1'	+	+	+	+	++	++	++	++
PDA + Supernatant of SPO3	+	+	-	-	++	++	0	0
PDA + Supernatant of BEU1	-	-	-	-	0	0	0	0
PDA + Supernatant of SR1'	+	+	+	+	++	++	++	++
PDA + Supernatant of SEU1	+	-	-	-	0	0	0	0
PDA + Supernatant of EC3	+	+	+	+	++	++	++	++
PDA + Supernatant of EUS1	+	+	+	+	0	0	0	0
PDA without supernatant	+				++			

+: Mycelial growth, -: No growth even after 15 days, + +: Fungus sporulation , 0: No sporulation even after 15 days,

Table 5. *Minimum inhibitory concentrations (MIC) in percentage of supernatant in culture medium*

Supernatant	Germination after			Growth	Sporulation
	24 hours	48 hours	72 hours		
SP6	20%	25%	25%	25%	25%
SFr1'	*	*	25%	*	*
SPO3	20%	20%	20%	20%	20%
BEU1	10%	10%	10%	10%	10%
SR1'	25%	25%	25%	*	*
SEU1	15%	20%	20%	15%	10%
EC3	15%	20%	20%	*	*
EUS1	25%	25%	25%	*	10%

*: Without MIC

4. Conclusion

Actinomycetes are widely distributed in the environment and known for the diverse biologically active molecules they produce. Several Moroccan ecosystems were explored. This study aimed to isolate and select actinomycetes bacteria which could produce antifungal substances. Among all isolates, eight of them (SP6, Sfr1', SPO3, BEU1, SR1' SEU1, EC3 and EUS1) have showed a significant inhibitory activity against *R. stolonifer*.

The obtained supernatant from culture of these actinomycete strains had the ability to inhibit at least one phase of the development cycle (spore germination, mycelial growth and sporulation) of this pathogenic fungus at varying degree. The growth of fungal mycelium decreases with the increase in the concentration of compound extracted from Actinomycete strains. Similar results have been investigated by various authors [2], [28], [29] who reported that the crude extract of antifungal compounds was active against *R. stolonifer*.

In this assay, isolate BEU1 have presented higher inhibitory activities than that exhibited by other isolates, the contrary, isolates SR1', SFr1' and EC3 have stimulated fungal growth and sporulation, Similar results were obtained by Beaulieu and Lerat [30]. thus, extracts of actinomycetes can be exploited in two ways, the first way is to use actinomycetes as a means of biological control against plant fungal diseases such as leak caused by *R. stolonifer*, and the second way is to exploit other physiological properties of actinomycetes (Secretion of enzymes, production of plant hormones, induction of defense mechanisms in plants etc..) to increase the plant growth.

These results show the interest that may have actinomycetes in biological control against phytopathogenic diseases and incites to deepen studies on bioactive metabolites produced by the active strains. Thus, it is necessary to:

- Optimize of bioactive metabolite production;
- Identify and characterize their principle active;
- Assess their impact in field or in warehouse in order to use them as natural fungicides to control *R. stolonifer* which causes diseases in plants, and subsequently reducing the dependence on the synthetic fungicides.

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