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Siderophore-extracts from some *Pseudomonas* strains and their antifungal activity against phytopathogenic fungi of *Phaselus vulgaris* L.

Slimane Mokrani[1,2], **Nabti El-hafid**[1]✉

[1]Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria.

[2]Department Agronomy, Laboratory of Research on BiologicalSystems and Geomantic (L.R.S.B.G), University of Mustapha Stumbouli, P.O. Box 305, 29000 Mascara, Algeria

✉**Corresponding author:**

Department Agronomy, Laboratory of Research on BiologicalSystems and Geomantic (L.R.S.B.G), University of Mustapha Stumbouli, P.O. Box 305, 29000 Mascara, Algeria

Email: nabtielhafid1977@yahoo.com

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ABSTRACT

Pseudomonas spp are largely known to produce siderophores that mainly ensure the Fe(++) chelation. Thus, this mechanism may induce an indirect inhibitory effect of plant pathogenic fungi. In this current study, we try to highlight the siderophores production, by 20 *Pseudomonas* strains, using the CAS agar medium. Followed, by siderophores extraction through two methods (liquid-liquid and Amberlite XAD4 resin). An antifungal activity with the obtained pure siderophore-extracts were tested against three

phytopathogenic fungi of *Phaseolus vulgaris L* (*Fusarium oxysporum f.* sp. *phaseoli, Stemphylium botryosum* and *Sclerotinia sclerotiorum*). Furthermore, inhibitory effects were compared by two techniques (disk and well diffusion). Results of siderophores production revealed the appearance of orange halos around growth, indicating "Fe" chelation by 13 (65%) of *Pseudomonas* strains on CAS agar medium, from which, 4*Pseudomonas* strains (P8, P9, P12 and P19) showed hyper siderophores production. The comparison between the both extraction methods revealed that Amberlite XAD4 resin extracts are more effective than liquid-liquid extracts. Whereas, the antagonistic methods did not reveal significant differences between disk and well diffusion methods. Consequently, this lead to conclude that *in vitro* trials with pure siderophore-extracts are potential inhibitors of phytopathogenic fungi.

Key words: *Pseudomonas* spp, *Phaseolus vlgaris* L, phytopathogenic fungi, siderophore extracts, antifungal activity.

1. INTRODUCTION

Among the important nutrient component, iron is one of the almost essential microelements certainly for every living cells because it's none abundance in the environment, particularly in soils (Pahari and Mishra, 2017). Microorganisms have consequently developed specific and efficient iron acquirements systems to assure their survival, which include the secretion of siderophores (Renshaw et al., 2002; Ortíz-Estrada et al., 2012). Siderophores are secondary metabolites of low molecular weight, between 200 and 2000 daltons. Whose function is to solubilize, chelate and extract ferric iron from many elements and organic complexes, and for that reason perform it accessible to microorganisms (Neilands, 1995; Ahmed and Holmström, 2014). They are produced by *bacteria*, *fungi, and plants (*Rajkumar et al., 2012; Goswami et al., 2016). These molecules have a high affinity for iron (III), and are secreted when microorganisms are underneath iron stress, permanency playing an essential function into host-pathogen interaction (Hissen et al., 2005). Furthermore, they are endowed with excellent properties in collection to iron scavenging, such as composition of complexes with other metals or antimicrobial activity (Cornelis and Matthijs, 2007; Shanmugaiah et al., 2015). Also, siderophores contribute to the mobilization of iron and other molecules, and are implicated in virulence processes. Recently, a vigorous relation between siderophores and oxidative stress tolerance has been also highlighted *(*Albelda-Berenguer et al., 2019*)*.

Particularly, soil phytopathogenic plant microbes and commonly beneficial microorganisms called PGPR (Plant Growth Promoting Rhizobacteria) compete to survive in naturally combined communities and numerous environments (Stubbendieck and Straight, 2016). More especially, *Pseudomonas* spp are potential agents for the biological control which suppress plant diseases through protecting plants from several fungal infection (Ganeshan and Manoj Kumar, 2005). This high capacity of Pseudomonas to inhibit phytopathogens is attributed to their capacity to produce various secondary metabolites (Nagarajkumar et al., 2004; Mishra and Arora, 2018). These include antifungal compounds like Pyrrolnitrin which is active against *Rhizoctonia* spp, *Fusarium* spp, and other plant pathogenic fungi (Ligon et al., 2000). Different species belonging to *Pseudomonas* genus like *P. stutzeri* produce extracellular enzymes like chitinase and laminase capable of lysing the mycelia of *Fusarium solani* (Srivastava and Shalini, 2008). Also, some *Pseudomonas* species can also produce levels of HCN (Hydrogen cyanide) that are toxic to certain pathogenic fungi (Rijavec and Lapanje, 2016). Whereas, siderophores are important extracellular compounds implicated directly and indirectly in suppression effects (O'sullivan and O'Gara, 1992; Rachid and Ahmed, 2005; Gamalero and Glick, 2011; Yasmin et al., 2017; Qessaoui et al., 2019). *Pseudomonas* spp siderophore positive strains were reported to inhibit different plant pathogens (Cheng et al., 1995; Sayyed and Patel, 2011; Kirienko et al., 2018). However, this lead to investigate the inhibitory effects of pure siderophore-extracts of *Pseudomonas* against plant phytopathogenic fungi.

This study was focused on characterization of the antifungal activity of siderophore-extracts from *Pseudomonas* strains against phytopathogenic fungi of *Phaseolus vulgaris* L (common bean). Also, to compare the antifungal activity of siderophore-extracts using two methods (liquid-liquid and Amberlite XAD4 resin). However, the possible variation of the inhibitory effects, according to the antifungal activity techniques (disk and well diffusion).

2. MATERIALS AND METHODS

2.1. Bacterial strains

20 *Pseudomonas* strains were characterized and identified in the previous study by biochemical and physiological tests (Mokrani, 2019). Identification was carried out by determination of lecithinase production, gelatin liquefaction, KOH solubility, and utilization of L-arabinose, D-xylose, sodium tartrate, D-alanine, L-tryptophan, sorbitol and L-arginine (Goszczynska et al., 2000). Grow at 4 °C and 41 °C was determined according to Rhodes (1959). Species identification was established according to the dichotomous keys

and LOPAT test as described by Krid et al. (2011). Numerical treatment of identification data was performed by online ABIS (Advanced Bacteria Identification Software).

Table 1 represented soil type, plant and bacterial identification of *Pseudomonas* strains. From all strains, 17 bacteria were isolated from rhizospheric soil of *Phaseolus vulgaris* L and Allium cepa L and 3 strains from bulk soil.

Table 1 Soil type, plant and bacterial strains identification.

Bacterial strain codes	ID	Soil (B/R)	Plant
P1	*P. pseudomallei*	R	*Phaseolus vulgaris* L
P2	*P. plantarii*	R	*Phaseolus vulgaris* L
P3	*P. cichorii*	R	*Phaseolus vulgaris* L
P4	*P. corrugota*	R	*Phaseolus vulgaris* L
P5	*P. alcaligenes*	R	*Phaseolus vulgaris* L
P6	*P. stutzeri*	R	*Phaseolus vulgaris* L
P7	*P. plantarii*	R	*Phaseolus vulgaris* L
P8	*P. cepatia*	R	*Phaseolus vulgaris* L
P9	*P. pseudomallei*	R	*Phaseolus vulgaris* L
P10	*P. cepatia*	R	*Phaseolus vulgaris* L
P11	*P. fluorescens* Biovar 2	R	*Allium cepa* L
P12	*P. fluorecens* Biovar 2	R	*Allium cepa* L
P13	*P. fluorescens* Biovar 2	R	*Allium cepa* L
P14	*P. stutzeri*	R	*Allium cepa* L
P15	*P. fluorescens* Biovar 2	R	*Allium cepa* L
P16	*P. fluorescens* Biovar 3	R	*Allium cepa* L
P17	*P. aeruginosa*	R	*Allium cepa* L
P18	*P. fluorescens* Biovar 2	B	
P19	*P. stutzeri*	B	
P20	*P. cichorii*	B	

ID: identification; B: bulk soil; R: rhizospheric soil; P: *Pseudomonas*

2.2. Fungal isolates

Three phytopathogenic fungi were isolated from greenhouse in Tighanif-Mascara (35°24' N 0°19'E). Fungal isolates were recuperated from infected *Phaseolus vulgaris* L plants. The isolates were: *Fusarium oxysporumf.* sp. *phaseoli*, *Sclerotinia sclerotiorum* (Mokrani et al., 2018b); and *Stemphylium botryosum* (Mokrani et al., 2019b). Fungi isolates were observed macroscopically (Alsohaili and Bani-Hasan, 2018) and microscopically (Su et al., 2012).

2.3. Siderophores detection

Pseudomonas strains were grown in King's B agar medium and incubated at 28-30 °C/24-30 h. Siderophores were detected by streaking the bacteria on Petri plate's containing CAS agar. Then, followed by incubation at 30°C/72 h. The formation of orange halos around the colonies indicates siderophores synthesis (Yeole and Dub, 2000; Louden et al., 2011).

CAS agar was prepared according to Alexander and Zuberer (1991) from four solutions that were sterilized separately before mixing them. The Fe-CAS indicator solution (solution 1) was prepared by mixing 10 ml of 1 mM $FeC1_3.6H_20$ (in 10 mM HC1) with 50 ml of an aqueous solution of CAS (1.21 mg/ml). The mixformed (dark purple) was homogenized with HDMA solution constituting volume of 40 ml (1.82 mg/ml). This resulted in a dark blue solution which was sterilized by autoclaving and then cooled to 50°C.Preparation of the solution 2 or buffer solution was realized by adding 30.24 g of PIPES in 750 ml of a saline solution containing 0.3 g $KHPO_4$, 0.5 g NaCl and 1.0 g NH_4C1. The pH was adjusted to 6.8 with 50% KOH and water was added to a final volume of 800 ml. The solution was autoclaved after adding 15 g of agar, then cooled to 50°C. Solution 3 contained the following elements (in 70 ml water): 2 g glucose, 2 g mannitol, 493 mg $MgSO4.7H_20$, ll mg $CaCl_2$, 1.17 mg $MnSO_4.H_20$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4.5H_20$, 1.2 mg $ZnSO4.7H_20$ and 1.0 mg $Na_2MoO_4.2H_20$. After preparation and cooling to 50 °C of Solution 3, it was then mixed with solution 2 and solution 4 composed of 30 ml of 10% (w/v) casamino acids. The indicator solution was added to the final mixture.

2.4. Siderophores production

Bacterial cultures were prepared on a nutrient broth that was incubated at 28-30 °C/3 days. After incubation, bacterial cultures were filtrated. Then, 10 ml of each bacterial filtrate were mixed with 0.01 mg of iron, 0.01 g of ferric chloride and 6.6 g of ammonium sulphate. The mixtures formed were shaken overnight at 4°C and passed through a filter paper (formed filtrated mixture).

2.4.1. Liquid/liquid extraction

Siderophores were extracted by the liquid/liquid method according the modified technique described by (Bertrand, 2009). Siderophores were firstly extracted from the filtrated mixture obtained after siderophores production four times with 5 ml of benzyl alcohol. The organic phase formed was dried by the addition of magnesium sulfate and filtrated on hydrophilic cotton. The procedure was completed by adding 12.5 ml of diethyl. Siderophores were secondly extracted four times with 2 ml of ultrapure water. The aqueous phases formed were washed with 5 ml of diethyl ether. 2-3 ml of the siderophore-extracts obtained were sterilized using Millipore membrane filter (0.45 µm).

2.4.2. Amberlite XAD-4 resin extraction

For extraction of siderophores 1 g of Amberlite XAD4 resin (Aldrich) was added to the filtrated mixture; and the whole mix obtained were shaken for 17 h. The Amberlite XAD4 resin was recovered by filtration on fritted glass and washed four times with 5 ml of ultrapure water. 2 ml of methanol was used for siderophores extraction. This procedure was carried out four times. Finally, 2 ml of pure siderophore extract formed were sterilized thru 0.45 µm Millipore membrane filter (Bertrand, 2009).

2.5. Antifungal activity of siderophore extracts

2.5.1. Well diffusion method

This technique was performed following the modified method described by Wafaa and Mostafa (2012). Agar disk of each phytopathogenic fungus was deposed in the center of the Petri plate containing PDA medium. Simultaneously, 50-100 µl of siderophore-extracts were introduced in four wells made at a distance of 2-3 cm from the fungus. Incubation of Petri dishes was realized at 25°C/6 days.

2.5.2. Disk diffusion technique

The Disk diffusion technique consists in placing an agar disk of each phytopathogenic fungi in the center of a Petri plate containing PDA medium and four sterile paper disks filled with 50-100 µl of siderophore-extracts at a distance of 2-3 cm from the fungus. The Petri plates were incubated at 25°C/6 days (Liu et al., 2019).

2.5.3. Data expression

The antifungal activity of siderophore-extracts were characterized by partial or total inhibition of the radial growth of the phytopathogenic fungi. The results were expressed by the distance growth between each fungus and the siderophore antagonistic extract. Thus, antifungal activity exerted was estimated by calculating the inhibition percentage of mycelia growth of four replicates compared to the control (Mohammadi and Aminifard, 2013):

$$IP\ (\%) = (r\ control - r\ test)/r\ control * 100$$

Where: IP: inhibition percentage; r test: Maximum radial distance of fungus growth on a line towards the siderophore extract; r control: Maximum radial distance of fungus growth inoculated alone in the center of Petri plate.

2.6. Statistical analysis

The percentages of fungi inhibition were represented by mean ± standard deviation. Then, ANOVA/Tukey tests were carried out as appropriate. Furthermore, for comparing among siderophore extraction methods and antifungal activity techniques, data obtained were treated using the three way analysis of variance (ANOVA) at ($P \leq 0.05$) level of significance.

3. RESULTS

3.1. Fungal isolates

Figure 1 showed the macroscopic and microscopic observations of the three phytopathogenic fungi isolated from *Phaseolus vulgaris* L.

The colony of isolate *Fusarium oxysporum f.* sp. *Phaseoli* is yellow and cottony. The isolate *Stemphylium botryosum* is very characteristic showing black color colony surrounded by a whitish zone. Furthermore, the colony of the isolate *Sclerotinia sclerotiorum* is white and revealing the especially presence of 15-20 sclerotia. The microscopic aspects of the three fungi revealed branched and compartmentalized hyphae. At least, isolate *Stemphylium botryosum* showed specific mature conidia of faded color and light immature conidia.

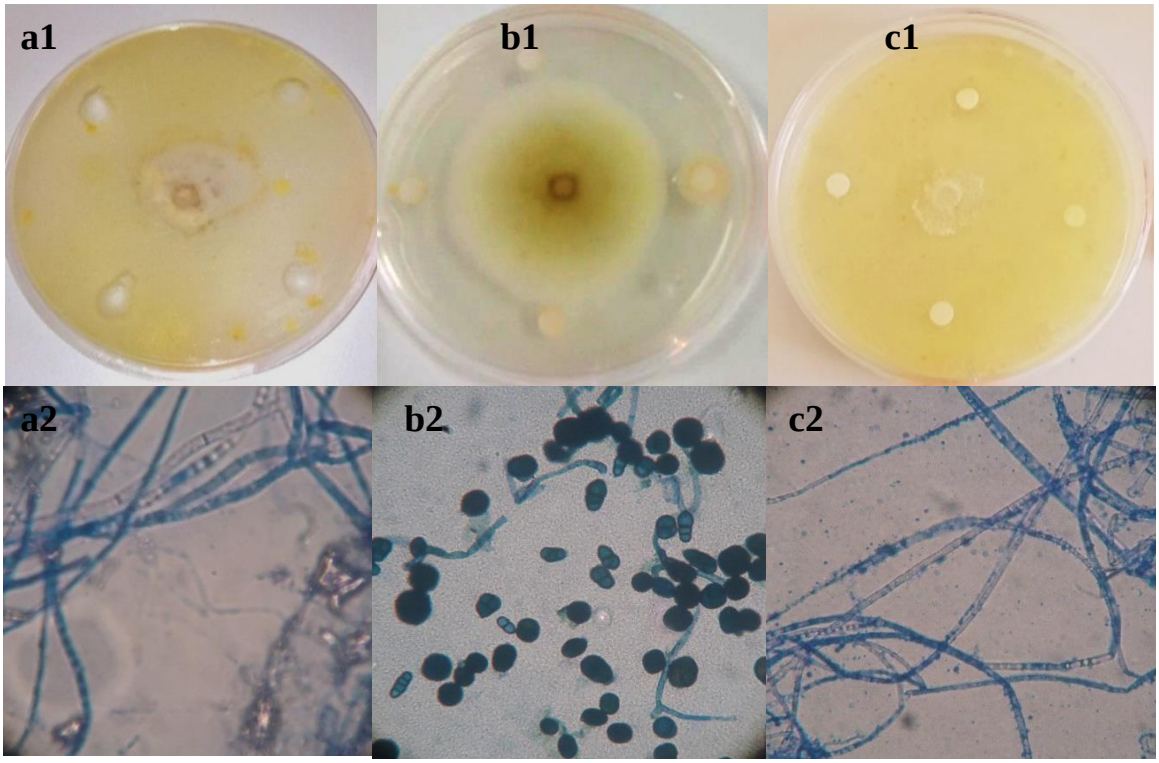

Fig. 1. Macroscopic and microscopic observations of phytopathogenic fungi isolates (a1: macroscopic aspect of *Sclerotinia sclerotiorum*; b1: macroscopic aspect of *Stemphylium botryosum*; c1: macroscopic aspect of *Fusarium oxysporum* f. sp. *phaseoli*; a2: microscopic aspect of *Sclerotinia sclerotiorum*; b2: microscopic aspect of *Stemphylium botryosum*; c2: microscopic aspect of *Fusarium oxysporum* f. sp. *phaseoli*).

3.2. Siderophores detection

Qualitative siderophores production revealed orange halos around growth, indicating Fe chelation. 13 (65%) of Pseudomonas strains produced siderophores in CAS agar medium, at the end 72 h of incubation. Estimation of siderophores synthesis varied from low, moderate and high production (Table 2).

Tab. 2. Qualitative siderophores production

Strains	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
SID	+	+	-	-	+	-	+	++	+++	-
Strains	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
SID	-	+++	+	+	+	-	+	+	++	-

SID: siderophores; +: low production; ++: moderate production; +++: high production; P: *Pseudomonas*.

Four strains (P8, P9, P12 and P19) revealed hyper production of siderophores characterized by highest halos around growth (Figure 2). This qualitative production test resulted in the screening of these strains for the siderophores extraction and antifungal activity tests.

3.3. Antifungal activity of siderophore extracts

Taken together, Amberlite XAD4 resin and liquid-liquid extracts exerted antifungal inhibition effects by both well and disk diffusion methods (Figure 3). In addition, direct confrontation of siderophore-extracts and phytopathogenic fungi on PDA medium showed

variable inhibition zones against the three fungi isolates *Fusarium oxysporum f. sp. phaseoli*, *Sclerotinia sclerotiorum* and *Stemphylium botryosum*.

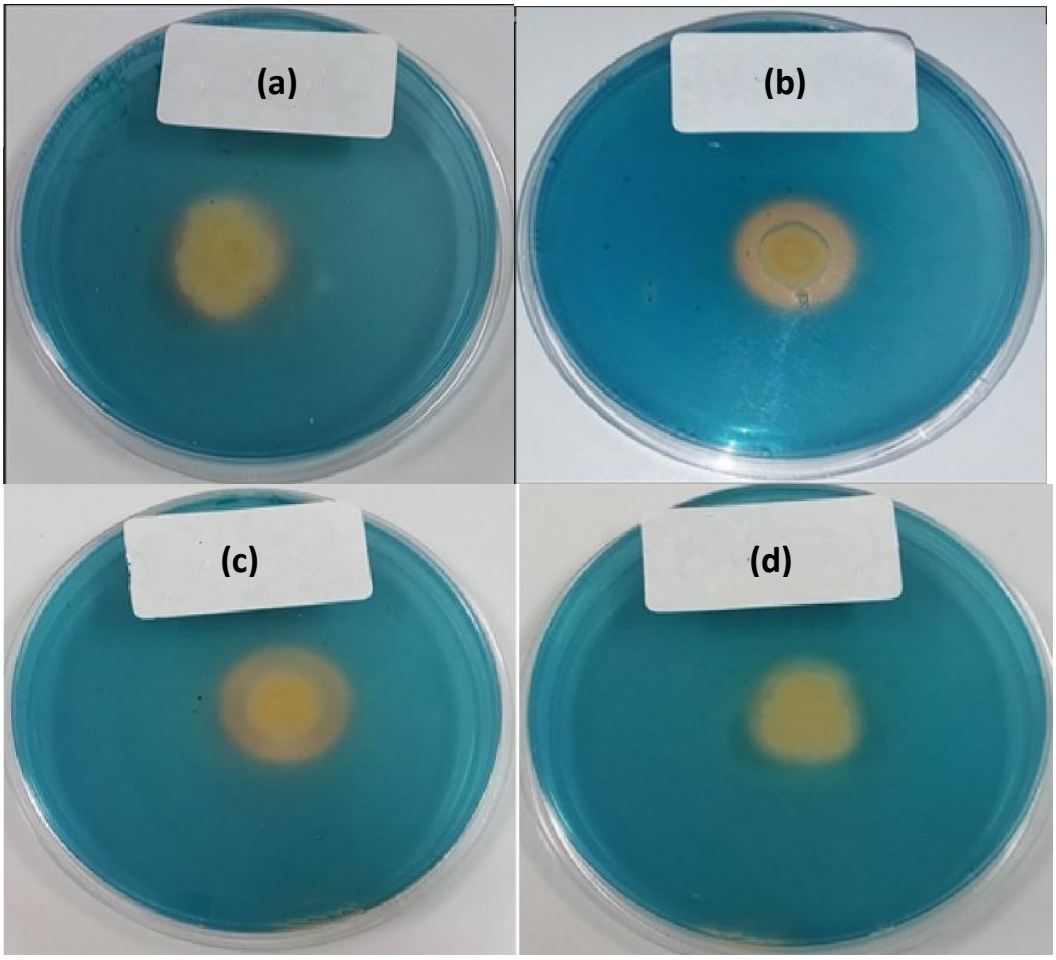

Fig. 1. Screening of *Pseudomonas* strains for siderophores production in CAS medium. (a: strain P8; b: strain P9; c: strain P12; d: strain P19).

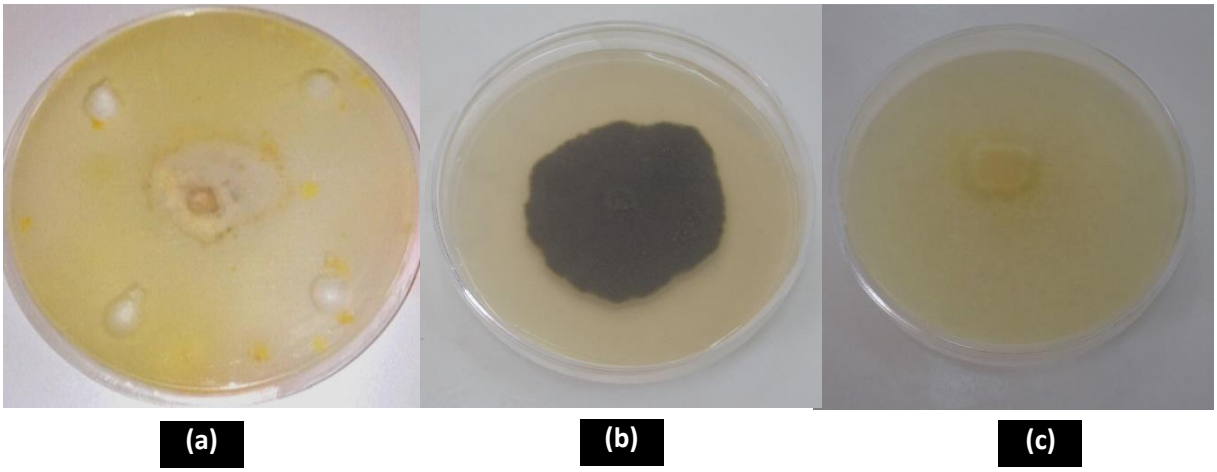

Fig. 2: Antagonistic activity of siderophore extracts against the phytopathogenic fungi (a: liquid-liquid extract of strain P8 against Sclerotinia sclerotiorum by well diffusion method; b: liquid-liquid extract of strain P9 against Sclerotinia sclerotiorum by disk diffusion method; c: Amberlite XAD4 resin extract of strain P19 against Sclerotinia sclerotiorum by disk diffusion method).

3.3.1. Antifungal activity of Amberlite XAD4 resin extracts

Antifungal activity of the siderophore extracts obtained by Amberlite XAD-4 resin method against the three phytopathogenic fungi showed significant differences compared to the control (Figure 4). Firstly, the disk diffusion technique of the four strains exerted a high antifungal activity against isolates *Sclerotinia sclerotiorum*, *Stemphylium botryosum* and *Fusarium oxysporum f.* sp. *phaseoli* representing inhibition percentages ranging from 66.00±0 to 90.75±0.96; 90.00±1.63 to 93.00±0.82 and 71.25±0.5 to 88.00±1.41, respectively. Secondly, well diffusion technique revealed similarly high significant inhibition percentages of 72.0±0.82 to 86.75±1.75; 87.25±0.96 to 94.0±0 and 72.25±0.5 to 84.5±1.29, in the same order.

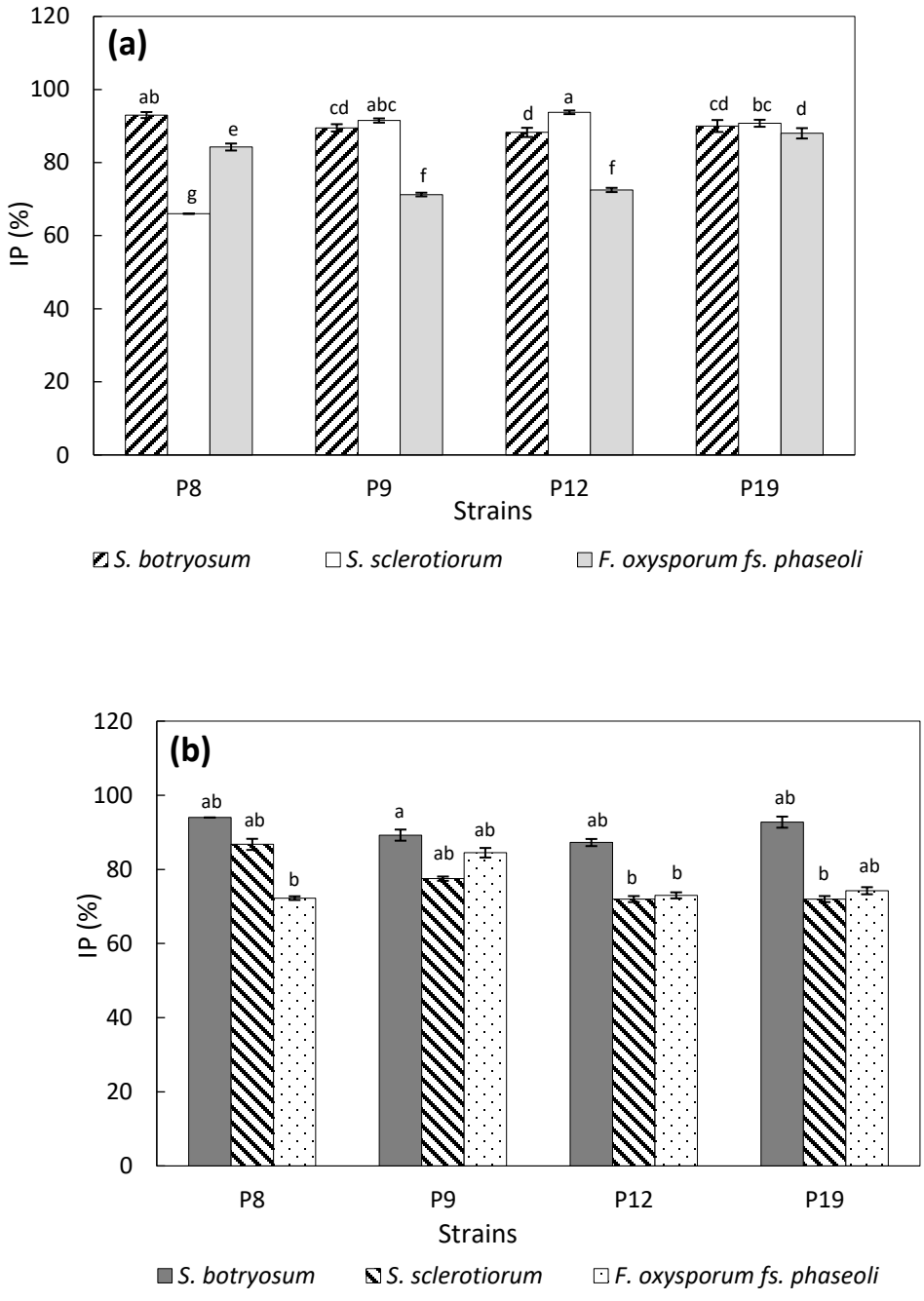

Fig. 3: Inhibition percentages of siderophore extracts by Amberlite XAD4 resin method against phytopathogenic fungi (a: disk diffusion technique; b: well diffusion technique). Each bar represents the mean percentage inhibition of each extract evaluated ± standard deviation. ANOVA/Tukey tests ($p<0.05$) were carried out as appropriate. Same letters indicate no significant statistical difference among means of the same treatment group.

3.3.2. Antifungal activity of liquid-liquid extracts

After incubation, all liquid/liquid extracts showed significant difference against the three phytopathogenic fungi compared to the control (Figure 5). For the disk diffusion technique, Amberlite XAD4 resin extracts of the four *Pseudomonas* strains exhibited moderate to low antifungal activity against *Fusarium oxysporum f.* sp. *phaseoli*, *Stemphylium botryosum and Sclerotinia sclerotiorum*, representing inhibition percentages varying from 10.25±0.96 to 25.75±0.50; 6.00±0.82 to 10.00±0.82 and 5.25±0.5 to 30.00±0, respectively. For the well diffusion technique, the highest antifungal activity unregistered ranged from 11.25±1.5 to 25.0±0.82 against *Sclerotinia sclerotiorum*.

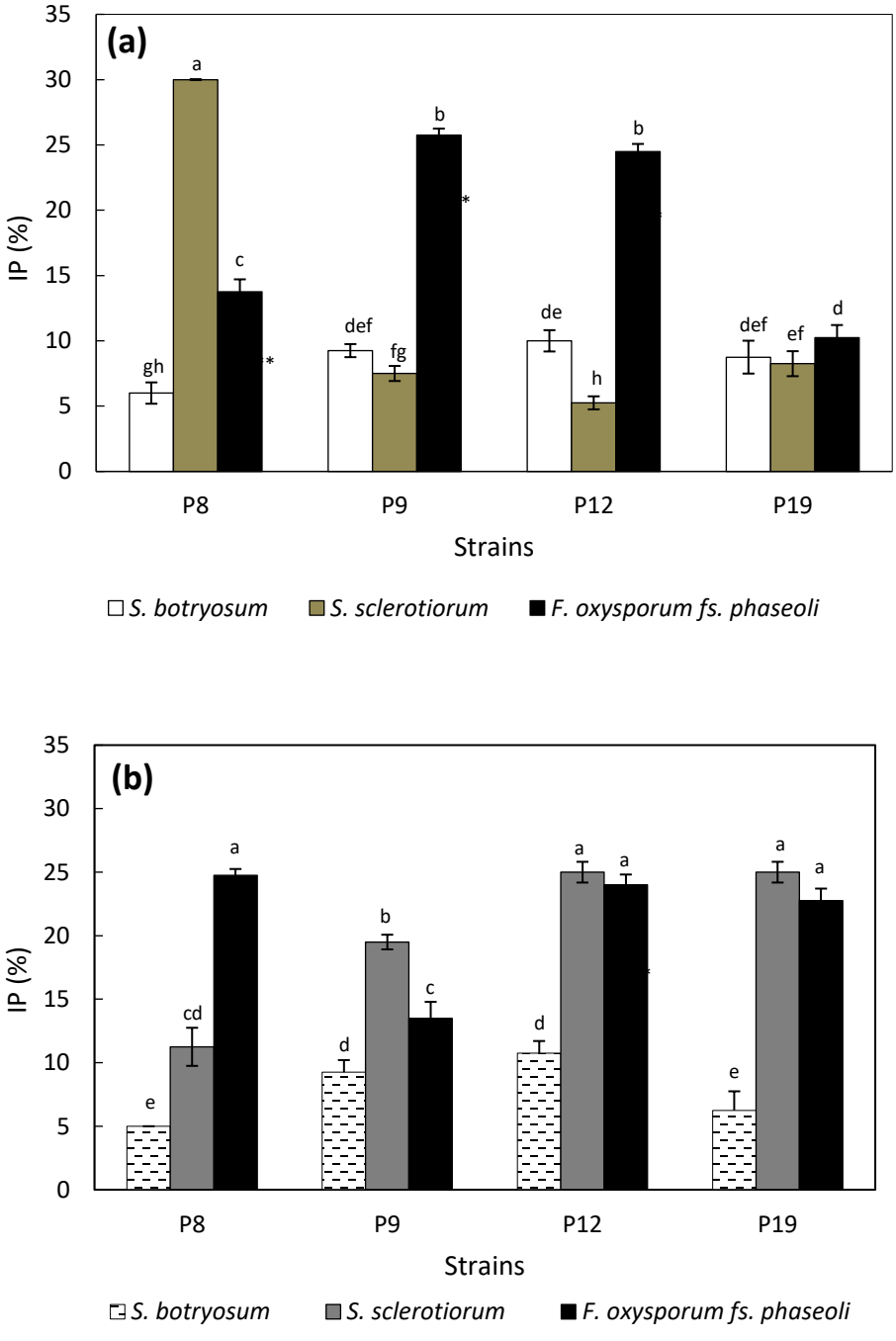

Fig. 4: Inhibition percentages of siderophore extracts by liquid-liquid method against phytopathogenic fungi (a: disk diffusion technique; b: well diffusion technique). Each bar represents the mean percentage inhibition of each extract evaluated ± standard deviation. ANOVA/Tukey tests ($p<0.05$) were carried out as appropriate. Same letters indicate no significant statistical difference among means of the same treatment group.

3.3.3. Antifungal activity interactions

Statistical analysis using three-way ANOVA analysis confirmed that the antifungal activity for the most treatments were significant (Table 3). Performed analysis showed that variation of the antifungal activity was in relation to fungi, siderophore extraction method, and antifungal activity technique. Consequently, most their interactions were significant at $P \leq 0.05$.

Tab. 3. Three-way ANOVA analysis of different siderophore extraction methods, against fungi isolates using various antagonistic techniques.

Source strain	DF	F value				R^2
		P8	P9	P12	P19	
F	2	6.367	1.591	2.305	1.185	95.5%
SEM	1	0.4898	0.6522	2.558	2.16	
AAT	1	80537	73190	75771	43657	
F*SEM	2	3.429	2.843	1.8	0.945	
F*AAT	2	1853	604.7	1311	353.4	
SEM*AAT	1	167.6	0.2348	901.9	766.1	
F*SEM*AAT	2	1454	830.3	857.4	328.6	
Source strain	DF	P value				
		P8	P9	P12	P19	
F	2	0.0043	0.2177	0.1143	0.3174	
SEM	1	0.4885	0.4246	0.1185	0.1503	
AAT	1	P<0.0001	0.0001	P<0.0001	P<0.0001	
F*SEM	2	P=0.0434	0.0714	P=0.1799	P=0.3981	
F*AAT	2	P<0.0001	0.0001	P<0.0001	P<0.0001	
SEM*AAT	1	P<0.0001	0.6309	P<0.0001	P<0.0001	
F*SEM*AAT	2	P<0.0001	P<0.0001	P<0.0001	P<0.0001	

F: Fungi; SEM: Siderophore extraction methods; AAT: Antifungal activity techniques; DF: Degree of freedom.

Furthermore, graphical representation of three-way ANOVA test mentioned clearly that antifungal activity of Amberlite XAD4 resin extracts were more effective than liquid-liquid extracts (Figure 6). Whereas, comparison between disk and well diffusion techniques, revealed no significant differences.

4. DISCUSSION

Pseudomonas species producing sidorophores are good candidates for the inhibition of several phytopathogenic microorganisms. Particularly, their siderophore-extracts can act more effectively in suppressing of phytopathogenic fungi.

Fungal microbial diseases are the major causes of severe global economic losses to agricultural crops (Sayyed et al., 2013). Particularly, plant diseases are the source of 80-100% yield loss of common bean. From which, fungi cause the most damage among all the transmittable seed-borne diseases (Kator et al., 2016). On the other hand, *Pseudomonas* bacteria are ubiquitous in agricultural soils and have various metabolic characteristics activating their effectiveness as biocontrol agents of plant pathogens(Weller, 2007). Especially, several works had reported that *Peudomonas* spp. are efficient siderophore producer microorganisms (Raaska et al., 1993;Bultreys et al., 2001;Tank et al., 2012;De Villegas et al., 2002; Christina Jenifer et al., 2015). These molecules are very important for the growth and survival of bacteria in soil (Rajkumar et al., 2010). In the rhizosphere, the concentration of siderophores is estimated to range from a few nano molar moles (Le Houedec et al., 2018) to a few micro molar moles (Essén et al., 2006). They serve as signal molecules controlling expression in the production of the siderophore itself (Lamont et al., 2002), siderophores are also important to the virulence of many pathogens (Ratledge and Dover, 2000; Holden and Bachman, 2015).

Current findings revealed that pure siderophores of *Pseudomonas* species obtained using liquid-liquid and Amberlite XAD4 resin extraction methods are endowed with a high antifungal inhibition effect against bean phytopathogenic fungi. For instance, it has been reported that siderophores exhibited prominent antagonistic activity against *Fusarium oxysporum* with a maximum inhibition rate of 95.24%. Furthermore, in the absence of FeCl3 siderophores can inhibit 11 different phytopathogenic fungi. Electron microscopy observations revealed that siderophore solution inhibit fungi spore germination, and affect also spores and mycelia structures(Yu et al., 2017). On the other hand, there are several works showing the potential effect of siderophore-extracts as antifungal compounds. For example, Maindad et al. (2014) reported the antifungal properties of the partially and completely

purified siderophore from *Acinetobacter calcoaceticus* HIRFA32 by *in vitro* trials. Indicating their probable effects in suppressing of phytopathogenic fungus *Fusarium oxysporum*. Additionally, siderophore crude extracts of fluorescent *Pseudomonas* spp isolated from paddy rhizosphere revealed antifungal activity against various phytopathogenic fungi (Sivasankari et al., 2008). Also, purified siderophores and *Pseudomonas* cultures had revealed good antifungal activity against plant deleterious fungi, namely, *Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Fusarium oxysporum,* and *Sclerotium rolfsii* (Manwar et al., 2004). Especially, siderophore-extracts from fluorescent and non-florescent *Pseudomonas* exerted inhibition percentage ranging from 38.1% to 83.5% against *Sclerotinia* sp, *Fusarium* sp and *Alernaria* sp., (Angel et al., 2013). Furthermore, Sulochana et al. (2014) reported that partial siderophore-extracts of *Pseudomonas aeruginosa JAS-25* purified by gel filtration chromatography using Sephadex G25 tested by well plate assay and microphotography method were active against spore germination of *Aspergillus niger, Fusarium udum* and *Fusarium oxysporum f.* sp. *ciceri*.

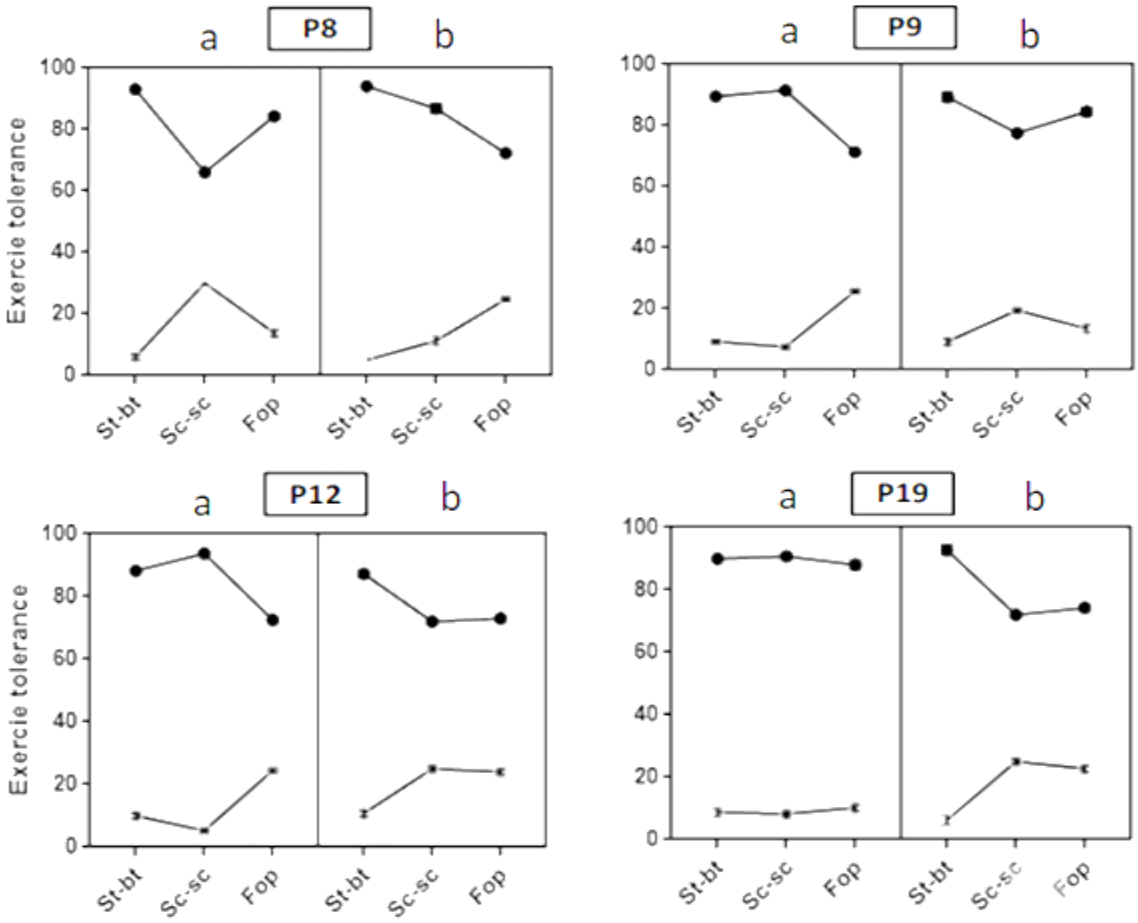

Fig. 5: Graphical representation of three-way ANOVA analysis of different siderophore extracts, against fungi isolates using various antagonistic techniques (a: disk diffusion technique; b: well diffusion technique; ⊗: liquid-liquid extracts; •: Amberlite XAD4 resin extracts; St-bt: *Stemphylium botryosum*; Sc-sc: *Sclerotinia sclerotiorum*; Fop: *Fusarium oxysporum* f. sp. *phaseoli*; P: *Pseudomonas*).

Pseudomonas species exert the antifungal activity through the production of extracellular lytic enzymes, siderophores, salicylic acid, antibiotics, and volatile metabolites, such as hydrogen cyanide (Walker et al., 2001; Manwar et al., 2004). Whereas, as the antifungal activity was realized from purified siderophore extracts; the observed inhibitory impacts can be explained solely due to the presence of these secondary metabolites. The indirect mode of action of siderophore-extracts in suppression of disease may be based on competition for iron with the pathogen (Whipps, 2001).Notably, the importance of siderophores is closely related to iron, which is an essential element for different biological processes (Crosa and Walsh, 2002; Ahmed and Holmström, 2014). Decreasing of siderophore levels affecting phytopathogenic microorganism's development is the inhibition result of vital cell processes like sporulation and nucleic acid production (Mathiyazhagan et al., 2004). In addition, this indirect mechanism by which siderophores

inhibit phytopathogenic microorganism is sequestration of iron making it unavailable for pathogenic fungi and thus inhibiting their growth (Scher at al., 19982;Rokhbakhsh-Zamin et al., 2011).

Siderophore extraction methods can vary and affect largely the effectiveness of the antifungal activity test. This can be explained by the difference between the degree and the purity of siderophore extracts. Which was higher in the case of extraction by Amberlite XAD4 resin method. Zhang et al. (2018) reported that Amberlite XAD resins are efficient extraction techniques of organic compounds from aqueous media. For example, the extraction of the primary siderophore "Petrobactin" synthesized by *Bacillus anthracis Str.* requests iron starvation (Koppisch et al., 2005).

None significant results observed between well and disc diffusion techniques, this could be related to the utilization of equal siderophore-extracts volume. Confirmatory screening test involves extraction of the active siderophore constituents from the supernatant. Where, disc and well diffusion methods are more suitable in this category test (Odeyemi et al., 2012). The most known and basic antibiosis study is the disk-diffusion technique. Other methods are used especially for antifungal testing (Balouiri et al., 2016).

5. CONCLUSION

The current study focused on the determination of siderophore-extracts antifungal activity of *Pseudomonas* species. This revealed that *Pseudomonas* strains (P8, P9, P12 and P19) showed high inhibitory effects on mycelium growth of three phytopathogenic fungi of *Phaseolus vulgaris* L: *Stemphylium botryosum, Sclerotinia sclerotiorum* and *Fusarium oxysporum f.* sp. *phaseoli*. Two simple techniques applied, liquid/liquid and Amberlite XAD-4 resin, were effective to extract pure siderophore compounds. However, extracts by XAD4 Amberlite resin was significantly more active in inhibiting growth of the three fungi. Also, the antifungal activity technique, well and disc diffusion, do not affect the inhibitory impacts observed. Characterized methods in this study could be used as simple and rapid techniques for detection and determination of siderophore-extracts antifungal properties.

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All data associated with this study are present in the paper.

REFERENCE

1. Ahmed, E., Holmström, S.J., 2014. Siderophores in environmental research: roles and applications. Microbial biotechnology. 7, 196-208.
2. Albelda-Berenguer, M., Monachon, M., Joseph, E., 2019. Siderophores: From natural roles to potential applications. Advances in applied microbiology. 106, 193-225.
3. Alexander, D.B., Zuberer, D.A., 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of soils. 12, 39-45.
4. Alsohaili, S.A., Bani-Hasan, B.M. 2018. Morphological and Molecular Identification of Fungi Isolated from Different Environmental Sources in the Northern Eastern Desert of Jordan. Jordan Journal of Biological Sciences. 11, 329-337.
5. Angel Jenifer, M.R., Reena, A., Aysha, O.S., Valli, S., Nirmala, P., Vinothkumar, P., 2013. Isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens. Int. J. Curr. Microbiol. App. Sci. 2, 59-65.
6. Balouiri, M., Sadiki, M., Ibnsouda, S.K. 2016. Methods for in vitro evaluating antimicrobial activity: A review. Journal of

pharmaceutical analysis. 6, 71-79. https://doi.org/10.1016/j.jpha.2015.11.005
7. Bertrand, S., 2009. Les sidérophores de *Scedosporium apiospermum*: identification, synthèse et applications. Doctoral dissertation, université d'Angers.
8. Bultreys, A., Gheysen, I., Maraite, H., de Hoffmann, E., 2001. Characterization of Fluorescent and Non fluorescent Peptide Siderophores Produced by *Pseudomonas syringae* Strains and Their Potential Use in Strain Identification. Applied and Environmental Microbiology. 67, 1718-1727. https://doi.org/10.1128/aem.67.4.1718-1727.2001
9. Cheng, C.M., Doyle, M.P., Luchansky, J.B., 1995. Identification of *Pseudomonas fluorescens* strains isolated from raw pork and chicken that produce siderophores antagonistic towards foodborne pathogens. Journal of food protection. 58, 1340-1344. https://doi.org/10.4315/0362-028x-58.12.1340
10. Christina Jenifer, A., Aruna, Sharmili, S., Anbumalarmathi, J., Umamaheswari, K., Shyamala, K., 2015. Studies on siderophore production by microbial isolates obtained from aquatic environment. European Journal of Experimental Biology. 5, 41-45.
11. Cornelis, P., Matthijs, S., 2007. *Pseudomonas*Siderophores and their Biological Significance. Microbial Siderophore. In: Varma, A., Chincholkar, S.B. (Eds.), Microbial Siderophores. Soil Biology. Springer, Berlin, Heidelberg, pp.193-203. https://doi.org/10.1007/978-3-540-71160-5_9
12. Crosa, J.H., Walsh, C.T., 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev. 66, 223-249. https://doi.org/10.1128/mmbr.66.2.223-249.2002
13. De Villegas, M.E.D., Villa, P., Frías, A., 2002. Evaluation of the siderophores production by *Pseudomonas aeruginosa*PSS. Revista latinoamericana de microbiología. 44, 112-117.
14. Essén, S.A., Bylund, D., Holmström, S.J., Moberg, M., Lundström, U.S., 2006. Quantification of hydroxamate siderophores in soil solutions of podzolic soil profiles in Sweden. Biometals. 19, 269-282. https://doi.org/10.1007/s10534-005-8418-8
15. Ganeshan, G., Manoj Kumar, A., 2005. *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. Journal of Plant Interactions. 1, 123-134. https://doi.org/10.1080/17429140600907043
16. Gamalero, E., Glick, B.R., 2011. Mechanisms used by plant growth-promoting bacteria. In: Maheshwari, D.K. (Ed.), Bacteria in Agrobiology: Plant Nutrient Management. Berlin, Heidelberg, Germany: Springer Verlag, pp. 17-46. https://doi.org/10.1007/978-3-642-21061-7_2
17. Goswami, D., Thakker, J.N., Dhandhukia, P.C., 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food and Agriculture. 2, 1-19. https://doi.org/10.1080/23311932.2015.1127500
18. Goszczynska, T., Serfontein, J.J., Serfontein, S., 2000. Introducion to practical phytobacteriology: A Manual for Phytobacteriology. Plant Protection Research Institute, Pretoria, pp. 61-71
19. Hissen, A.H., Wan, A.N., Warwas, M.L., Pinto, L.J., Moore, M.M., 2005. The *Aspergillus fumigates* siderophore biosynthetic gene sidA, encoding L-ornithine N5-oxygenase, is required for virulence. Infection and immunity. 73, 5493-5503. https://doi.org/10.1128/iai.73.9.5493-5503.2005
20. Holden, V.I., Bachman, M.A., 2015. Diverging roles of bacterial siderophores during infection. Metallomics. 7, 986-995. https://doi.org/10.1039/c4mt00333k
21. Kator, L., Ogo-Oluwa, A.T., Kemi, A.B., 2016. Isolation and Identification of Seed Borne Fungi of Common Bean (Phaseolus vulgaris L.) from Selected Markets in Makurdi International. J Appl Agri Sci. 2, 75-78. https://doi.org/10.11648/j.ijaas.20160205.11
22. Kirienko, D.R., Kang, D., Kirienko, N.V., 2018. Novel Pyoverdine Inhibitors Mitigate *Pseudomonas aeruginosa* Pathogenesis. Frontiers in microbiology. 9, 3317. https://doi.org/10.3389/fmicb.2018.03317
23. Koppisch, A.T., Browder, C.C., Moe, A.L., Shelley, J.T., Kinkel, B.A., Hersman, L.E., Lyer, S., Ruggiero, C.E., 2005. Petrobactin is the primary siderophore synthesized by *Bacillus anthracis* str. Sterne under conditions of iron starvation. Biometals. 18, 577-585. https://doi.org/10.1007/s10534-005-1782-6
24. Krid, S., Gharsallaoui, M., Triki, M. A., Rhouma, A., 2011. Epidemiologie et etiologie de *Pseudomonas savastanoi* pv. *savastanoi*, agent causal de la tuberculose de l'olivier en Tunisie. Revue ezzaitouna. 12, 1-10.
25. Lamont, I.A., Beare, P.A., Ochsner, U., Vasil, A.I., Vasil, M.L., 2002. Siderophore-mediated signaling mediates virulence factor production in *Pseudomonas aeruginosa.*Proc Natl Acad Sci. 99, 7072-7077. https://doi.org/10.1073/pnas.092016999
26. Le Houedec, S., Thibault de Chanvalon, A., Mouret, A., Metzger, E., Launeau, P., Gaudin, P., Lebeau, T., 2018. 2D Image Quantification of Microbial Iron Chelators (Siderophores) Using Diffusive Equilibrium in Thin Films Method. Analytical chemistry. 91, 1399-1407. https://doi.org/10.1021/acs.analchem.8b04021
27. Ligon, J.M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann, D., Kempf, H.J., Pée, K. H.V., 2000. Natural products with antifungal activity from Pseudomonas biocontrol bacteria. Pest Management Science: formerly Pesticide Science. 56, 688-695. https://doi.org/10.1002/1526-4998(200008)56:8<688::aid-ps186>3.0.co;2-v
28. Liu, D., Yan, R., Fu, Y., Wang, X., Zhang, J., Xiang, W, 2019. Antifungal, plant growth-promoting and genomic properties of an endophytic actinobacterium Streptomyces sp. NEAU-

S7GS2. Frontiers in microbiology. 10, 1-16. https://doi.org/10.3389/fmicb.2019.02077

29. Louden, B.C., Haarmann, D., Lynne, A.M., 2011. Use of blue agar CAS assay for siderophore detection. Journal of microbiology and biology education: JMBE. 12, 51-53. https://doi.org/10.1128/jmbe.v12i1.249
30. Maindad, DV. Kasture, V.M., Chaudhari, H., Dhavale, D.D., Chopade, B.A., Sachdev, D.P., 2014. Characterization and Fungal Inhibition Activity of Siderophore from Wheat Rhizosphere Associated *Acinetobacter calcoaceticus* Strain HIRFA32. Indian J Microbiol. 54, 315–322. https://doi.org/10.1007/s12088-014-0446-z
31. Manwar, A.V., Khandelwal, S.R., Chaudhari, B.L., Meyer, J.M., Chincholkar, S.B., 2004. Siderophore production by a marine *Pseudomonas aeruginosa* and its antagonistic action against phytopathogenic fungi. Applied biochemistry and biotechnology. 118(1-3), 243-252. https://doi.org/10.1385/abab:118:1-3:243
32. Mathiyazhagan, S., Kavitha, K., Nakkeeran, S., Chandrasekar, G., Manian, K., Renukadevi, P., Krishnamoorthy, A.S., Fernando, W.G.D., 2004. PGPR mediated management of stem blight of *Phyllanthus amarus* (Schum and Thonn) caused by *Corynespora cassiicola* (Berk and Curt) Wei. Archives of phytopathology and plant protection. 37, 183-199. https://doi.org/10.1080/03235400410001730658
33. Mishra, J., Arora, N.K., 2018. Secondary metabolites of fluorescent pseudomonads in biocontrol of phytopathogens for sustainable agriculture. Applied soil ecology. 125, 35-45. https://doi.org/10.1016/j.apsoil.2017.12.004
34. Mohammadi, S., Aminifard, M.H., 2013. Inhibition of grey mould *in vitro* and *in vivo* with essential oil of fennel (*Foeniculum vulgare* L.). Journal of Central European Agriculture. 14, 420-431. https://doi.org/10.5513/jcea01/14.1.1217
35. Mokrani, S., Belabid, L., Nabti, E., 2018b. Antifungal Activities of *Pseudomonas* Strains Against Indigenous Phytopathogenic Fungi of *Phaseolus Vulgaris* L. Isolated from a Greenhouse in Western Algeria. Res J Plant Pathol. 2, 1-7.
36. Mokrani, S., 2019. Recherche de bactéries PGPR/B isolées de l'ouest algérien et leurs effets de biofertilisation et de lutte biologique à l'égard de quelques microorganismes phytopathogènes de *Phaseolus vulgaris* L. Thèse de doctorat, université de Béjaia.
37. Mokrani, S., Bejaoui, B., Belabid, L., Nabti, E. 2019b. Potential of *Pseudomonas stutzeri* strains isolated from rhizospheric soil endowed with antifungal activities against phytopathogenic fungus *Stemphylium botryosum*. Asian Journal of Agriculture. 3, 47-54.
38. Nagarajkumar, M., Bhaskaran, R., Velazhahan, R., 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiological Research. 159, 73-81. https://doi.org/10.1016/j.micres.2004.01.005
39. Neilands, J.B., 1995. Siderophores: structure and function of microbial iron transport compounds. Journal of Biological Chemistry. 270, 26723-26726. https://doi.org/10.1074/jbc.270.45.26723
40. Ortíz-Estrada, G., Luna-Castro, S., Piña-Vázquez, C., Samaniego-Barrón, L., León-Sicairos, N., Serrano-Luna, J., De La Garza, M., 2012. Iron-saturated lactoferrin and pathogenic protozoa: could this protein be an iron source for their parasitic style of life?. Future microbiology. 7, 149-164. https://doi.org/10.2217/fmb.11.140
41. O'sullivan, D.J., O'Gara, F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiology and Molecular Biology Reviews. 56, 662-676. https://doi.org/10.1128/mmbr.56.4.662-676.1992
42. Odeyemi, O.A., Ahmad, A., Usup, G., 2012. In-vitro antimicrobial activity of *Aeromonas* spp isolated from estuary using different screening protocols. International Journal of Pharmaceutical Sciences and Research. 3, 428-433.
43. Pahari, A., Mishra, B.B., 2017. Characterization of siderophore producing Rhizobacteria and its effect on growth performance of different vegetables. Int. J. Curr. Microbiol. App. Sci. 6, 1398-1405. https://doi.org/10.20546/ijcmas.2017.605.152
44. Qessaoui, R., Bouharroud, R., Furze, J.N., El Aalaoui, M., Akroud, H., Amarraque, A., Van Vaerenbergh, J., Tahzima, R., Mayad, E.H., Chebli, B., 2019. Applications of New Rhizobacteria *Pseudomonas* isolates in Agroecology via Fundamental Processes Complementing Plant Growth. Scientific reports. 9, 1-10. https://doi.org/10.1038/s41598-019-49216-8
45. Rajkumar, M., Ae, N., Prasad, M.N.V., Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends in biotechnology. 28, 142-149. https://doi.org/10.1016/j.tibtech.2009.12.002
46. Rajkumarn, M., Sandhya, S., Prasad, M.N.V., Freitas, H., 2012. Perspectives of plant-associated microbes in heavy metal phytoremediation. Biotechnology advances. 30, 1562-1574. https://doi.org/10.1016/j.biotechadv.2012.04.011
47. Raaska, L., Viikari, L., Mattila-Sandholm, T., 1993. Detection of siderophores in growing cultures of *Pseudomonas* spp. Journal of Industrial Microbiology. 11, 181-186. https://doi.org/10.1007/bf01583720
48. Ratledge, C., Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. A. Rev. Microbiol. 54, 881-941.

49. Rachid, D., Ahmed, B., 2005. Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*. African Journal of Biotechnology. 4, 697-702.
50. Renshaw, J.C., Robson, G.D., Trinci, A.P.J., Wiebe, M.G., Livens, F.R., Collison, D., Taylor, R.J., 2002. Fungal siderophores: structures, functions and applications. Mycol Res. 106, 1123-1142.
51. Rhodes, M.E., 1959. The characterization of Pseudomonas fluorescens.Journal of General Microbiology. 21, 221-263.
52. Rijavec, T., Lapanje, A., 2016. Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. Frontiers in microbiology. 7, 1-14. https://doi.org/10.3389/fmicb.2016.01785
53. Rokhbakhsh-Zamin, F., Sachdev, D., Kazemi-Pour, N., Engineer, A., Pardesi, K.R., Zinjarde, S.S., Dhakephalkar, P.K., Chopade, B.A. 2011. Characterization of plant growth promoting traits of *Acinetobacter* species isolated from rhizosphere of *Pennisetum glaucum*. J Microbiol Biotech. 21, 556-566.
54. Sayyed, R.Z., Patel, P.R., 2011. Biocontrol potential of siderophore producing heavy metal resistant *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 vis-a-vis organophosphorus fungicide. Indian journal of microbiology. 51, 266-272. https://doi.org/10.1007/s12088-011-0170-x
55. Sayyed, R.Z., Chincholkar, S.B., Reddy, M.S., Gangurde, N.S., Patel, P.R., 2013. Siderophore Producing PGPR for Crop Nutrition and Phytopathogen Suppression. In: Maheshwari, D.(Ed.), Bacteria in Agrobiology: Disease Management. Disease Management. Springer, Berlin, Heidelberg, pp. 449-471. https://doi.org/10.1007/978-3-642-33639-3_17
56. Scher F.M., Baker R., 1982. Effect of Pseudomonas putida and asynthetic iron chelator on induction of soil suppressiveness to fusarium wilt pathogens, Phytopathology, 72(12), 1567–1573.
57. Scher F.M., Baker R., 1982. Effect of *Pseudomonas putida* and asynthetic iron chelator on induction of soil suppressiveness tofusarium wilt pathogens. Phytopathology. 72, 1567-1573. https://doi.org/10.1094/phyto-72-1567
58. Shanmugaiah, V., Nithya, K., Harikrishnan, H., Jayaprakashvel, M., Balasubramanian, N., 2015. Biocontrol mechanisms of siderophores against bacterial plant pathogens. In: Bastas, K.K. (Ed.), Sustainable Approaches to Controlling Plant Pathogenic Bacteria. Boca Raton, FL: CRC Press, pp. 167-190.
59. Sivasankari, B., Hemala Devi, R., Daniel, T., 2008. Evaluation of Antifungal Activity of Siderophore Produced by Fluorescent *Pseudomonas* sp. Isolated from Paddy Rhizosphere. Journal of Pure and Applied Microbiology. 2, 595-598.
60. Srivastava, R., Shalini, R., 2008. Antifungal activity of *Pseudomonas fluorescens* against different plant pathogenic fungi. EJEAFChe. 7, 2789-2796.
61. Stubbendieck, R.M., Straight, P.D., 2016. Multifaceted interfaces of bacterial competition. Journal of bacteriology. 198, 2145-2155. https://doi.org/10.1128/jb.00275-16
62. Su, Y.Y., Qi, Y.L., Cai, L., 2012. Induction of sporulation in plant pathogenic fungi. Mycology. 3, 195-200.
63. Sulochana, M.B., Jayachandra, S.Y., Kumar, S.K.A., Dayanand, A., 2014. Antifungal attributes of siderophore produced by the *Pseudomonas aeruginosa* JAS-25. Journal of basic microbiology. 54, 418-424.
64. Tank, N., Rajendran, R., Patel, B., Saraf1, M., 2012. Evaluation and biochemical characterization of a distinctive pyoverdin from a Pseudomonas isolated from chickpea rhizosphere. Brazilian Journal of Microbiology. 43, 639-648.
65. Wafaa, M.H., Mostafa, A.E.S., 2012. Production and optimization of *Pseudomonas fluorescens* biomass and metabolites for biocontrol of strawberry grey mould. American Journal of Plant Sciences. 3, 836-845.
66. Walker, R., Innes, C.M., Allan, E.J., 2001. The potential biocontrol agent *Pseudomonas antimicrobica*inhibits germination of conidia and outgrowth of *Botrytis cinerea*. Lett. Appl. Microbiol. 32, 346–348.
67. Weller, D.M., 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology. 97, 250-256.
68. Whipps, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. J Exp Bot. 52, 487–511. https://doi: 10.1093/jexbot/52.suppl_1.487.
69. Yasmin, S., Hafeez, F.Y., Mirza, M.S., Rasul, M., Arshad, H.M., Zubair, M., Iqbal, M., 2017. Biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. Frontiers in microbiology. 8, 1-23. https://doi.org/10.3389/fmicb.2017.01895
70. Yu, S., Teng, C., Liang, J., Song, T., Dong, L., Bai, X., Jin, Y., Qu, J., 2017. Characterization of siderophore produced by *Pseudomonas syringae* BAF.1 and its inhibitory effects on spore germination and mycelium morphology of *Fusarium oxysporum*. J Microbiol. 55, 877–884.
71. Yeole, R.D., Dube, H.C., 2000. Siderophore-mediated antibiosis of rhizobacterial fluorescent pseudomonads against certain soil-borne fungal plant pathogens. Journal of Mycology and Plant Pathology. 30, 335-338.
72. Zhang, Q.W., Lin, L.G., Ye, W.C., 2018. Techniques for extraction and isolation of natural products: a comprehensive review. Chinese medicine. 13, 1-26.