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Efficacy of Secondary Metabolites Produced by *Bacillus Amyloliquefaciens* on the Inhibition of *Zymoseptoria Tritici*

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Abstract- The strain I3 of *Bacillus amyloliquefaciens*, which was isolated from soft wheat leaves, has revealed, *in vitro* and *in vivo,* a high antagonistic potential against septoria leaf blotch of wheat. In order to investigate the existence of antifungal molecules secreted by strain I3, the filtrates of this strain were tested for their inhibitory activity on the germination of pycnidiospores of the two strains of *Zymoseptoria tritici,* G1-1 and A5-1 isolated from soft wheat and durum wheat, respectively. The antibiosis assays showed a high level of inhibitory activity, with inhibition rates ranging from 94% to 99% compared to the control after 96 hours of incubation. Filtrate analysis by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS), have identified several families of lipopeptides reported as antifungal molecules (iturins, fengycins, and surfactins); and polyketides (macrolactins, chlorotetaines, and bacillaenes) which would also be responsible for the antagonistic activity against *Z. tritici*. A solid-liquid extraction method of these secondary metabolites from the confrontation zones between Bacillus and pathogenic strains, identified the same families of lipopeptides and polyketides with a higher relative abundance compared to the filtrates of the liquid-liquid extraction process.

Keywords: nephelometry, antibiosis, HPLC-MS, secondary metabolites, bacillus amyloliquefaciens I3, zymoseptoria tritici.

I. Introduction

Plant protection by Bacillus strains against pathogenic organisms are based on several modes of action, including the antibiosis mechanism, which is dependent on the production of different secondary metabolites that have a toxic effect against the pathogenic organisms. This mechanism is the most widely known and may be the most important mechanism used by Bacillus as plant growth promoting rhizobacteria to reduce the pathogen's infestation in the host plant's tissues [1]. The mechanism of antibiosis is direct inhibition of pathogen growth through the production of metabolites with antimicrobial properties [2,3]. Some Bacillus species, such as *Bacillus amyloliquefaciens*, can use up to 8.5% of their genetic material to synthesize a wide range of antimicrobial compounds, including lytic enzymes, antibiotics, polyketides, and a range of lipopeptides synthesized by non-ribosomal mechanisms [4,5]. The cyclic lipopeptides (surfactins, iturins and fengycins), are particularly interesting by the fact of being secreted at bio-effective levels in the natural conditions of the rhizosphere [6,7,8]. Also, bacillaenes, macrolactines, and chlorotetaines are polyketides with a high range of antibacterial and antifungal activities [4,9]. This antibiotic arsenal and the high ability to colonize roots probably explain the high biocontrol potential of the Bacillus genus *in vitro* and under natural conditions, [10,11,12,13].

In this study, we evaluated by nephelometry the effect of three filtrates - prepared with *B. amyloliquefaciens* I3 cultured alone or in the presence of the strain A5-1 of *Z. tritici* isolated from soft wheat and the strain G1-1 of *Z. tritici* isolated from durum wheat) - on the inhibition of pycnidiospores germination of septoria leaf blotch pathogen. Furthermore, the inhibiting/stimulating effects of the G1-1 and A5-1 strains on the secondary metabolite production in liquid and agar media was evaluated. HPLC-MS analyses of the secondary metabolites produced in *B. amyloliquefaciens* I3 filtrates identified cyclic lipopeptides and polyketides that could document the antagonistic activity.

II. Materials and Methods

a) Culture of Bacillus amyloliquefaciens I3

The bacterium was isolated from soft wheat leaves in a field located in the northern part of Morocco and exhibited high antagonistic activity against *Z. tritici* [14,15,16]. The identity of the bacterium was confirmed in the Laboratory of Exact and Natural Sciences at the University of Reims-France.

b) Antifungal activity of Bacillus amyloliquefaciens I3 filtrates

❖ *Preparation of filtrates and pathogen suspensions*

B. amyloliquefaciens I3 filtrates were obtained from the cultures growing in PDB media

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(Potato Dextrose Broth). Sixteen pre-cultivated pastilles of *B. amyloliquefaciens* I3 in PDA media (Potato Dextrose Agar) were placed in 200 ml of PDB for 48 hours. Cultures of *B. amyloliquefaciens* were also prepared in confrontation with G1-1 and A5-1 strains of *Z. tritici*. The cultures were incubated in the dark under agitation at 25°C for five days and then sterilized by filtration (0.45 μm (Minisart filters/Sigma-Aldrich)).

The suspensions of *Z. tritici* strains (G1-1 and A5-1) were obtained after seven days of incubation in the dark at 18°C on PDA media. The suspension concentration was adjusted to 10^6 pycnidiospores/ml.

❖ *Antibiotic activity quantification*

The three filtrates (F1 prepared with I3, F2 prepared with I3 and G1-1, and F3 prepared with I3 and A5-1) and the control F4 (the filtrate was substituted by sterile distilled water) were tested in three dilutions (d1=1/10, d2=1/2, and d3=9/10) on both *Z. tritici* strains G1-1 and A5-1. Depending on the dilution, the filtrate was mixed with 100 μl aqueous suspension of *Z. tritici* supplemented with PDB media for a final volume of 1 ml. The final suspension was deposited a 96-well plate with 300 μl per well. For each modality, three biological repetitions with three technical repetitions were performed. The control F4 was prepared with sterile MilliQ water.

The antibiosis effect was characterized by nephelometry (Chronos-NEPHELO star plus, BMG LABTECH) at 25°C. The number of analysis cycles was 16, with 6 hours between two successive cycles and 80% of laser intensity. Before each measurement, the plate was shaken for 300 seconds at 150 rpm.

❖ *Statistical analysis*

SPSS 21 statistic software was applied for turbidity data analysis. Statistical analyses were established at three factors: the first corresponded to the filtrates (F1, F2, F3, and the control F4), the second factor indicated the dilutions (d1, d2, and d3) and the third factor denoted the *Z. tritici* strains (A5-1 and G1-1). ANOVA was applied for the analysis of variation of means, while the Duncan test was used for the comparison of means at $p = 0.05$.

c) *Production and identification of secondary metabolites*

❖ *Culture preparation*

In petri plates containing the 24-hour-old *Z. tritici* culture, 10 μl of the pre-cultivated *B. amyloliquefaciens* culture on PDA media for 48 hours was added to the centre of the plates. These latter were incubated at 25°C in the dark for four days.

The liquid cultures were prepared according to the same protocol described in the previous section.

❖ *Secondary metabolites extraction*

The secondary metabolites were extracted from the agar media by mixing three agar fragments randomly collected from the inhibition areas or the I3 culture with 2 ml of methanol in assay tubes. The mixture was homogenized using vortex and incubated at 4°C for 4 hours, then centrifuged for 10 min at 4000 rpm. The supernatant was recovered and purified by filtration (Millipore of 0.45 μm).

The liquid-liquid extraction was carried out by precipitation of the secondary metabolites at pH=2 (adjusted with hydrochloric acid (HCl)) and then centrifugation (20 min at 8000 rpm) followed by two successive rinses of the precipitate (ultra-pure water at pH=2), methanol extraction, and filtration (Millipore of 0.2 μm). The extract was dried by rotavapor and re-suspended in 1 ml of 0.01 M PBS.

Regarding the PBS buffer, 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (Kcl), 1.4 g of dibasic sodium phosphate (Na_2HPO_4), and 0.24 g of di-potassium phosphate (K_2HPO_4) were added to one litter of ultra-pure water. Then, the pH was adjusted at 7.4.

❖ *Lipopeptides and polyketides identification by HPLC/MS*

The extracts were analyzed by HPLC-MS (Thermo Scientific) using a C18 column. After, a 10 μl injection of each extract (diluted at 1/100), the elution was conducted in a binary solvent system (solvent A: water + 0.1% formic acid and solvent B: acetonitrile + 0.1% formic acid) with the following gradient: 30% solvent B for 5 min, from 30% to 45% solvent B for 5 min and from 45% to 100% solvent B for 25 min, (flow rate was 0.5 ml/min at 40 °C). The detected lipopeptides and polyketides were identified according to their molecular weight.

III. Results

a) *Inhibition of pycnidiospores germination by Bacillus amyloliquefaciens filtrates*

All the tested filtrates showed an antifungal effect against the pycnidiospores of *Z. tritici* G1-1. These results were confirmed by the nephelometry turbidity measurements, as demonstrated in figures 1, 2, and 3. The pycnidiospore's germination rate was reduced progressively with the decrease of the filtrates dilution factor. Also, the ANOVA revealed a highly significant difference at $p=0.05$ between filtrates and dilutions. The average comparison by Duncan test classified the filtrates (F1, F2, and F3) and the control (F4) into four homogeneous groups. Filtrates F1, F2, and F3 showed very high levels of inhibition compared to the control (F4). However, it should be noted that F1, prepared with only *B. amyloliquefaciens* I3 caused a high inhibition of pycnidiospores germination compared to F2 (prepared with I3 and *Z. tritici* G1-1) and F3 (prepared with I3 and

Z. tritici A5-1) filtrates on the three tested dilutions (1/10, 1/2 and 9/10). After 96 hours of incubation, the dilutions d2, and d3 of both F1 and F2 filtrates inhibited 95% (101.2 KNTU) of pycnidiospores. The dilution d1 of F1 inhibited 82% (358.6 KNTU) of pycnidiospores germination compared to the control, while the same dilution of F2 did not exceed 42% of inhibition (1148.7 KNTU) (Figures 1 and 2). The dilutions d1, d2, and d3 of F3 expressed inhibition levels of 53% (942.5 KNTU), 64% (711.1 KNTU), and 99% (190 KNTU) respectively (Figure 3).

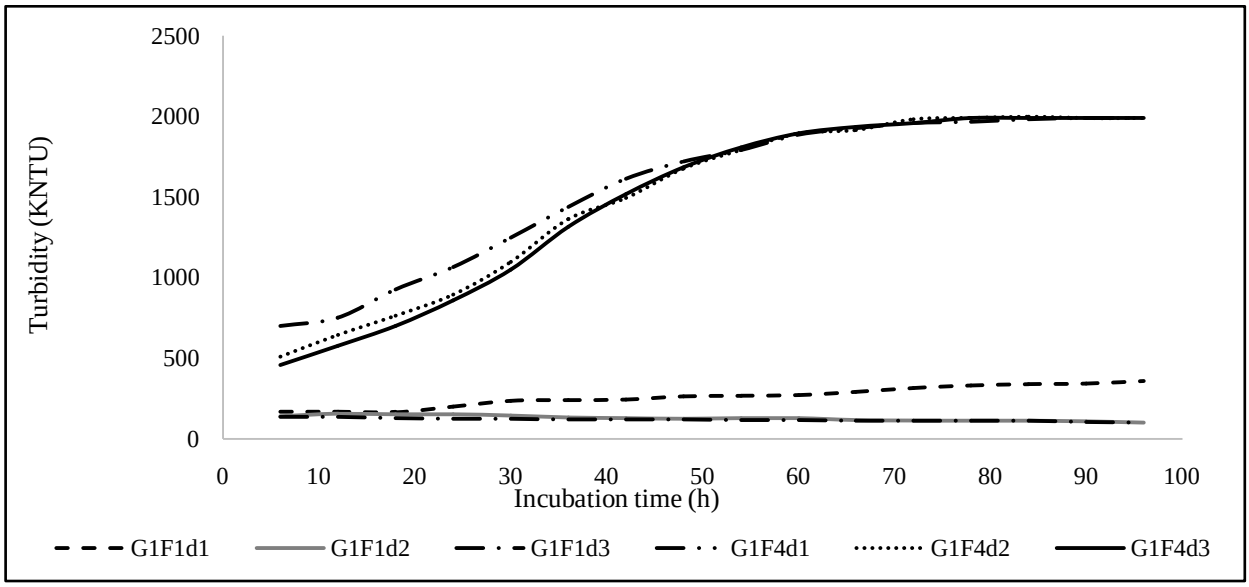

Figure 1: Kinetic of *Zymoseptoria tritici* G1-1 pycnidiospores germination in the presence of the F1 filtrate (prepared with *Bacillus amyloliquefaciens* I3) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4). NTU: Nephelometric Turbidity Unit.

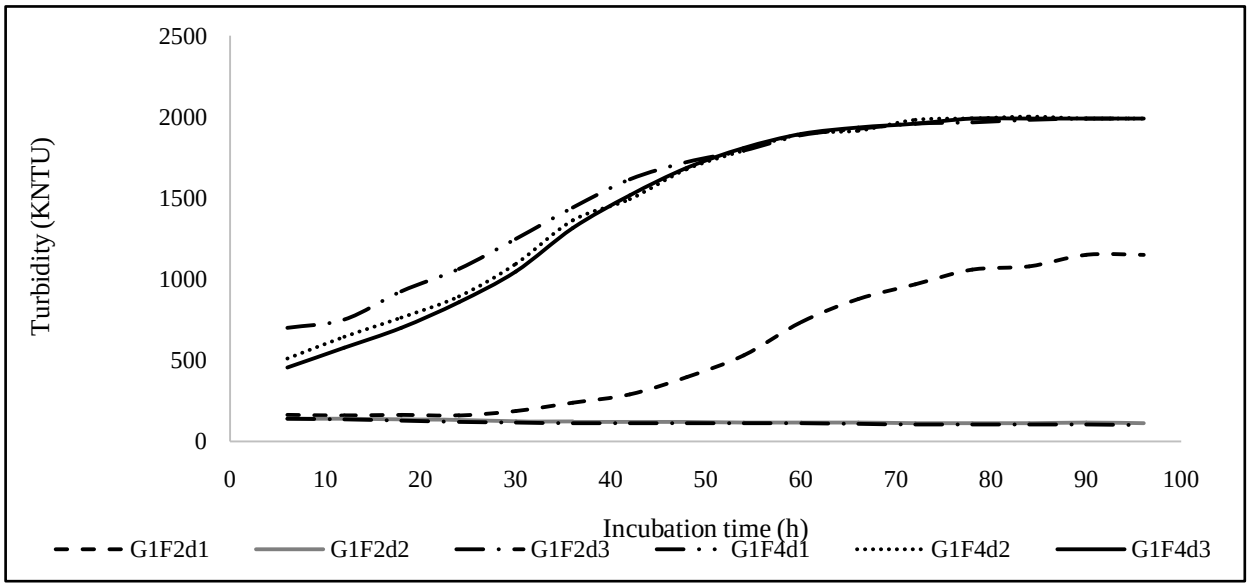

Figure 2: Kinetic of *Zymoseptoria tritici* G1-1 pycnidiospores germination in the presence of the F2 filtrate (prepared with *Bacillus amyloliquefaciens* I3 and *Zymoseptoria tritici* G1-1) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4).

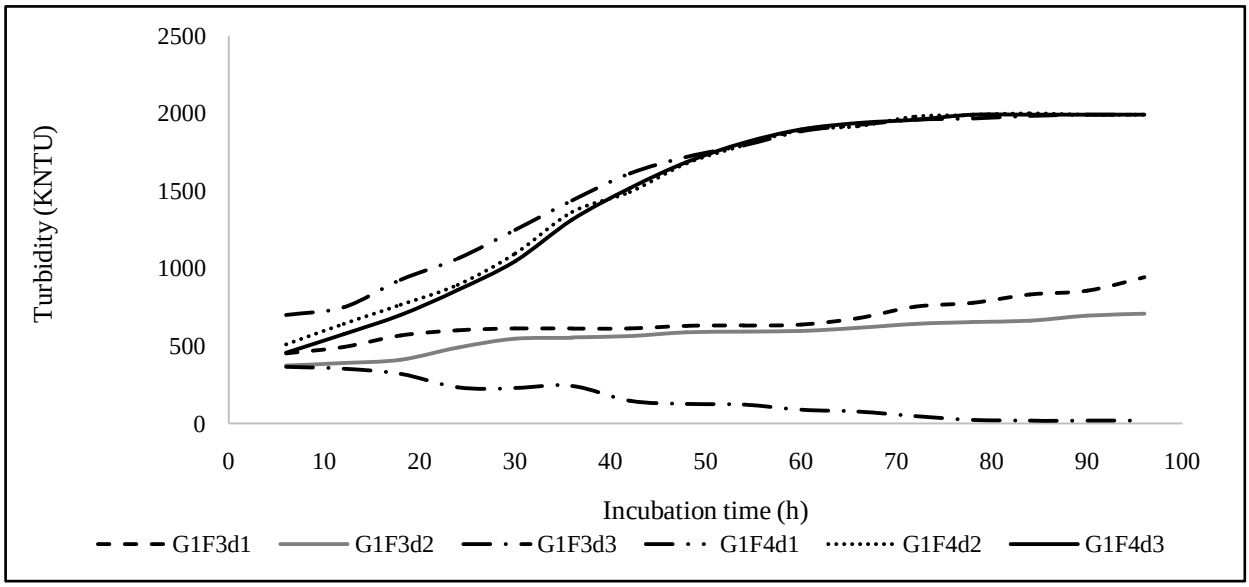

Figure 3: Kinetic of *Zymoseptoria tritici* G1-1 pycnidiospores germination in the presence of the F3 filtrate (prepared with *Bacillus amyloliquefaciens* I3 and *Zymoseptoria tritici* A5-1) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4).

Regarding the *Z. tritici* A5-1, the antagonistic effect of the I3 strain on the germination rate of *Z. tritici* A5-1 was then studied by nephelometry using the I3 filtrates F1, F2, and F3 (figures 4, 5, and 6). ANOVA analysis of turbidity data showed that the inhibitory effect of *B. amyloliquefaciens* I3 filtrates, without or with *Z. tritici* G1-1 and A5-1 strains, depended on the tested dilutions. After 96 hours, dilution d1=1/10 caused an inhibition rate of pycnidiospores germination around 88% (244583 NTU), 33% (1335620 NTU), and 51% (991081 NTU) for F1, F2, and F3 filtrates, respectively. For the medium dilution (d2=1/2), the inhibition percentages were about 93% (132210 NTU), 90% (201164 NTU), and 57% (796998 NTU) for F1, F2, and F3 filtrates, respectively. The dilution d3=9/10 exhibited the highest inhibition rates reaching the 94% (108850 NTU) for F1 and F2, and 72% (566249 NTU) in the case of F3 (Figures 4, 5 and 6).

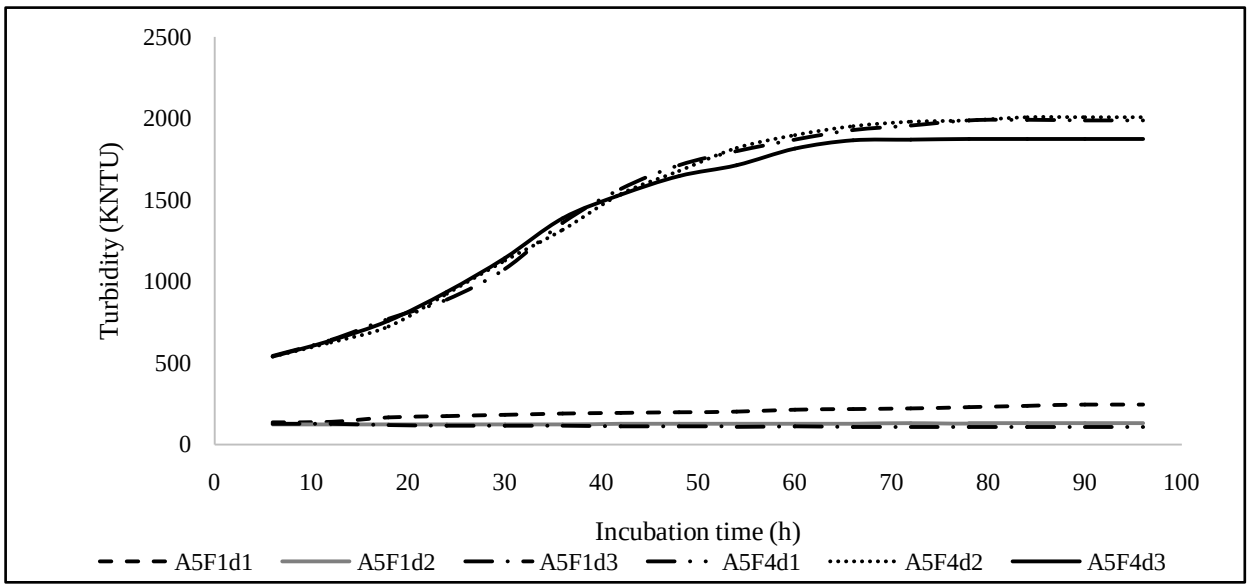

Figure 4: Kinetic of *Zymoseptoria tritici* A5-1 pycnidiospores germination in the presence of the F1 filtrate (prepared with *Bacillus amyloliquefaciens* I3) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4).

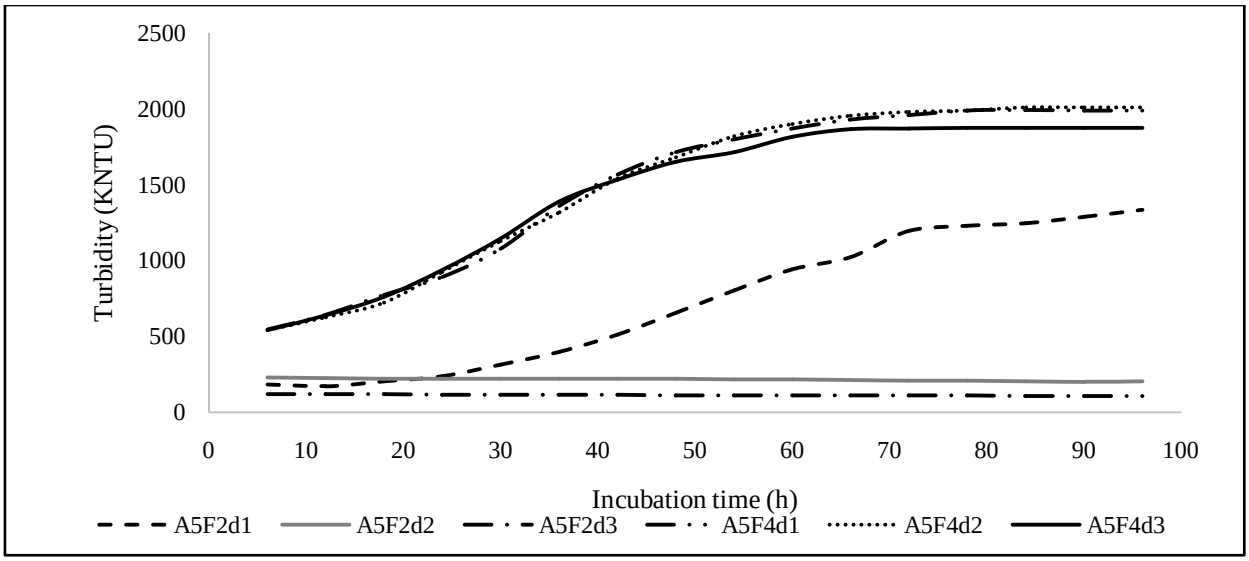

Figure 5: Kinetic of *Zymoseptoria tritici* A5-1 pycnidiospores germination in the presence of the F2 filtrate (prepared with *Bacillus amyloliquefaciens* I3 and *Zymoseptoria tritici* G1-1) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4).

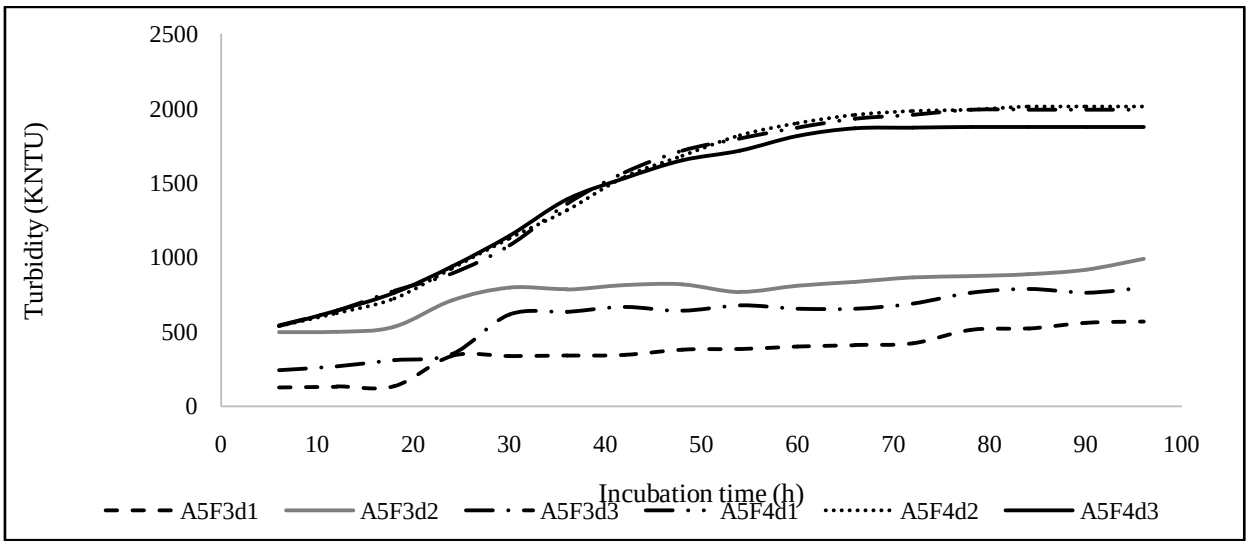

Figure 6: Kinetic of *Zymoseptoria tritici* A5-1 pycnidiospores germination in the presence of the F3 filtrate (prepared with *Bacillus amyloliquefaciens* I3 and *Zymoseptoria tritici* A5-1) at the three dilutions (1/10, 1/2 and 9/10) in comparison with the control (F4).

b) Antifungal compound identification

The HPLC-MS characterization of the metabolic composition of both agar culture and liquid filtrate extracts has revealed the presence of several families of cyclic lipopeptides and polyketides (see table 1). Comparing the molecular weights to already known lipopeptides [17,18,19], three major families of cyclic lipopeptides (surfactins, fengycins, and iturins) and three families of polyketides (macrolactins, chlorotetaines, and bacillaenes) were identified in the extracts prepared from inhibition zones of *Z. tritici* in agar media and liquid filtrates of *B. amyloliquefaciens* I3 without or with *Z. tritici* G1-1 and A5-1 strains. The identified lipopeptides include surfactins C12 and C15 with molecular weights of 1015.4 Da (Dalton) and 1057.5 Da, respectively. Two variants of iturins were also produced by *B. amyloliquefaciens* I3, named iturins A or mycosubtilin with a chain of 14 and 15 carbon atoms (C14, C15) whose molecular weights were 1043.6 Da and 1057.6 Da respectively, and iturins B C14 and C15 with a molecular weight of 1065.6 Da and 1079.3 Da, respectively. The macrolactins produced by the strain I3 are the following: D, A28, 7-o-succinyl-A, and 7-o-malonyl-A, with molecular weights ranging from 510.5 Da to 628.6 Da. The two identified chlorotetaine isoforms Cl35 and Cl37 had molecular weights of 289.2 Da and 291.1 Da, respectively. Also, two bacillaenes were identified, bacillaene A (582.5 Da) and bacillaene B (582.4 Da). Regarding the fengycin family, the only homologous produced by strain I3 was fengycin A with

17 carbon atoms and a molecular weight of 1498.8 Da. The families of iturins, macrolactins, bacillaenes, and chlorotetaines were the most abundant compared to the other families. However, extraction from the inhibition zones on agar media allowed higher intensities of the molecules produced by I3 strain - in the presence and the absence of *Z. tritici* G1-1 and A5-1 strains - compared to liquid culture extraction. The mass-spectrum and the identified families of lipopeptides and polyketides are presented in Table 1.

Table 1: Antifungal metabolite production of *Bacillus amyloliquefaciens* I3 detected by HPLC-MS.

Metabolite families	Identified molecules	Molar mass (Da)	Peak area (x10^5)					
			Agar media			Liquid media		
			I3	I3+G1	I3+A5	I3	I3+G1	I3+A5
Iturins	A C14	1043.6	9.05	3.32	2.70	7.94	1.17	0.39
	A C15	1057.6	5.52	1.09	1.04	3.21	0.45	0.568
	B C14	1065.6	5.42	4.32	1.41	2.29	0.534	3.12
	B C15	1079.3	77.7	6.27	2.61	8.01	21.5	2.64
Bacillaenes	A	582.5	2.89	2.67	1.37	1.89	1.80	2.94
	B	582.4	10.6	1.77	5.57	6.78	2.67	2.15
Chlorotetaines	Cl35	289.2	7.95	1.77	1.40	2.10	0.95	0.47
	Cl37	291.1	8.20	1.67	8.66	3.30	0.99	0.61
Macrolactins	D	628.6	74.5	4.06	1.07	7.05	2.82	0.23
	A28	425.4	1.61	16.7	-	2.40	3.39	-
	7-o-malonyl-A	524.6	21.9	2.44	0.90	8.45	3.66	0.31
	7-o-succinyl-A	510.5	2.80	9.78	-	4.74	0.57	0.52
Fengycin	A C17	1498.8	0.30	0.12	-	0.21	0.11	-
Surfactins	C12	1015.4	0.29	0.21	0.22	0.19	0.05	-
	C15	1057.5	0.20	0.19	-	0.11	0.10	0.28

IV. Discussion

The results of the inhibition assays of pycnidiospores of *Z. tritici* strains by *B. amyloliquefaciens* I3 (pycnidiospores of both *Z. tritici* strains, obtained from soft and durum wheat) have shown the presence of antifungal metabolites that are involved in the antagonistic activity of *B. amyloliquefaciens* I3 against *Z. tritici* (antibiosis).The three tested filtrates (F1, F2, and F3) induced inhibition of *Z. tritici* pycnidiospores germination up to 99%. However, dilutions d2 and d3 (1/2 and 9/10) showed inhibition rates ranging from 94% to 99% compared to the negative control F4. The identified property of *B. amyloliquefaciens* I3 to inhibit *Z. tritici* illustrate a new significant antifungal activity of strain I3. Our finding is consisted with reports by Zhang et al., [20] and Dimkic et al., [21] describing a high antagonistic effect of the crude extract of *B. amyloliquefaciens* TF28 lipopeptides against *F. oxysporum*, *B. cinerea, and Pythium sp*. Similar results were obtained by Sun et al., [19] who demonstrated that the *B. amyloliquefaciens* ES-2 filtrate inhibited significantly the growth of several phytopathogenic fungi such as the following: *Penicillium italicum*, *Fusarium culmorum*, *Botrytis cinerea*, *Magnaporthe grisea*, and *Erysiphe graminis hordei*. Considering the same context, Xu et al., [22] confirmed the severe toxicity of the *B. amyloliquefaciens* SQR9 filtrate on *F. oxysporum* conidia. Likewise, the filtrate of *B. amyloliquefaciens* CNU114001 inhibited the germ tube elongation of *B. cinerea*. The same bacteria showed a broad-spectrum of antagonistic activity against 12 phytopathogenic fungi (*Alternaria panax*, *B. cinerea*, *Colletotrichum acutatum*, *C. orbiculare*, *Corynespora cassicola*, *F. oxysporum*, *Phytophthora capsici*, *P. digitatum*, *Rhyzoctonia solani*, *Stemphylium lycopersici*, *Pyricularia grisea*, and *Sclerotinia sclerotiorum*) [23].

To characterize the responsible compounds involved in this inhibition, several identification methods were used, and the most important was based on chromatographic techniques. The HPLC-MS results of the different types of *B. amyloliquefaciens* I3 extracts, in both the presence and absence of *Z. tritici* A5-1 and G1-1, showed the production of many molecules with very high antimicrobial activity, including iturins, macrolactins, bacillaenes, chlorotetaines, fengycins, and surfactins. However, iturins A and B were identified in all extracts from I3 alone or in confrontation to *Z. tritici* G1-1/A5-1 strains with a very high relative abundance. Similar results were obtained in several previous investigations, among them the study of Arrebola et al., [24] which showed that iturin A produced by *B. amyloliquefaciens* PPCB004 affected *A. citri*, *Botryosphaeria sp*., *C. gloeosporioides*, *Fusicoccum aromaticum*, *Lasiodiplodia theobromae*, *P. crustosum*, and *Phomopsis persea* while the other lipopeptides - fengycins and surfactins -produced by this antagonist did not have a major effect on all studied pathogens. Inhibition of these seven fungal species could be added to nine other fungal pathogens affected by iturin A produced by different strains of Bacillus, as described by Hsieh et al., [25]. According to Jacques, [26], the fungitoxic activity of iturins is due to their ability to

penetrate membranes. Pathak, [27] proved that fengycins have a high fungitoxic activity, specifically against filamentous fungi. They also contribute to the formation of a complex with sterols, which suggests the ability of fengycines to interact with membrane lipids. The antifungal activity of fengycins is enhanced by the presence of surfactins [28,19], and iturins [29]. In this regard, Raaijmakers et al., [30] reported that surfactins contribute to the formation of a stable biofilm on host surfaces, protecting bacteria against antibiosis and competition of other microorganisms. While surfactins are not very active directly on fungal pathogens. The secretion of these lipopeptides promotes the colonization of root tissues by bacteria, which is a necessary condition for the constant availability of antifungals and the successful biocontrol of plant pathogens [5,31]. Furthermore, Xu et al., [22] demonstrated that bacillomycin D (a type of iturin) produced by *B. amyloliquefaciens* SQR9 contributes to biofilm formation in addition to its antifungal activity against *F. oxysporum in vitro* and *in vivo*. This may explain the low production of fengycins and surfactins molecules in the confrontation between *B. amyloliquefaciens* I3 and *Z. tritici* G1-1 and A5-1. In addition to lipopeptides, polyketides (macrolactins, bacillaenes, and chlorotetaines) were identified with high intensities in the different studied extracts. Thus, the compounds are also responsible for the inhibition of *Z. tritici* due to their high intensities when confronted with *Z. tritici* strains. Furthermore, the results obtained from the two studied extraction techniques have shown no difference in the metabolites produced in both the presence and absence of *Z. tritici*. However, in extracts prepared from I3 alone, the relative abundance of iturins, chlorotetaines, bacillaenes, and macrolactins was more significant compared to the other extracts prepared from I3 confronted to G1-1 and A5-1.

V. Conclusion

The *B. amyloliquefaciens* I3 filtrates tested in this study demonstrated high antifungal activity against *Z. tritici*. This effect was correlated to the importance and the diversity of the identified antifungal metabolites in the filtrates. These interesting results obtained in this study justify the need to proceed for purification of these metabolites and to test them against Septoria and other diseases of wheat *in planta*. If these metabolites are effective in plants, it would be useful for conducting advanced research to develop biopesticides from these molecules and testing them in other pathosystems.

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Conflicts of interest

The authors declare no conflicts of interest.

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