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Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization

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ABSTRACT

In this study, the biocontrol ability and colonization behavior of a highly antagonistic *Streptomyces mutabilis* strain, named IA1, recently isolated from a Saharan soil, were assessed on wheat seedlings. In this study we showed that strain IA1 secretes IAA and GA3 and is able to enhance growth of wheat seedlings. Using DOPE-FISH coupled with confocal laser scanning microscopy (CLSM), we observed moreover that following caryopsis inoculation and plant growth the strain can colonize the rhizoplane, the surface of caryopsis as well as the endorhiza, crossing from the rhizodermis up to the vascular system. Interestingly, further visualizations revealed that the actinobacterial strain could also be endophytic inside the caryopsis up to the endocarp layer (the dried fruit part, not the seed part of grain). Disease caused by *Fusarium culmorum* was further evaluated on seedlings and results showed that coated seeds with strain IA1 can reduce both disease occurrence (64.7%) and decrease severity (79.6%). This study showed that strain IA1 derived from a Saharan soil could protect a temperate crop from *F. culmorum* seedling blight, promote growth and colonize various niches on the surfaces of the phytosphere (roots, seeds) as well as plant endosphere compartments.

1. Introduction

Various beneficial bacteria support their host plants by increasing growth and/or by protecting them against pathogenic diseases (Bakker et al., 2007; Lugtenberg and Kamilova, 2009). The majority of these plant-associated bacteria are derived from the soil environment of the plants, including the rhizosphere, the laimosphere, and/or the spermosphere. Other sources can also be the caulosphere, phyllosphere, anthosphere as well as the carposphere as parts of the phytosphere environment, demonstrating the various sources of beneficial microbes (Compant et al., 2011). Among these bacteria, a subset can also enter inside plants and establish an intimate interaction with their hosts. These so-called endophytes proliferate in various plant tissues as well as organs without inducing pathogenicity symptoms (Rosenblueth and Martínez-Romero, 2006; Hallmann and Berg, 2007; Compant et al., 2010a).

Microbiological, microscopy, genetic as well as genomic analyses have provided clues about some specific plant–endophyte interactions. However, there are still many plant beneficial microorganisms which have been poorly investigated and which represent an interesting resource for application as biofertilizers or as biopesticides. Most researchers have focused on microorganisms derived from temperate regions. However, microorganisms derived from extreme environments such as desert soils may represent a promising resource for new types of microorganisms (Barrow et al., 2008; Compant et al., 2010b; Yekkour et al., 2012). Due to their enhanced resistance against environmental stresses, the application of these microorganisms as biofertilizers and/or biopesticides in crop production, even under temperate conditions, could confer various advantages as compared to microorganisms applied so far, particularly under changing climate conditions (Barrow et al., 2008; Compant et al., 2010b).

Some microorganisms, which are prominently encountered in harsh environments such as in arid soils of the Sahara desert, are *Actinobacteria* (Sabaou et al., 1998; Meklat et al., 2013) and they have been shown to spontaneously colonize plants in this desert environment as endophytes (Goudjal et al., 2013). Several studies on the

Abbreviations: DOPE-FISH, double labeling of oligonucleotide probes for fluorescence in situ hybridization; CLSM, confocal laser scanning microscopy; ISP, International *Streptomyces* Project; IAA, indole-3-acetic acid; GA3, gibberellic acid.

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diversity of *Actinobacteria* in desert environments have elucidated several novel species as well as new antagonistic compounds, which are active against various phytopathogens (Zitouni et al., 2004a, 2004b; Badji et al., 2007; Boudjella et al., 2010; Meklat et al., 2012). Furthermore, effective biocontrol strains have been identified (Yekkour et al., 2012) as well as strains stimulating plant growth (Goudjal et al., 2013). Nevertheless, plant colonization behavior of *Actinobacteria* derived from desert environments, particularly under temperate conditions, has been addressed rarely. We previously demonstrated, however, that strain IA1, isolated from desert soil in Algeria, exhibited strong activity against a wide range of plant pathogenic fungi and that for the first time actinomycin D was found in a *Streptomyces mutabilis* related species (Toumatia et al., 2015).

The aim of the present study was to analyze the ability of strain IA1 to produce phytohormones, such as auxins and gibberellins, to stimulate wheat seedlings growth as well as to protect wheat against *Fusarium culmorum*, a serious fungal pathogen prevalent in the north of Algeria, responsible of extensive damping-off and various blights (Yekkour et al., 2015). A particular aim was to elucidate the colonization behavior of this strain of wheat grown under temperate conditions using DOPE-FISH (double labeling of oligonucleotide probes for fluorescence in situ hybridization) tool coupled with confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Bacterial strain

The strain IA1 was recently isolated by our research team from a Saharan soil of Algeria and investigated with regard to its strong antifungal properties (Toumatia et al., 2015). Based on both phenotypic characteristics and phylogenetic analysis of the 16S rRNA gene sequences, the strain IA1 was closely related to *S. mutabilis* as shown in Fig. 1.

2.2. Preparation of the bacterial inoculum suspension for plant assay

The strain IA1 was cultivated on yeast extract–malt extract agar (ISP-2, International *Streptomyces* Project) medium (Shirling and Gottlieb, 1966) and incubated at 30 °C for 10 days. Actinobacterium spore suspensions were obtained by scraping the surfaces of plates. Then, independent homogenization in sterile distilled water was carried out and the suspension was filtered through a sterile gauze. Spore concentrations were then adjusted to 10^8 CFU ml^{-1} using the hemacytometer counting method.

2.3. Production of phytohormones

The ability to produce the plant growth regulators indole-3-acetic acid (IAA) and gibberellic acid (GA3) was determined by analyzing the extracts of the strain IA1. For this, one-milliliter aliquots of the spore suspensions of strain IA1 were transferred to 250 ml-Erlenmeyer flasks containing 50 ml of yeast extract-tryptone broth (YT) (yeast extract, 5 g l^{-1}; tryptone, 10 g l^{-1}; NaCl 5 g l^{-1}; pH 7.2) supplemented with 5 mg ml^{-1} of L-tryptophan (Sigma) for the detection of IAA, and on the ISP-2 broth for the detection of gibberellins. Flasks were cultured on a rotary shaker (200 rpm) at 30 °C for 5 days and supernatant cultures were harvested by centrifugation at 6000 rpm for 15 min. IAA and GA3 were then extracted with ethyl acetate from culture supernatants by a modified method of Goudjal et al. (2013) and Berríos et al. (2004), respectively. Ethyl acetate extract was evaporated to dryness in a rotary evaporator at 40 °C, then re-dissolved in 500 μl of methanol prior to HPLC analysis. The HPLC quantitation was performed in a Jasco® plus system (Japan) equipped with an Interchim reverse-phase C-18 column (15 μm particle size; 7.8 × 300 mm) and UV-detection at 280 and 208 nm for IAA and GA3, respectively. As mobile phase, a methanol–water linear gradient under two-step of 20–50% methanol (0–5 min) and 50–100% methanol (5–35 min) was used at a flow rate of 1 ml min^{-1}. Final quantitation was performed with reference to external calibration curve using standard IAA and GA3 purchased from Sigma-Aldrich (France).

2.4. Plant growth-promoting abilities of strain IA1

Soft wheat (*Triticum aestivum* L.) variety Hidhab HD 1220 was chosen as cereal plant model for this study. Seeds were supplied by the Technical Institute of Field Crops of Algeria. Effects of strain IA1 on wheat seedlings were assessed using the following method. Prior to use, seeds were surface sterilized (5% w/v NaClO; 0.2% w/v Tween 20; 3 min) then rinsed three times with sterile distilled water. Seeds were subsequently soaked in the IA1 spore suspension (10^8 CFU ml^{-1}; 60 min) before an air-drying (2 h) under a laminar flow hood. They

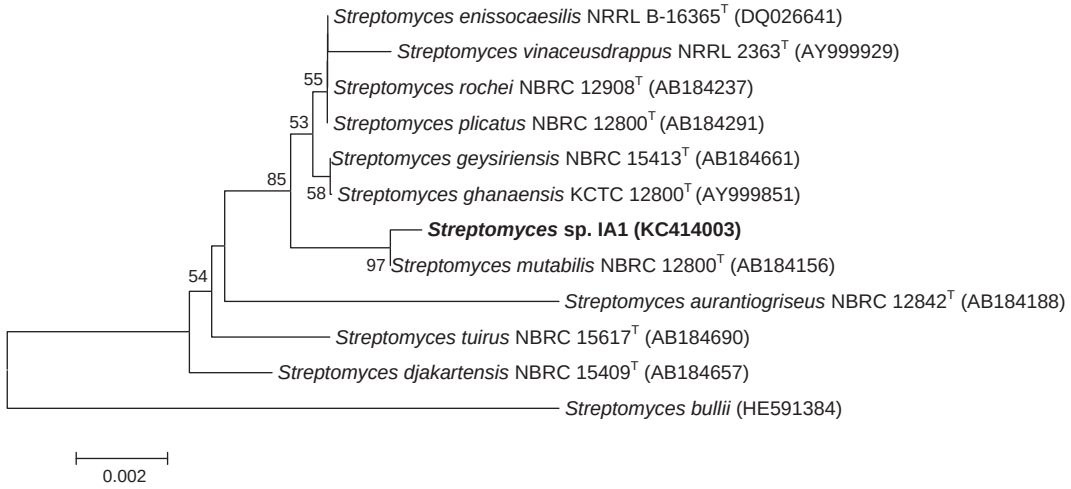

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the relationship between strain IA1 and related type-species of the genus *Streptomyces*. Analysis was carried out using software included in the MEGA version 6.0 package (Tamura et al., 2013). The distance matrix was calculated as described by Jukes and Cantor (1969) and the tree was inferred by neighbor-joining (Saitou and Nei, 1987). The numbers at the nodes indicate the levels of bootstrap support (Felsenstein, 2005) based on neighbor-joining analyses of 1000 resampled data sets (only values >50% are shown). Bar, 0.002 nt substitution per nt position. *Streptomyces bullii* $C2^T$ was used as outgroup. Sequences used in this analysis were those previously obtained by Toumatia et al. (2015) for strain IA and those available in the public sequence databases EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains (Kim et al., 2012)), for the related *Streptomyces* type-species.

were then placed into 9 cm Petri dishes (20 seeds per plate) containing a filter paper watered with 3 ml of sterile distilled water. For control experiment, seeds were soaked in sterile distilled water. Experiments were conducted in randomized design and repeated thrice. Plates with seeds were then placed in a phytotronic growth chamber (16 h photoperiod; 20–25 °C night–day; 70% relative humidity) and plants were allowed to grow during 10 days. Emergence of seedlings was scored (%) and shoot length of each seedlings was measured.

2.5. DOPE-FISH microscopy and visualization of the niches of colonization of strain IA1

For visualization of the bacterial colonization of IA1 strain, the roots, caryopsis, and leaves of wheat seedlings were harvested 10 days post inoculation and cut in small parts. Fixation was carried out overnight at 4 °C in a paraformaldehyde solution (4% in PBS 7.2) in Eppendorf tubes, before that samples be rinsed thrice with PBS. Treatment with a lysozyme solution (1 mg ml^{-1} in PBS) was then applied to the samples for 10 min at 37 °C before dehydration in an ethanol series (25, 50, 75 and 99.9%; 15 min each step). DOPE-FISH was performed using probes from Genecust (Luxembourg) labeled at both the 5′ and 3′ positions. An EUBmix (equivalent mixture of EUB338, EUB338II, EUB338III; Amann et al., 1990; Daims et al., 1999) coupled with a FLUOS fluorochrome and HGC69a probe specific to *Actinobacteria* (HGC69a; Küsel et al., 1999) coupled with Cy5 were used. NONEUB probe (Wallner et al., 1993) coupled with Cy5 or FLUOS was also used independently as a negative control. Hybridization was done at 46 °C for 2 h with 10–20 μl solution (containing 20 mM Tris–HCl pH 8.0, 0.01% w/v SDS, 0.9 M NaCl, formamide at the concentration suited to the probe, and 10 ng μl^{-1} of each probe) applied to each plant sample placed on slides in a 50-ml moist chamber (also housing a piece of tissue imbibed with 5 ml hybridization buffer). Post-hybridization was conducted at 48 °C for 30 min with a post-FISH pre-warmed solution containing 20 mM Tris–HCl pH 8.0, 0.01% (w/v) SDS, 5 mM EDTA pH 8.0 and NaCl at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before air drying for at least one day in the dark. This method was used for root surface colonization assay. In parallel to the root colonization study, the possibility that strain IA1 could be endophytic was evaluated by cutting longitudinally or transversally, using razor blades and after dehydration steps, roots, caryopses and leaves of young wheat seedlings. Hybridization, post hybridization processes, rinse steps were the same as described before.

For microscopic examination, samples on slides were covered with coverslips before observation under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G)laser FV10-LAHEG230-2). X, Y, Z pictures of samples were taken at 405, 488, 633 nm at 10×, 20× or 40× objectives and then merged (RGB) using Image J software. Z Project Stacks was then used to create the pictures. All experiments have been repeated five independent times on different plant parts with comparable results on each time 10 plants pooled.

2.6. Biocontrol properties of strain IA1 against F. culmorum

The ability of strain IA1 to protect wheat seedlings against *F. culmorum* was evaluated using a pathogenic *F. culmorum* strain formerly isolated in field from infested cereals and PCR-identified (Yekkour et al., 2015).

For the fungal inoculum preparation, *F. culmorum* was cultivated on potato dextrose agar (PDA) at 25 °C during 7 days before macroconidia were harvested by scrapping from the culture surfaces, then homogenized and filtered as described above for the bacterial strain. The concentration was adjusted to 10^5 macroconidia ml^{-1} using hemacytometer counting method.

Effects of strain IA1 on *F. culmorum* seedling blight of wheat were assessed, using the same protocol described above for evaluating the strain IA1-plant growth improvement capacity, excepted that seeds, after strain IA1 inoculation, were placed into Petri dishes containing a filter paper pre-inoculated with 3×10^5 macroconidia of *F. culmorum* (Yekkour et al., 2012). For control experiment, seeds were soaked in sterile distilled water and the filter paper was moistened with 3 ml of sterile distilled water. Experiments were conducted in randomized design and repeated three times. Plates were then placed in a phytotronic growth chamber in the same conditions described above. Visible disease symptoms on stems were scored 10 days post-inoculation according to the scoring system described by Khan et al. (2006). Seedling blight score was the product of lesion length (cm) by lesion color. The lesion color scale was: 0, no disease; 1, very slight brown necrosis; 2, slight/moderate brown necrosis; 3, extensive brown necrosis; and 4, extensive black necrosis.

Table 1
Effects of the strain IA1 on seedling emergence and shoot length[1].

Treatment[2]	% emerged plants	Shoot length (in mm)
Control	91.67 ± 3.34[a]	90.4 ± 6.95[b]
IA1	86.87 ± 4.41[a]	114.93 ± 7.86[a]

Means with the same letter in the same column are not significantly different at $P = 0.05$.
[1] Seeds were inoculated with actinobacterium strain IA1.
[2] The data shown are mean values of three replicates ± SE.

2.7. Data analysis

All data were subjected to analysis of variance (ANOVA). Mean separation was accomplished by Newman and Keuls multiple range test, and significance was evaluated at the probability level of $P = 0.05$.

3. Results

3.1. Plant growth promoting ability by of strain IA1

Using seedling assays, results showed that strain IA1 did not exhibit any significant improving of plant establishment (Table 1). However, shoot length of seedlings was increased in case of seeds pre-treated with strain IA1 by more than 27% (Table 1).

To further determine mechanisms involved in plant growth promotion, IAA and GA3 productions by strain IA1 were evaluated. Data showed that strain IA1 produced both IAA and GA3 under controlled conditions of fermentation with amounts rating of 74.39 ± 1.46 and 94.24 ± 23.66 μg ml^{-1}, respectively (Table 2).

3.2. Visualization of niches of colonization of strain IA1

Following seed inoculation, colonization of the strain IA1 was visualized 10 days post-inoculation on the rhizoplane and inside the endorhiza as well as inside tissues of caryopses of plants, using EUB338mix-FLUOS and HGC69a probe coupled with cy5 fluorochrome allowing visualization of the bacterium as fluorescent yellow/orange colored. Some root zones were colonized rarely by strain IA1 (Fig. 2–b). Neither root tip colonization nor root emergence site colonization was observed for the *S. mutabilis* strain IA1 on the rhizoplane of the root systems of the wheat seedlings. Root elongation zone was colonized rarely (Fig. 2a–b). At the root hair zone level, strain IA1 was however detected as hyphae forms colonizing intensively the surfaces of root hairs (Fig. 2c).

Table 2
Ability of strain IA1 to secret phytohormones IAA and GA3.

Strain	IAA (μg ml^{-1})	GA3 (μg ml^{-1})
IA1	74.39 ± 1.46	94.24 ± 23.66

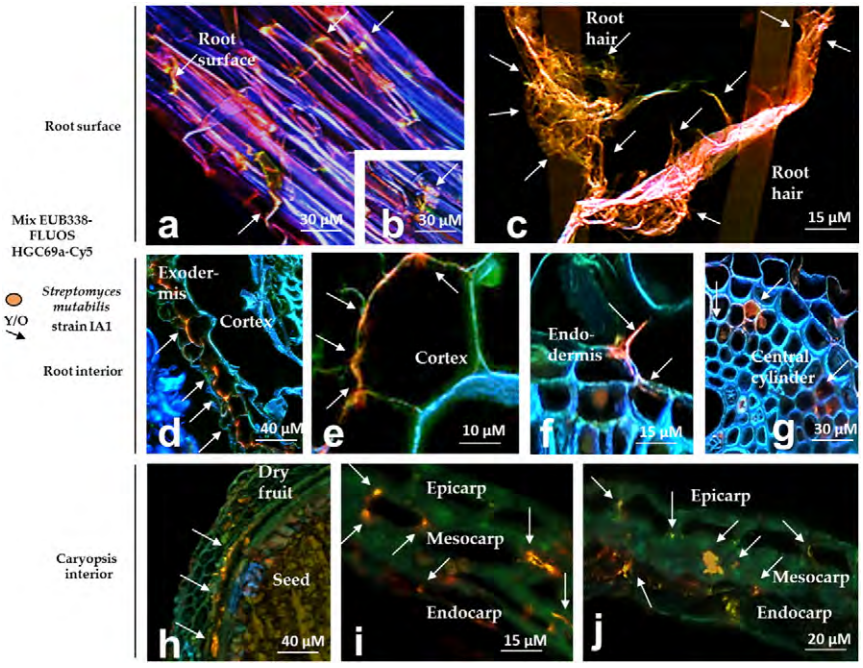

Fig. 2. Confocal scanning microscopy with DOPE-FISH hybridization of seedling parts 10 day post inoculation with strain IA1 showing, using EUB338mix-FLUOS and HGC69a-Cy5 probes, niches of colonization of strain IA1 appearing yellow/orange color due to probe mix and fluorochromes. a–b) Root surface, c) root hairs, d) exodermis, e) cortex, f) endodermis, g) central cylinder, h) caryopses with dried fruit parts and seed, i–j) dried fruit parts of the caryopsis.

Strain IA1 was additionally detected colonizing root internal tissues such as the exodermis (Fig. 2d), cortex (Fig. 2e), endodermis (Fig. 2f) as well as the central cylinder close to xylem vessels (Fig. 2g). *S. mutabilis* strain IA1 was also visualized inside the caryopses (Fig. 2h–j). Short hyphae were visualized particularly inside parts of the dry fruit part of caryopses (Fig. 2h–j), e.g. in the epicarp, mesocarp and endocarp cell layers (Fig. 2i–j). Inside the seed part of the caryopses, neither spores, nor germinated spores or hyphae forms of strain IA1 were visualized (Fig. 2i).

In comparison to seedling parts colonized strain IA1, control plants showed no bacteria on the root surfaces (Fig. 3a–c), inside root internal tissues (Fig. 3d) as well as inside caryopses (Fig. 3e–f). In some cases, few rare bacteria were visualized, however, that may be due to naturally occurring endophytes of caryopses (see Fig. 3e). Use of negative NONEUB probe on both colonized seedling parts did not detect colonization by strain IA1 (Fig. 4a–d). Same results were obtained with the use of the same probe on samples from control treatment (Fig. 4e–h).

3.3. Plant protection by strain IA1 against Fusarium seedling blight

Results of this study showed additionally that strain IA1 can protect wheat seedlings against *F. culmorum* on plants (Table 3). Untreated seedlings (control) as well as IA1 treated plants exhibited no disease symptoms, while pre-infested seedlings with *F. culmorum* (noted FC) showed a high significant disease impact (100% of diseased seedlings). Compared to the treatment FC, the pre-infested and treated plants with the actinobacterium strain (noted FC-IA1) exhibited a significant decrease on plant disease occurrence (64.74% of reducing) and severity (79.6% of score reducing).

4. Discussion

Streptomyces species have often been described in the literature for their important antifungal properties (Valanarasu et al., 2009; Duraipandiyan et al., 2010; Yekkour et al., 2012), which was attributed

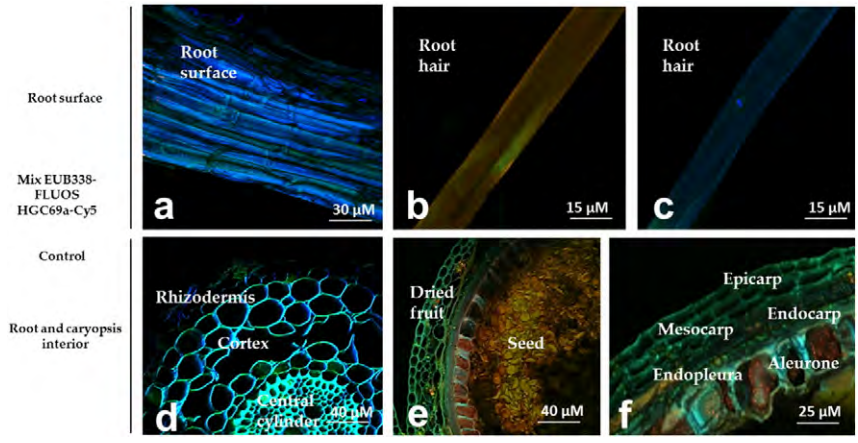

Fig. 3. Confocal scanning microscopy with DOPE-FISH hybridization of seedling parts 10 day post control inoculation showing, using EUB338mix-FLUOS and HGC69a-Cy5 probes, no hybridization of probes. a) Root surfaces, b–c) root hairs, d) root internal tissues, e) caryopses with dried fruit parts and seed, f) dried fruit parts of the caryopsis.

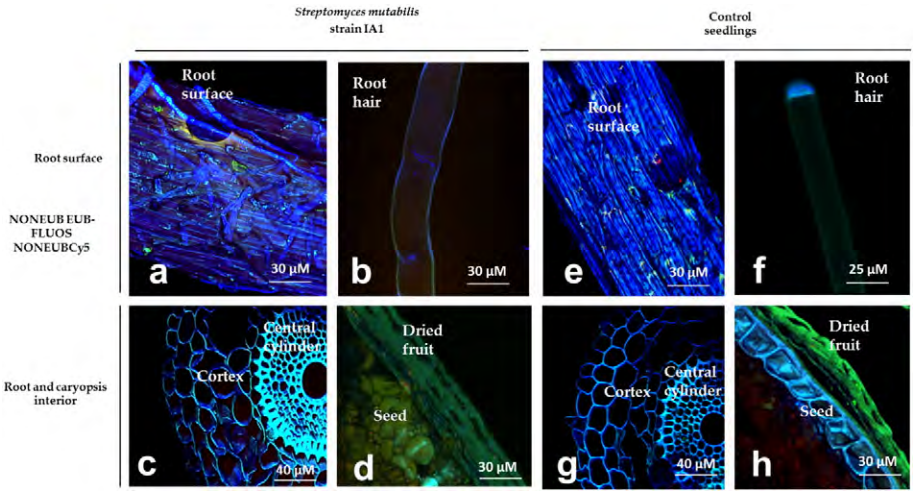

Fig. 4. Confocal scanning microscopy with DOPE-FISH hybridization of seedling parts 10 day post inoculation with strain IA1 (a–d) or control treatment (e–h) showing, using NONEUB-FLUOS and NONEUB-Cy5 probes. a and e) Root surface, b and f) root hairs, c and g) root internal tissues, d and h) caryopses with dried fruit parts and seeds.

to their abilities to secrete active metabolites (Solecka et al., 2012). Several studies have described the isolation of new *Actinobacteria* strains from Saharan soils with important antagonistic features such as the production of novel antibiotics (Sabaou et al., 1998; Zitouni et al., 2004a, 2004b; Badji et al., 2007; Boudjella et al., 2007, 2010). Additional studies have shown that some *Streptomyces* members can stimulate plant growth (Sardi et al., 1992; Goudjal et al., 2013; Goudjal et al., 2014). This stimulation has been frequently associated to the ability of these strains to produce phytohormones, mainly auxins and gibberellins (Strzelczyk and Pokojska-Burdziej, 1984; Tokala et al., 2002; Goudjal et al., 2013).

The strain *S. mutabilis* IA1 exhibited strong activity against a wide range of plant pathogenic fungi, protected plants against *Fusarium* wilt of flax, and chocolate spot disease of field bean and produces large amount of actinomycin D first found in *S. mutabilis* related species (Toumatia et al., 2015). In this study we showed that strain IA1 can both stimulate wheat seedling growth, and protect them against *Fusarium* seedling blight.

Interestingly, the application of the strain stimulated shoot growth by more than 27%. It can be speculated that the ability of the strain to produce appreciable amounts of phytohormones such as IAA and GA3 plays, at least in part, a role in this growth improvement (Babalola, 2010), although other mechanisms can also be responsible of plant growth promotion.

Numerous studies have described plant colonization by *Actinobacteria* (Coombs and Franco, 2003; Merzaeva and Shirokikh, 2006). In the present study, the colonization behavior of one strain isolated from Saharan soil was monitored on wheat seedlings. Although the strain was isolated from a desert environment, it could not be expected before that it could colonize wheat plants on the surfaces as well as inside plants under climate chamber conditions. In this study we demonstrated, however, that the Saharan isolate could colonize the root surfaces, especially root hairs and cross from the rhizodermis to the central cylinder. In our study, we demonstrated further that endophytism in caryopsis by strain IA1 was restricted to the dried parts of the caryopsis, e.g. the fruit part. Coombs and Franco reported in 2003 that a *Streptomyces* sp. could colonize seed inside caryopsis, as they found the strain tagged with a *gfp* marker in the endosperm. In our study, only the dry part of the fruit could be colonized by the Saharan soil isolate, suggesting that endophytic colonization of wheat by *Actinobacteria* depends of the actinobacterial strain.

The *S. mutabilis* IA1 strain had an important protective effect on wheat seedlings against the *F. culmorum*, causal agent of seedling blight. When the seeds were pre-infested with the fungus, the treatment with strain IA1 significantly reduced the disease occurrence on seedlings by a ratio of 2/3 compared to the untreated ones. Furthermore, regarding the disease severity, strain IA1 significantly reduced seedling blight symptoms by almost 80%. Similarly, Shimizu et al. (2001) reported an induced disease resistance response on rhododendron seedlings after treatment with an actinomycin D producing strain, *Streptomyces* R-5, and also showed its strong suppressive impact on the fungus *Pestalotiopsis sydowian*, the causal agent of *Pestalotia* disease of rhododendron. As a whole, results of the biocontrol experiment confirmed previous studies, which demonstrated the ability of some Saharan *Streptomyces* strains to control some phytopathogenic diseases (Errakhi et al., 2007; Loqman et al., 2009; Yekkour et al., 2012; Goudjal et al., 2014).

The strain isolate from Saharan soil exhibited therefore promising biocontrol properties against the cereal pathogenic *F. culmorum* and was also able to colonize various parts of young wheat plants with the use of diverse niches to colonize plant internal tissues such as the roots, and caryopsis. Although more investigations are needed to understand better the interaction between *S. mutabilis* IA1 and wheat plants and confirm consistence of biocontrol effectiveness of IA1 under field conditions. This study shows that the strain, isolated from a Saharan soil, could colonize wheat plants, outside of its natural environment.

Table 3
Effects of the strain IA1 on seedling blight[1].

Treatment[2]	% Plants with disease	Disease score
Control	0 ± 0^{c}	0 ± 0^{c}
IA1	0 ± 0^{c}	0 ± 0^{c}
FC	100 ± 0^{a}	3.88 ± 0.3^{a}
FC-IA1	35.26 ± 7.57^{b}	0.79 ± 0.06^{b}

Means with the same letter in the same column are not significantly different at $P = 0.05$.
[1] Seeds were co-inoculated with *F. culmorum* (FC) and actinobacterium strain IA1.
[2] The data shown are mean values of three replicates ± SE.

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