Efficiency of *Saccharothrix algeriensis* NRRL B-24137 and Its Produced Antifungal Dithiolopyrrolones Compounds to Suppress *Fusarium oxysporum*-Induced Wilt Disease Occurring in Some Cultivated Crops

Rabiâa Merrouche[1] · Amine Yekkour[1,2] · Lynda Lamari[1] · Abdelghani Zitouni[1] · Florence Mathieu[3] · Nasserdine Sabaou[1]

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Abstract *Saccharothrix algeriensis* NRRL B-24137 (SA) is a well-studied actinobacterium strain for its ability to produce numerous bioactive dithiolopyrrolone derivatives with appreciable antifungal properties. Our study aimed to investigate the possible usefulness of SA and its produced compounds to control several formae speciales of *Fusarium oxysporum*, which affect different important crops. Such is the case of *F. oxysporum* f. sp. *lini* (FOLi), *F. oxysporum* f. sp. *lentis* (FOLe), *F. oxysporum* f. sp. *ciceris* (FOC) and *F. oxysporum* f. sp. *lycopersici* (FOLy) which cause devastating wilt to flax, lentil, chickpea and tomato, respectively. Antagonistic properties of the strain SA have been primarily evaluated *in vitro*. This screening showed the significant antifusarium activity of the actinobacteria strain against FOLi, FOC, FOLe and FOLy. Consecutively, strain SA and FOLi soil development has been assessed. The SA soil treatment permitted an important decrease (threefold) in the FOLi infestation. Moreover, the actinobacteria soil density seemed maintained after 7-week treatment at an appreciable level of 44×10^6 CFU/gram of dry soil (gds). Through different pot experiments, soil pre-treatment with the SA strain significantly reduced the disease incidence of FOLi, FOLe, FOC and FOLy by 71.0, 73.3, 61.2 and 59.7%, respectively. However, the treatment with pure dithiolopyrrolones appeared less effective with about 50% of FOLi disease impact reduction, which thus suggested the involvement of more than direct antibiosis in the whole SA biocontrol performance. Globally, significant correlation between the *in vitro* antagonistic properties of SA and its capacity to reduce the disease occurrence ($r = 0.81$, $P \leq 0.05$) has been highlighted.

Keywords Antifusarium activity · Biocontrol · Dithiolopyrrolones · *Saccharothrix algeriensis* NRRL B-24137 · Vascular wilt fungi

1 Introduction

Fusarium oxysporum is a common devastating soilborne pathogen causing wilt symptoms to various plant hosts and is widely distributed in different soil types worldwide [1]. A large number of formae speciales (f. sp.) affect particular hosts, such as *F. oxysporum* f. sp. *lini*, which is pathogenic to flax. Pathogenic isolates of *F. oxysporum* cause severe damage and yield losses across a wide range of plant families, including many economically important crop species such as tomato, chickpea and lentil [2–4]. The pathogen can persist in the soil for several years through the production of conidia and chlamydospores [5] and is thus difficult to control. Traditionally, crop rotation has been proved to be an effective strategy to control many soilborne diseases, but because most of these pathogens can survive for a long period of time (10–15 years), the effectiveness of crop rotation is limited once a disease outbreak occurs [6]. Soil disinfection using various chemicals [7] is also a traditional practice, but must phase out because of environmental and human health concerns [8]. As no single control measure is fully effective to reduce *F. oxysporum*-induced wilt to cultivated plants, inte-

✉ Nasserdine Sabaou
sabaou@yahoo.fr

1 Laboratoire de Biologie des Systèmes Microbiens, Ecole Normale Supérieure de Kouba, Alger, Algeria

2 Centre de Recherche en Phytotechnie, Institut National de Recherche Agronomique d'Algérie, B.P. 37, Mehdi Boualem Baraki, Alger, Algeria

3 Laboratoire de Génie Chimique UMR 5503 (CNRS/INPT/UPS), INP de Toulouse/ENSAT, Université de Toulouse, 1, avenue de l'Agrobiopôle, Castanet-Tolosan Cedex, France

gration of different complementary options is still needed for sustainable disease management including cultural practices (soil solarization, sowing date, use of fungi-free seed and more resistant cultivars) and use of efficient biological control agents [9,10].

In recent years, the biological control of plant pathogens has become an increasingly promising alternative to chemical control in the management of soilborne disease [11,12].

Numerous studies have demonstrated a reduction in diseases incidence in different crops after supplementing the soils with antagonistic microorganisms such as *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp. or biochemicals [13,14].

Microorganisms can control soilborne pathogens through competition, antibiosis, parasitism and the induction of plant disease resistance [15].

Among these microorganisms, actinobacteria have also demonstrated effective biocontrol ability against various soilborne fungi such as *Fusarium* spp. [4]. This biocontrol ability was frequently related to their antibiosis properties [16,17]. In fact, numerous actinobacteria species produce a large number of antifungal compounds and other bioactive secondary metabolites [18,19]. Within the actinobacteria group, *Streptomyces* spp. have been investigated predominantly because of the commercial interest shown on their great antibiotic-producing capacity. Currently, two *Streptomyces*-based biofungicides have been approved and marketed: Mycostop® (*Streptomyces griseoviridis* strain K61) and Actinovate® (*Streptomyces lydicus* strain WYEC108) are being used as seed treatment for controlling damping-off and wilting mainly due to soilborne *Fusarium* [20,21].

However, many of non-streptomycete actinobacteria taxa, such as *Saccharothrix,* have been less explored [22] and evaluation of their biocontrol ability against *Fusarium* wilt is not reported anywhere.

Such is the case of *Saccharothrix algeriensis* NRRL B-24137 (SA), a Saharan soil deviated strain extensively studied in the last decade by our research team for its ability to produce numerous bioactive metabolites, belonging to pyrrothine class of compounds, named dithiolopyrrolone derivatives [23–25]. These compounds exhibited appreciable activities against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi, but have not been tested against plant pathogenic fungi such as *Fusarium* spp. [23,24].

Concerning their chemical structure, all the SA-produced dithiolopyrrolone derivatives are characterized by a unique pyrrolinonodithiole nucleus with difference in side chain *N*-acyl groups (*R*) (Fig. 1) [23,24,26].

As a whole, the present work was carried out to evaluate for the first time the effectiveness of the well-documented *Saccharothrix algeriensis* and its produced dithiolopyrrolones to control *Fusarium* wilt occurring in various cultivated plants.

2 Materials and Methods

2.1 Microorganisms and Inoculates Preparation

The strain *Saccharothrix algeriensis* NRRL B-24137 (SA) was maintained at 4 °C on slants of International *Streptomyces* Project 2 (ISP-2) medium (10-g malt extract, 4-g yeast extract, 4 g glucose, 18 g agar, 1000 ml distilled water, pH 7.0) [27].

Four *Fusarium oxysporum* formae speciales, isolated from different infested plants and causing various wilt diseases, were provided by the culture collection unit of the Department of Plant Pathology, Blida University, Algeria. It included *Fusarium oxysporum* f. sp. *lini* (FOLi), *F. oxysporum* f. sp. *lentis* (FOLe), *F. oxysporum* f. sp. *ciceris* (FOC) and *F. oxysporum* f. sp. *lycopersici* (FOLy) infecting flax, lentil, chickpea and tomato, respectively. All fungal strains were maintained at 4 °C on PDA medium (200-g potato infusion, 20 g dextrose, 20 g agar, 1000 ml distilled water, pH 6.0).

For the preparation of inoculate suspensions, strain SA and *Fusarium* isolates were subcultured in a manner to obtain an optimum sporulation (ISP-2 medium for 12 days at 30 °C and PDA medium for 10 days at 25 °C, respectively). Both actinobacteria and fungi propagules were harvested by scraping from the culture surface with a glass slide, homogenized in sterile distilled water and filtered through a double layer of sterile gauze. The concentrations were adjusted to 10^8 of colony-forming units (CFU)/ml for actinobacteria and 10^4 conidia/ml for each fungal isolate [16].

2.2 Antifusarium Activity

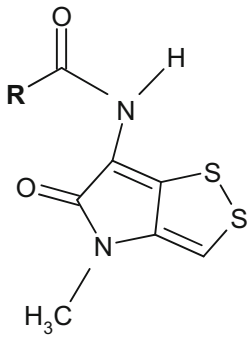
The antagonistic activity of the strain SA against *F. oxysporum* formae speciales (cited above) was evaluated on ISP-2 medium by the streak method. Plates were inoculated with actinobacterial culture by a single streak and incubated at 30 °C for 10 days. Then, target fungi were seeded in streaks perpendicular to the actinobacterial strain. The antifungal activity was evaluated by measuring the distance of inhibition between target fungi and the actinobacterial colony margins, after incubation at 30 °C for 36 h [28].

2.3 Dithiolopyrrolones Obtainment

The dithiolopyrrolones used in this study have been characterized in our previous works, and their spectroscopic and spectrometric data have been fully described [23–25]. Thus, all the considered dithiolopyrrolones have been produced and HPLC purified with respect to the same procedure.

Briefly, a mature slant culture of the strain SA was inoculated into 500-ml Erlenmeyer flasks, each containing 100 ml of a basal semi-synthetic medium (SSM, containing 10 g glu-

Fig. 1 Structure of some dithiolopyrrolone antibiotics produced by *Saccharothrix algeriensis* NRRL B-24137



R : CH_3	Acetyl-pyrrothine (thiolutin)
R : 	Benzoyl-pyrrothine
R : H	Formyl-pyrrothine
R : $CH{=}C(CH_3)_2$	Senecioyl-pyrrothine
R : $CH{=}CH{-}CH{=}CH(CH_3)$	Sorbyl-pyrrothine
R : $C(CH_3){=}CH(CH_3)$	Tigloyl-pyrrothine
R : $CH_2CH_2CH_2CH_3$	Valeryl-pyrrothine

cose, 2 g $(NH_4)_2SO_4$, 2 g NaCl, 0.5 g KH_2PO_4, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCO_3$, 2-g yeast extract, 1000 ml distilled water, pH 7.0) [26]. The inoculated cultures were put on a rotary shaker (240 rpm; 30 °C) for 5 days. For purification of the produced dithiolopyrrolones, a total of 20 l of 5-day-old culture were used as previously described [23,24]. Briefly, the mycelium was first removed and the culture broth was extracted with dichloromethane. The extracts were partially purified using preparative thin-layer chromatography (Merck Art. 5735, Kiesselgel 60HF 254–366; 20 × 20 cm) with ethyl acetate/methanol (100:15 v/v) solvent system. Two antifungal-active bands named (AJ, $R_f = 0.52$ and PS, $R_f = 0.59$) were obtained. After elution with methanol, these bands were subjected to a semi-preparative HPLC purification with a reverse-phase C18 column (UP5ODB, 250 × 7.8 mm, Waters, Milford, MA). The samples were analysed by linear gradient elution of methanol–water (40–100% for 30 min), a flow rate of 1.5 ml/min and UV detection at 220 and 390 nm. Each peak fraction corresponding to a dithiolopyrrolone was repetitively collected and reinjected in the HPLC system until total purification (near 100%) and then air-dried.

Finally, the dithiolopyrrolone identification was carried out by comparing combined data from their retention times and mass (electron impact MS—EIMS—recorded at 70 eV with a Nermag R-10-10C spectrometer) spectra, to those previously described [23–25].

2.4 Seeds Material and Soil

Seeds of susceptible plants to wilt diseases caused by *Fusarium oxysporum* formae speciales (cited above) were used in this study. The plants investigated were: flax (*Linum usitatissimum* L.; variety Hera), chickpea (*Cicer arietinum* L.; variety ILC482), lentil (*Lens culinaris* L.; variety ILL4400) and tomato (*Solanum lycopersicum* L.; variety Bolivar).

Saharan soil (sterilized and non-sterilized) was used in this study. The soil presented a sandy texture (92.24% of sand), basic pH (8.4) with a reduced moisture content (3.2%) and a low amount organic carbon (0.2%), and it was non-salt (electrical conductivity = 0.14 mS/cm). For sterilization, the soil was autoclaved three times for 20 min at 120 °C with 24-h intervals.

2.5 Assessment of the SA and FOLi Development in the Soil

The number of SA and FOLi propagules in the soil was assessed during 9 weeks after an initial inoculation of 10^8 colony-forming units (CFU)/gds (gram of dry soil) and/or 10^4 conidia/gds, respectively. Microbial densities were determined by placing serial dilutions of soil (0.1 ml/plate) on nutrient agar (5 g peptone, 2-g yeast extract, 1-g meat extract, 5 g NaCl, 20 g agar, 1000 ml distilled water, pH 7.0). The formed colonies were counted after 3- and 7-day incubation at 20 °C for FOLi and SA, respectively. To avoid unwanted microbial development, the medium was supplemented with 50 mg/l chloramphenicol, 25 mg/l kanamycin and 10 mg/l streptomycin for counting fungi, whereas 50 mg/l of cycloheximide was added for counting actinobacteria.

2.6 Biocontrol Assay

The effect of soil treatment with the SA strain on the development of *Fusarium* vascular wilt of flax, lentil, chickpea and tomato, respectively, caused by *Fusarium oxysporum* f. sp. *lini*, *F. o. lentis*, *F. o. ciceris* and *F. o. lycopersici*, was assessed during 7 weeks in pot experiments as follows: surface sterilized seeds (5% NaClO; 3 min) of each susceptible plants were sown in pots (15 seeds/pot, 1 cm depth) containing sterilized soil (100 g/pot) pre-inoculated with the corresponding wilt-causing *F. oxysporum* formae speciales (10^4 conidia/gds) and with SA spore suspension (10^8 CFU/gds) [17]. Before being transferred to the growth chamber (22 ± 2 °C, 12-h 8000 lux light period and 70–80% relative humidity), the pots were kept in darkness for 3 days at 15 °C to support seed germination.

The same procedure was used to evaluate the control ability of the HPLC-purified dithiolopyrrolones (identified as thiolutin, formyl-pyrrothine, tigloyl-pyrrothine, senecioyl-

pyrrothine, benzoyl-pyrrothine, valeryl-pyrrothine and sorbyl-pyrrothine) against *Fusarium* wilt of flax. The solution of each antibiotic was prepared in sterile tube by dissolving pure compound into water in such a manner to treat fungi infested soil with a dose of 5 mg per 100 gds. This dose represents the lowest concentration with significant effect. The concentration has been selected after preliminary experiments (data not shown) which showed that concentrations less than 5 mg were less effective, whereas concentrations greater than 5 mg did not improve the protection of the plant.

All plants were maintained by pot watering as needed (soil moisture kept at 15%) with sterilized tap. Pots inoculated with each fungal pathogen (10^4 conidia/gds) acted as control.

2.7 Data Analysis

All experiments were repeated three times. Pot experiments were conducted in a randomized design, and the data obtained were analysed by an analysis of variance (ANOVA) using Newman and Keuls multiple range test for mean separation. Pearson's correlation coefficient (r) was also calculated. For all data, significance was evaluated at the probability level of $P \leq 0.05$.

3 Results and Discussion

3.1 Antifusarium Activity

Through the in vitro evaluation of SA antagonistic properties, the strain exhibited appreciable antifusarium activity with an inhibition distance of 12, 10, 09 and 06 mm towards FOLi, FOC, FOLe and FOLy, respectively. Members of the genus *Saccharothrix* were already shown to exhibit strong activity against various filamentous fungi such as *Ascochyta fabae*, *Aspergillus carbonarius*, *Fusarium culmorum*, *F. equiseti, Penicillium expansum* and *Umbelopsis ramanniana* [28,29].

On grapevine plants, inoculation of *Saccharothrix algeriensis* NRRL B-24137 exhibited interesting biocontrol ability to reduce grey mould symptoms due to *Botrytis cinerea* with effective root systems colonizing properties [30,31].

Furthermore, SA is known to produce several bioactive compounds (dithiolopyrrolones) which are putatively linked to the significant antifungal properties [23,24]. Similarly, other dithiolopyrrolones-producing microorganisms, such as those belonging to *Streptomyces* and *Xenorhabdus* genera, also exhibited strong activities against several filamentous fungi including *Botrytis*, *Fusarium*, *Plasmopara*, *Pythium*, *Rhizoctonia*, *Verticillium*, *Umbelopsis*, *Penicillium*, *Trichoderma* and *Phytophthora* [19,32].

3.2 Assessment of the SA and FOLi Development in the Soil

Considering its interesting antifusarium activity, the SA strain was further used in a pot experiment to assess its effect for suppressing FOLi soil persistence (Fig. 2). The treatment with the actinobacterial strain significantly decreased the FOLi density in the natural non-sterilized soil (Fig. 2a). In fact, after 9 weeks of treatment, the pathogen infestation decreased threefold in comparison with the control (non-treated soil) and reached 0.047×10^4 CFU/gds.
In contrast, SA density stayed stable from the seventh week of treatment at an appreciable level of 44×10^6

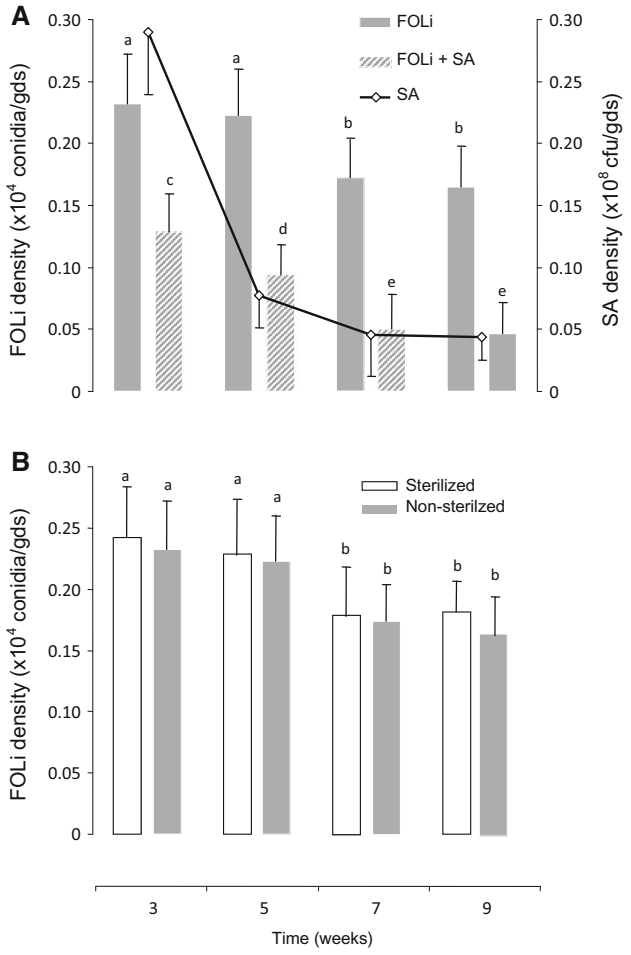


Fig. 2 Assessment of SA and FOLi development in the soil. **A** Suppressive capacity of SA soil treatment for FOLi infestation in non-sterilized soil. **B** Similar FOLi density evolution in both sterilized and non-sterilized soils without SA inoculation. Bars indicate standard deviation of the mean ($n = 3$). *Columns with the same letters* are not significantly different at $P \leq 0.05$. The initial soil inoculation was 10^8 CFU/gds and 10^4 conidia/gds for SA and FOLi, respectively. For sterilization, the soil was autoclaved three times for 20 min at 120 °C with 24-h intervals. SA, *Saccharothrix algeriensis*; FOLi, *Fusarium oxysporum* f. sp. *lini*

CFU/gds (Fig. 2a), indicating that the introduced actinomycete survived, colonized and competed with the fungi in the rhizosphere. Comparable results have been observed by Gopalakrishnan et al. [4] in a field experiment evaluating several *F. oxysporum* f. sp. *ciceris*-antagonistic *Streptomyces* as potential biocontrol agent of *Fusarium* wilt of chickpea where up to 10^6 values of artificially inoculated *Streptomyces* were enumerated from *F. oxysporum* f. sp. *ciceris*-infested rhizosphere.

Ryder and Jones [33] reported that the ability of the antagonistic microorganism to maintain a sufficient population density in the rhizosphere for a sufficient length of time is critical for the success of any biocontrol method.

Similar SA behaviour has been observed when the strain was inoculated in a non-FOLi-infested soil (data not shown). When the FOLi density evolution has been assessed in sterilized and non-sterilized soils, no significant difference was observed at the end of experiment (Fig. 2b). This suggests that the decrease in the pathogen density was mainly due to the presence of the SA strain, and the soil microflora did not interfere with the actinobacterial exhibited antagonism (Fig. 2a). Similar results were obtained against *F. oxysporum* f. sp. *albedinis* (pathogen of date palm) infestation, which was significantly decreased in soil treated with *Streptomyces* spp. [34].

Several authors have reported that either competition for carbon and antibiosis ability between soil-living microorganisms may be involved in the limitation of *Fusarium* germination propagules in the soil [35,36]: competition for minor elements also frequently occurs in soil. For example, competition for iron is one of the most observed limiting factor for growth of several pathogenic fungi [8]. Antibiosis resulting from the antagonism of the production by one microorganism of secondary metabolites toxic for other microorganisms is a very common phenomenon responsible for the biological activity of many biocontrol agents such as *Streptomyces* spp. and *Trichoderma* spp. [37,38]. A large diversity of metabolites, enzymes and volatile compounds have been described in the literature, and their role in suppression of several plant pathogens has been documented [39,40].

3.3 Assay of *S. algeriensis* Biocontrol Ability

With regard to SA interesting suppressive capacity for FOLi soil infestation, the subsequent potential of the actinobacterial strain to control FOLi-induced wilt to flax was evaluated. Moreover, since SA is known to produce appreciable quantities of several antifungal dithiolopyrrolones, antibiosis ability of these compounds to suppress the pathogen was also separately evaluated and then compared to the global strain biocontrol ability.

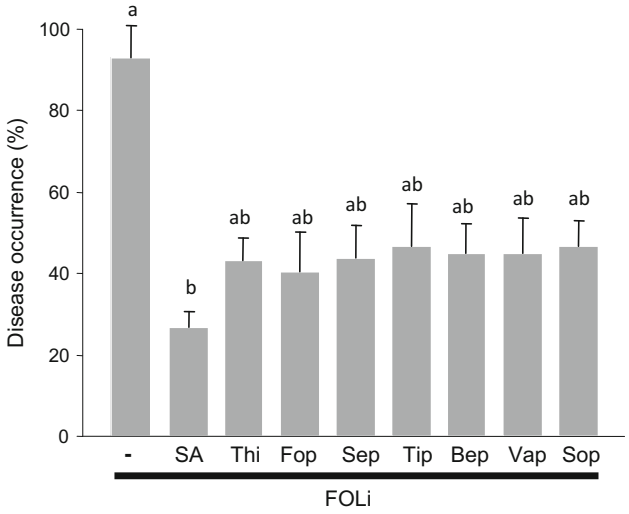


Fig. 3 Ability of soil treatment with SA and its produced dithiolopyrrolone antibiotics treatment to the soil to control FOLi-induced vascular wilt to flax. Disease occurrence was estimated 7-week post-planting. *Bars* indicate standard deviation of the mean ($n = 3$). *Columns with the same letters* are not significantly different at $P \leq 0.05$. The initial soil inoculation was 10^8 and 10^4 CFU/gds for SA and FOLi, respectively. Each antibiotic was used at a dose of 5 mg per 100 gds. SA, *Saccharothrix algeriensis*; FOLi; *Fusarium oxysporum* f. sp. *lini*; Bep, benzoyl-pyrrothine; Fop, formyl-pyrrothine, Sep, senecioyl-pyrrothine; Sop, sorbyl-pyrrothine; Thi, thiolutin; Tip, tigloyl-pyrrothine; Vap, valeryl-pyrrothine

The results showed significant reduction in the disease occurrence when the infested planting soil was treated with the SA strain or each of its produced dithiolopyrrolones (Fig. 3). However, the actinobacterial strain appeared more protective than the tested compounds (reduction in disease occurrence after 7 weeks of treatment of about 70 and 50%, respectively) when compared to the disease development plants grown in infested and non-treated soil (control). In comparison, reduction in *Fusarium* wilt incidence never exceeded 25% when commercial *Bacillus* spp., *Trichoderma* spp. or indigenous non-pathogenic isolates of *F. oxysporum* have been used [41], while an actinomycin D-producing strain, *Streptomyces* sp. IA1, isolated from a Saharan soil reduced wilt disease by more than 60% [17].

The SA strain has recently demonstrated to affect pathogen performance by more than direct antibiosis such as interference with pathogen signalling, stimulation of host plant defence (induced systemic resistance/ethylene and jasmonate signalling pathways) as well as competition for niches and nutrients [15,42].

In addition, the similar efficiency within the tested dithiolpyrrolones to control FOLi-induced wilt could be explained by compounds having very close chemical structure (same pyrrolinonodithiole nucleus and differs in only the side chain; Fig. 1), which therefore are speculated to express relatively close antibiosis ability.

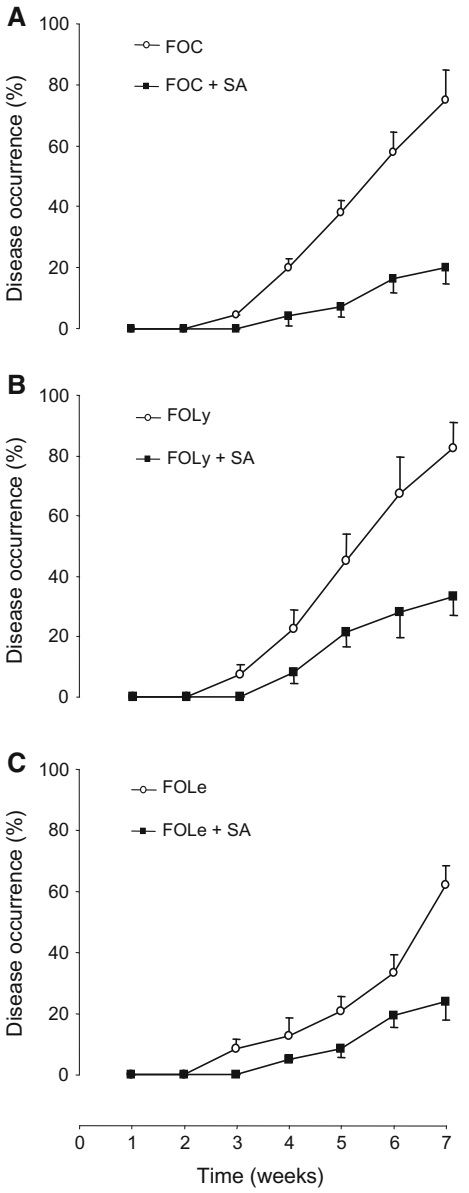


Fig. 4 Ability of SA soil inoculation to control the induced FOC, FOLy and FOLe vascular wilt to chickpea (**a**), tomato (**b**) and lentil (**c**), respectively. *Bars* indicate standard deviation of the mean ($n = 3$). The initial soil inoculation was 10^8 CFU/gds and 10^4 conidia/gds for SA and each considered fungi, respectively. SA, *Saccharothrix algeriensis*; FOC, *Fusarium oxysporum* f. sp. *ciceris*; FOLi; FOLy, *Fusarium oxysporum* f. sp. *lycopersici*; FOLe, *Fusarium oxysporum* f. sp. *lentis*

Since the soil treatment with SA strain exhibited significant ability to reduce the vascular wilt on the experimental flax/FOLi model, we further checked the possible usefulness of this treatment to control other common *F. oxysporum* f. sp. that affect economically important cultivated plant such as lentil, chickpea and tomato. The soil treatment with SA strain permitted to significantly reduce the pathogenic vascular wilt after 7 weeks in all experienced model, FOC/chickpea, FOLe/lentil and FOLy/tomato with 73.3, 61.2 and 59.7%, respectively (Fig. 4).

Our biocontrol assay exhibited effectiveness of SA to control various vascular wilt induced by several *F. oxysporum* f. sp. to cultivated plants.

Previous studies have also demonstrated potential use of some highly antagonistic actinobacteria strains recovered from Saharan soils to control soilborne diseases [16,17] although our assay is the first study highlighting potential usefulness of an non-streptomycete actinobacteria strain (SA) to control several *F. oxysporum* f. sp. that induce different devastating vascular wilt to cultivated plants. In the present investigations, a significant correlation between the *in vitro* antagonistic properties of SA strain and its capacity to reduce the disease occurrence has been recorded ($r = 0.81$, $P \leq 0.05$).

Further investigations are needed to ascertain the biocontrol effectiveness of *Saccharothrix algeriensis* against the vascular wilt fungi at greenhouse and field level with assessing the microorganism establishment and maintenance as prerequisite for potential introduction in an integrated pest management solution.

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