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MICROBE-PLANT INTERACTIONS BETWEEN *STREPTOMYCES* AND MODEL AGRICULTURAL PLANTS – *HORDEUM VULGARE* AND *LYCOPERSICON ESCULENTUM* (MICROTOM)

Aim. Microbe-plant interactions (MPI) constitute an important aspect of ecology because of their significant influence on plant's ability to withstand abiotic stress and infection. In comparison to proteobacteria and bacilli, the roles of streptomycetes in MPI remain poorly studied. Here, we elucidate some aspects of MPI between two model plant species, *Hordeum vulgare* and *Lycopersicon esculentum*, and several strains of *Streptomyces lividans* 1326 and *S. ghanaensis* ATCC14672. ***Methods***. Microbiology, microscopy and molecular genetics were combined to reveal the MPI. ***Results***. We demonstrate the colonization of *H. vulgare* and *L. esculentum* roots by different strains of *S. ghanaensis* deficient in production of either the antibiotic moenomycin or signaling molecule of the γ-butyrolactone type. The treatment of *H. vulgare* seeds with *S. lividans* spores increased the root biomass. Plants treated with 1,4-butyrolactone had no positive influence on plants, at milimolar concentrations this compound inhibited the root and shoot growth of *L. esculentum.* ***Conclusions***. Roots of two mono- and dicot plants are colonized by *Streptomyces*; reporter gene *uidA* is useful to monitor the colonization. Under our experimental conditions the ability to colonize plants by streptomycetes was not affected by the deficiency in antibiotic or butenolide production.

Keywords: *Streptomyces ghanaensis*, moenomycinA, low-molecular weight signal compounds, root colonization.

Members of the actinobacterial genus *Streptomyces*, as soil dwellers, are imprescriptible constituents of ecosystems. They excrete hydrolytic enzymes and bioactive compounds displaying various activities, including (but not limited to) metal-chelating, antibacterial and antifungal ones [1]. Through production of these substances streptomycetes can promote plant growth, facilitate roots colonization by nodule-forming bacteria and protect plants against phytopathogens [2–7]. Although ubiquity of streptomycetes in soil and rhizosphere is well-documented, there is little data on how secondary metabolism of the former shapes their ability to colonize plants. Most of *Streptomyces*-centered research has been done on model plant *Arabidopsis thaliana*. Particularly, colonization of *A. thaliana* roots by *Streptomyces lividans* was shown to provide protection of the former against fungal infection [8]. Recently *A. thaliana* colonization by endophytic streptomycetes has been reported as well as their responses to plant hormones [9]. These and other studies support the assumption that *Streptomyces* spp. can be important players in plant ecology.

In our work we decided to depart from studies on *A. thaliana* and focus on interaction of *Streptomyces* with agriculturally important plants. Particularly, monocot *Hordeum vulgare* and dicot *Lycopersicon esculentum* (Microtom) have been chosen. As bacterial partners we picked *S. lividans* strains because of proven ability to colonize plant roots. *S. ghanaensis* has also been included in the study; a collection of mutants for this species is available allowing us to address the roles of secondary metabolites in interactions with plants. The research was focused on three topics. First, we wanted to demonstrate that the streptomycetes under study indeed colonize plant roots and are able to produce antibiotics *in situ*. Second, effects of *Streptomyces* on plant growth were studied under different experimental setups. Third, we studied whether streptomycetes hormones of butenolide type, either

endogenously produced or artificially added, affect plants. The last research line was prompted by the fact that the root colonization intensity and pathogen inhibition by several non-streptomycetes depend on the production of low-molecular weight signaling compounds, LMWSC [10]. Butyrolactones and butenolides in streptomycetes are essential for secondary metabolism and morphogenesis [11, 12], yet their influence on other bacteria or plants was not studied.

Materials and methods

We used wild type moenomycin A (MmA) producer *Streptomyces ghanaensis* ATCC14672 and its derivatives d_aco and OB21e. In the d_aco mutant a key gene, aco_{gh} (*ssfg_07849*), for butenolide production is disrupted; in OB21e deletion of *moeGT1* glycosyltransferase gene completely blocks MmA production [13]. Wild type *S. lividans* 1326 and its butyrolactone-deficient *scbA*-mutant were chosen as positive control for root colonization [8]. Agriculturally important mono- and dicotyledonous plants, *Hordeum vulgare* L. *cv* Barke (Josef Breun GdbR, Herzogenaurach, Germany) and *Lycopersicon esculentum* Mill *cv* MicroTom (Bruno Nebelung GmbH, Everswinkel, Germany), respectively, were used. Prior to bacterial inoculation, the seeds were surface-sterilized as described in [10]. Briefly, after 2-3 days of germination on oatmeal agar plates at 30 °C, the seeds were washed and incubated in either 3 mL of *Streptomyces* spore suspension ($2{,}5\times10^9$ spores/ml) or 3 mL of 48-h liquid culture broth (OD=0,7) for 1–1,5 h [8]. The treated seeds were placed into sterile quartz sand or soil with mineral media (Hoagland's salt). The plants were incubated under 14 h light, 25°C/10 h dark, 20°C cycles and 70 % relative humidity [10]. For biometric analyses, two-weeks-old plants were washed from the sand, weight and length of wet shoots and roots were determined (overall we analyzed 36 plants, 6 plants for each strain). The statistical analysis of obtained data was carried out using Sigma Plot (*t*-test, ANOVA). The root colonization was studied using an established fluoresent *in situ* hybridization coupled to light scanning microscopy (FISH-LSM) approach [8].

Results and discussion

In this work we pursued several research directions. First, we tested different approaches towards visualization of MPI. Second, we studied the possibility that secondary metabolism of streptomycetes influence the efficiency of MPI. Third, we explored the influence of model butyrolactone on plant growth parameters. Below we describe our results along these four research lines and discuss the most promising future research directions.

Plant colonization, as portrayed by FISH-LSM and reporter beta-glucuronidase gene. All *S. ghanaensis* and *S. lividans* strains colonized root surface of *H. vulgare* and *L. esculentum* (Fig. 1). The hybridization signals were mainly located on root hairs. In comparison to mycelial suspensions of *S. ghanaensis* (grown in tryptic soy broth), spore inoculations (harvested from oatmeal agar) provided more extensive colonization of *H. vulgare* roots.

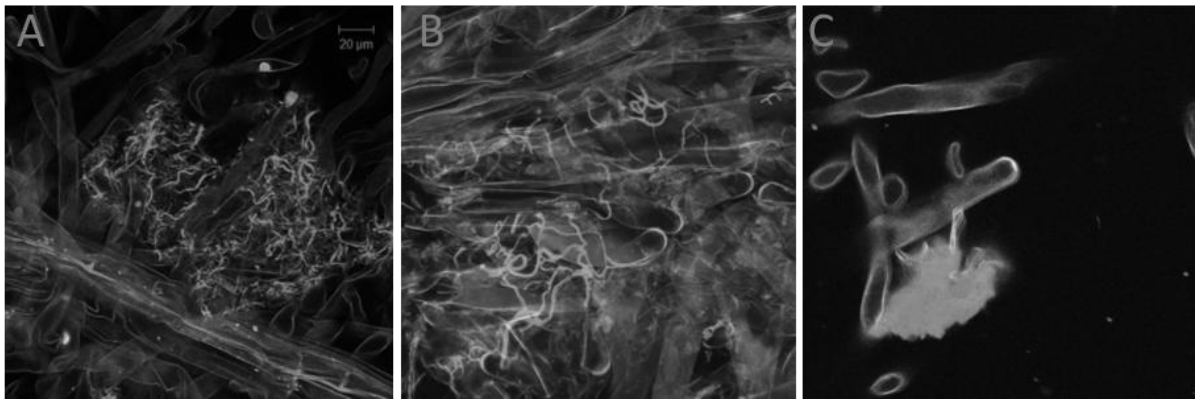

Fig. 1. FISH-LSM-screening of *H. vulgare* and *L. esculentum* roots colonization by *S. ghanaensis* and *S. lividans*: A – *S. ghanaensis* ATCC14672 mycelium on *H. vulgare* root hairs; B – *S. ghanaensis* ATCC14672 mycelium on *L. esculentum* root surface; C – *S. lividans* M707 mycelium on *H. vulgare* roots. Pink and blue-violet coloration is a result of fluorescence of two probes – Cy5EUBMix and Cy3HGC69 – owing to overlap of red and green light filters.

As FISH-LSM is time-consuming, requires expensive reagents and equipment, we therefore explored the possibility to use a *Streptomyces* codon-adapted β-glucuronidase reporter gene [14] as a faster and simpler way to detect *Streptomyces* mycelia *in situ*. For this purpose reporter plasmid pmoeE5script was used where *uidA* is fused to AdpA-regulated promoter of hexoisomerase gene *moeE5* involved in MmA biosynthesis [15].

An expression of glucuronidase activity from this promoter would therefore be an indicative of MmA production. The reporter plasmid was transferred conjugally from *E. coli* ET12567 (pUZ8002) into *S. ghanaensis* strains and spore suspensions of pmoeE5script$^+$ transconjugants were used to inoculate plant seeds. Expression of the reporter gene was visualized by soaking the roots in a 2 mM solution of 5-Br-4-Cl-3-indolyl glucuronide (X-Gluc). Plant roots of *H. vulgare* and *L. esculentum* treated with spores of three reporter-containing strains (wild type, d_aco, OB21e) turned blue upon application of X-Gluc solution. Two observations attracted our attention. First, OB21e strain, deficient in MmA production, colonized the root surface in a patchy pattern, leading to a mix of colorless and blue-colored root areas (Fig. 2). Perhaps, absence of MmA affects the interaction between the plant and OB21e.

Second, wild type *S. ghanaensis* colonized the *H. vulgare* roots as well as the d_aco strain did; colonization of *L. esculentum* roots by the aforementioned strains was less intense as compared to *H. vulgare* colonization. These results were reproducible in three independent experiments.

Influence of streptomycetes and simple butyrolactone analogue on plant growth parameters. *S. lividans* 1326 and *scbA* strains promoted the growth of *H. vulgare* in an axenic system, but not in soil, while *S. ghanaensis* strains affected *H. vulgare* and *L. esculentum* biometric parameters under neither condition. In case of *S. lividans* 1326 and M707, the biomass increase was observed for roots (Fig. 3). *Lycopersicon*-based experiments revealed no plant growth-promoting activity of *Streptomyces*.

Bacterial hormones can affect the plant growth as well as inhibit the development of phytopathogens. The simplest analogue of streptomycetes butyrolactones, 1,4-butyrolactone, is known to stimulate the antibiotic production in *S. hygroscopicus* [16]. In micromolar and low milimolar concentrations this chemical influenced neither MmA production by *S. ghanaensis* nor plant growth. However, at 10 mM it inhibited the shoot and root elongation of *L. esculentum*.

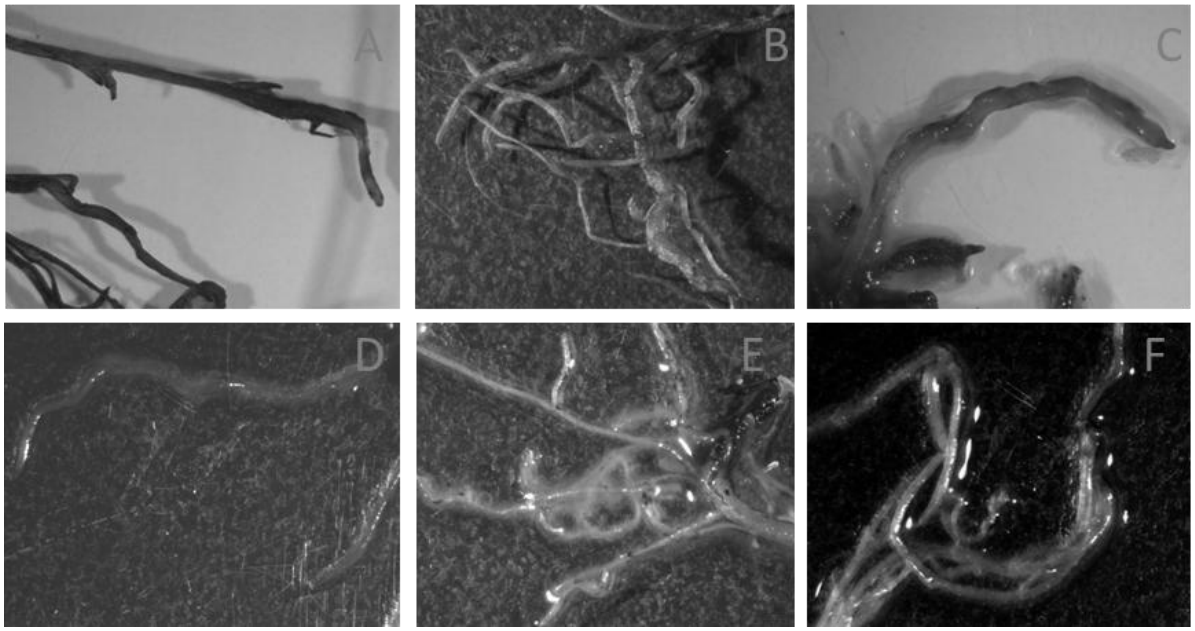

Fig. 2. Glucuronidase screening of *H. vulgare* (A, B, C) and *L. esculentum* (D, E, F) roots colonization by *S. ghanaensis* strains: A, D – wild type; B, E – d_aco; C, F – OB21E. A blue coloration of root surface is a result of the hydrolysis of X-Gluc to indigo by β-glucuronidase, which is expressed from a reporter gene in pmoeE5script-carrying streptomycete strains. The photos represent typical result of three independent experiments.

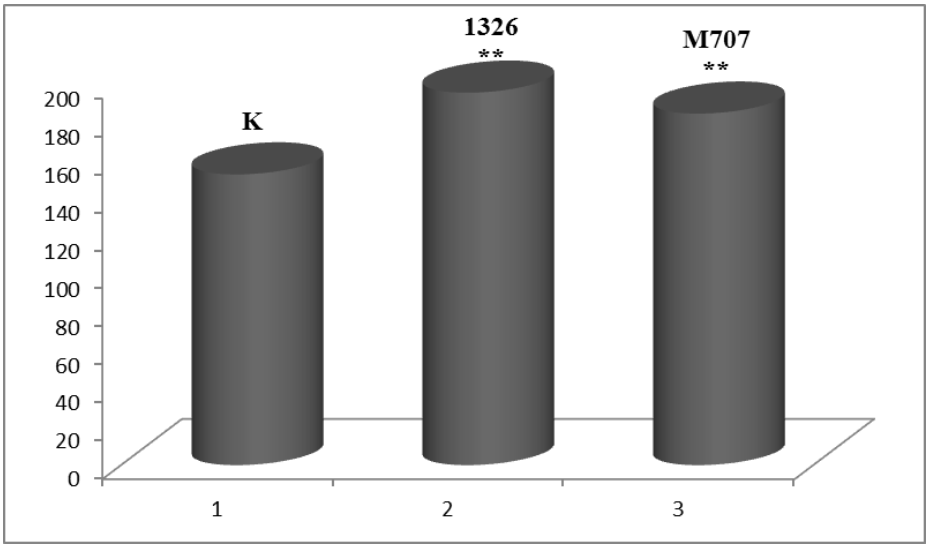

Fig. 3. The weight of roots (vertical axis, mg) of *H. vulgare* plants grown in axenic system from seeds treated with spore suspension of *S. lividans* 1326 (2) and *S. lividans* M707 (3); K (1) – untreated control samples. According to t-test ANOVA, the difference in the mean values of the treated groups in comparison to control group, is greater than would be expected by chance (p=0,996 and p=0,991, respectively); m (K)=8,177; m (1326)=10,562; m (M707)=8,019.

Conclusions

In this work we provided evidence that roots of two practically valuable mono- and dicot plants are colonized by *Streptomyces* species and showed the utility of reporter gene *uidA* to monitor the colonization. Secondary metabolism of *Streptomyces* is believed to be one of the main factors providing a competitive advantage for plants in the form of growth improvement or pathogen suppression [17]. Nevertheless, little evidence exists that streptomycetes indeed are capable of producing antibiotics in rhizosphere or *in planta* [18]. In our work we used a reporter system based on the promoter of the gene *moeE5* essential for initial steps in MmA biosynthesis. The fact that it is "switched on" on root surface indirectly implies that antibiotic production could occur there as well. We think that wider application of genetic reporters will help us to better understand the processes of secondary metabolism in rhizosphere, thus portraying a more complete picture of *Streptomyces*-plant interactions. Finally, we show that low concentrations of butenolide analog do not influence plant growth. These results are in agreement with the observation that butenolide-deficient mutant d_aco is not less successful than wild type in its ability to colonize rhizosphere.

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МІКРОБНО-РОСЛИННІ ВЗАЄМОДІЇ МІЖ *STREPTOMYCES* І МОДЕЛЬНИМИ СІЛЬСЬКОГОСПОДАРСЬКИМИ РОСЛИНАМИ – *HORDEUM VULGARE* І *LYCOPERSICON ESCULENTUM* (MICROTOM)

Мета. Мікробно-рослинні взаємодії (МРВ) – важливий екологічний аспект, унаслідок їхнього суттєвого впливу на здатність рослин протистояти абіотичну стресу та інфекції. Порівняно з протеобактеріями і бацилами, роль стрептоміцетів у МРВ залишається слабо вивченою. У цій роботі ми висвітлили деякі аспекти взаємодії двох модельних видів рослин, *Hordeum vulgare* і *Lycopersicon esculentum*, та кількох штамів *Streptomyces lividans* 1326 і *S. ghanaensis* ATCC14672. ***Методи***. Було поєднано мікробіологічні, мікроскопічні та молекулярно-генетичні підходи для виявлення МРВ. ***Результати***. Показано колонізацію коренів *H. vulgare* і *L. esculentum* штамами *S. ghanaensis* – дикого типу та мутантів з пошкодженою продукцією моеноміцину або сигнальної сполуки γ-бутиролактонного типу. Обробка насіння *H. vulgare* суспензією спор *S. lividans* викликала зростання біомаси кореня. Рослини, оброблені 1,4-бутиролактоном, не відрізнялися за морфометричними показниками від контролю. Однак, у мілімолярних концентраціях ця сполука пригнічувала ріст коренів і пагонів *L. esculentum.* ***Висновки***. Корені одно- та дводольних рослин колонізуються бактеріями роду *Streptomyces*; репортерний ген *uidA* корисний для моніторингу стрептоміцета на рослині. За використаних умов здатність до колонізації рослин стрептоміцетами не залежала від продукції останніми антибіотиків чи бутеноліду.

Ключові слова: *Streptomyces ghanaensis*, моеноміцин А, низькомолекулярні сигнальні молекули, колонізація кореня.