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ANTIMICROBIAL AND PLANT-GROWTH PROMOTING PROPERTIES OF RHIZOBACTERIA ISOLATED FROM WEED PLANT SENNA OCCIDENTALIS

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A B S T R A C T

The use of microorganisms to increase productivity and maintain soil fertility is an alternative sustainable agricultural practice to replace agrochemicals. The purpose of this study was to isolate microorganisms with high-antifungal, -antibacterial and -plant growth promoting activity from the rhizosphere of the weed *Senna occidentalis* native to West Africa. Out of 14 isolates, 5 inhibited the growth of *Fusarium oxysporum* f. sp. *radicis lycopersici* (*Forl*) ZUM2407 and one inhibited the growth of *Curtobacterium flaccumfaciens*. Based on the 16S rRNA gene sequence analysis, the isolates were identified as *Streptomyces hydrogenans* KS13AU, *Bacillus megaterium* KSO8AU, *B. subtilis* KS07AU, *B. subtilis* KS11AU, and *B. velezensis* KS04AU. The plant growth-promoting ability of the isolated bacteria as a single-strain and consortium inoculant was tested on spring wheat (*Triticum aestivum*). The isolate inoculants were applied on seeds as vegetative cells. The results showed that both forms of inoculation had a positive effect on spring wheat growth. However, the greatest effect on the spring wheat plant was observed when isolates were inoculated as a consortium. The biometric parameters of the plant, such as dry biomass, shoot length, and root length, increased significantly up to 30% compared to single strain inoculations.

Keywords: antagonistic activity, *Curtobacterium flaccumfaciens, Bacillus velezensis, Streptomyces hydrogenans.*

INTRODUCTION

The cereal crop is an important source of nutrition and has great economic importance in the world (Awika, 2011). Plant pathogens such as *Fusarium oxysporum*, *Fusarium graminearum, Alternaria solani* and *Botrytis cinerea* pose a serious threat to modern agriculture (Wang *et al.*, 2011; Keller *et al.*, 2014). Among these phytopathogens, Fusarium is one of the most ubiquitous pathogens that affect a wide range of agricultural plants, causing severe production losses worldwide that approximate 14.5% to 50% of harvested crops (Nelson *et al.,* 1983; Gilchrist and Dubin, 2002; Oerke, 2006).

Recently, there is great concern on the harmful use of antibiotics in crop production based on the resistance of bacteria to these antibiotics which sooner or later might be banned (Taylor and Reeder, 2020). Bacteria pathogens contribute to yield loss including bacterial wilt disease caused by *Curtobacterium flaccumfaciens* (Chen *et al.*, 2021). *C. flaccumfaciens* is a pathogenic bacterium infecting several cultivated and wild leguminous species such as common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), mung bean (*V. radiata*) and soybean (*Glycine max*), and causing great economic losses (Jeger *et al.,* 2018). Since 2019, *C. flaccumfaciens* has been included in the list of quarantine pathogens in many countries (EPPO, 2019).

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Taking into account the urgency of searching for new alternatives for effective environmentally disease control and to increase yields while producing quality, the use of soil microbial communities and the diversity of microorganisms living in plants under various conditions is one of the promising and effective approaches to solving ecological and economic problems with minimal damage to crop yields (Maksimov *et al.,* 2011). From a biological point of view, the rhizosphere is one of the rich microbial ecosystems and the main source of diversity and stability of the plant microbiome (Raaijmakers and Lugtenberg, 2013; Panke-Buisse *et al.,* 2015). Soils in this zone are often under more complex interactions between plants and microorganisms than in other zones, due to the presence of root exudates and nutrients secreted by the roots (Chaparro *et al.,* 2013). Microorganisms of various taxonomic groups have been found in the rhizospheres of different agricultural crops, among which many are capable of inhibiting pathogen growth, stimulating plant growth, development, and productivity (Chamkhi *et al.,* 2021; Hayat *et al.,* 2010).

Senna occidentalis is a drought resistant plant with significant antibacterial and antifungal properties (Hussain and Deeni, 1991). In many countries, *S. occidentalis* is used for various purposes, such as insecticides, as well as for the treatment of many diseases (Usha *et al.,* 2007). *S. occidentalis* is an abundant plant in nature, growing in dense populations. This plant is generally considered a weed due to its negative impact on the germination and development of surrounding cultivated plants (Da Silva and Vieira, 2019; Munif *et al.,* 2022). However, these plants often have a positive effect on soil microbial diversity and subsequent soil health and quality (Sturz *et al.,* 2001).

This work aimed to isolate microorganisms with high antibiotic activity against plant pathogens from the rhizosphere of *S. occidentalis* weed from west Africa, as well as their plant growth-promoting activity on spring wheat when applied as a single strain inoculant and microbial consortium.

MATERIALS AND METHODS

Sample collection: The soil from rhizosphere of *S. occidentalis* was collected in sterile test tubes and frozen at -20°C.

Microorganism isolation: Before use, the soil was pre-treated with 10% $CaCO_3$ (m/m) and incubated at 37°C for 3 days. Further, one gram of rhizosphere soil was added to a sterile test tube containing 9 mL PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 1.8 mM NaH_2PO_4, pH 7.4) with 5% (v/v) phenol. The mixture was incubated at 180 rpm in a shaker incubator for 30 min at 37°C. After serial dilutions, 0.1 mL of aliquot (10^3 dilution) was plated on Gause's synthetic agar medium amended with nystatin (100 µg/mL) and incubated at 28ºC ±1°C for 7 days for Actinomycetes isolation. Based on their morphology and pigmentation nature, colonies were selected and purified on International Streptomyces Project 3 (ISP-3) agar medium. For screening other bacterial genera such as Bacillus and Pseudomonas, 0.1 mL aliquot (10^4 dilution) was spread on LB agar amended with nystatin (100 µg/mL). Plates were then incubated at 28ºC for five days. The selected bacteria were purified on king's B agar medium.

Antagonistic ability: Antagonistic activity of isolated bacteria was performed using a dual culture assay on PDA medium. For this purpose, the plant pathogen *Fusarium oxysporum* f. sp. *radicis lycopersici (Forl)* stain ZUM2407 was placed at the centre of Petri dishes. The isolated bacteria were inoculated on the same agar plate at a distance of 2.5 cm from the growing pathogen. Plates were further incubated at 27°C for 7 days. Bacterial isolates with antagonistic activity against *Forl* ZUM2407 were selected and reassayed for their ability to inhibit the growth of phytopathogenic bacteria such as *Curtobacterium flaccumfaciens.* For that, 100 µL of an overnight culture of *C. flaccumfaciens* MM16 with an optical density (595 nm) 0.5 were plated on LB agar using a plate spreader, then bacterial isolates were co-inoculated on the same plate as a plug. The plates were incubated at 28°C for 3 days. The formation of a clear zone around the bacterial colonies was considered as antagonistic activity.

PCR identification: For molecular identification using the 16S rDNA gene, the total DNA was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The 16S rDNA gene was amplified from the bacterial genomic DNA using primers 27fm 5'-AGAGTTTGATCMTGGCTCAG-3' and 1522R 5'-AAGGAGGTGATCCAGCCGCA-3' (Lane *et al.,* 1985). The PCR products were separated by electrophoresis in 1.25% agarose gel. The target fragments were extracted and purified from the gel using the Evrogen® Cleanup Mini Kit (Evrogen, Russia) according to the manufacturer's protocol. The sequence analysis of the target fragment was performed by Evrogen® and the chromatograms obtained were analysed using the Clone

Manager 9.51 software package. Nucleotide sequences were analysed by BLAST sequence analysis against those from the NCBI database Nucleotide Collection for homology sequence comparison using the NCBI nucleotide BLAST (Basic Local Alignment Search Tool).

Plant growth-promoting ability: Plant growth-promoting ability of the isolated bacteria as a single and consortium inoculant on wheat was carried out under laboratory conditions, in plastic pots containing quartz sand, previously moistened with a nutrient plant solution. For this purpose, a variety Ulyanovsk 105 (Russia) spring wheat (*Triticum aestivum*) was used. The compatibility of microorganisms was checked in advance by the method of double culture in Petri dishes on king's B agar.

The bacteriological inoculum was prepared from a single strain and a consortium of all isolates grown overnight in LB medium. For this purpose, the cultures were centrifuged, washed three times and diluted with a PBS buffer to an optical density of 0.5 at 595 nm. Wheat seeds were surface sterilized according to Simons *et al.* (1996). The seeds were inoculated in bacterial suspensions for 15 minutes and dried in a laminar flow hood. For the control group, the seeds were inoculated in sterilized distilled water. Fifteen seeds were sown in pots containing a sterile quartz sand amended with plant nutrient solution [(PNS: 1.25 mM Ca $(NO_3)_2$; 1.25 mM KNO_3; 0.50 mM $MgSO_4$; 0.25 mM KH_2PO_4 and trace elements (0.75 mg/l KI; 3.00 mg/l H_3BO_3; 10.0 mg/l; $MnSO_4.H_2O$; 2.0 mg/l $ZnSO_45H_2O$; 0.25 mg/l $Na_2MoO_4.2H_2O$; 0.025 mg/l $CuSO_4.5H_2O$; 0.025 mg/l $CoCl_2.6H_2O$)]. Plants were grown in a climate chamber (MEMMERT AtmoCONTROL, Germany) with a 16/8h day-night light cycle, at 26 °C and 70% of humidity. After three weeks, plant biometric parameters were performed. For the reliability of the experiment, two replicas of each treated group were prepared.

Statistical analysis was performed using the statistical program originLab Pro SR1 127 b9.5.1.195 (originLab Corp. USA). The significant difference between groups was performed using one-way repeated measures ANOVA and Tukey's post hoc test at $p < 0.05$.

RESULTS

Based on the morphology and nature of the pigmentations, in total, 24 bacterial cultures were isolated and purified from the media mentioned above. All preselected strains were evaluated for the antagonistic activity against *Forl* ZUM2407 (Figure 1A, B and C) and *C. flaccumfaciens* **(**Figure 1D**)**. Five out of twenty-four bacterial isolates showed antagonistic activity against *Forl* ZUM2407, and one isolate showed against *C. flaccumfaciens*. Among isolated strains, KS11AU and KS04AU demonstrated a strong inhibition activity. Isolates, which had antagonistic properties, were then selected for further study.

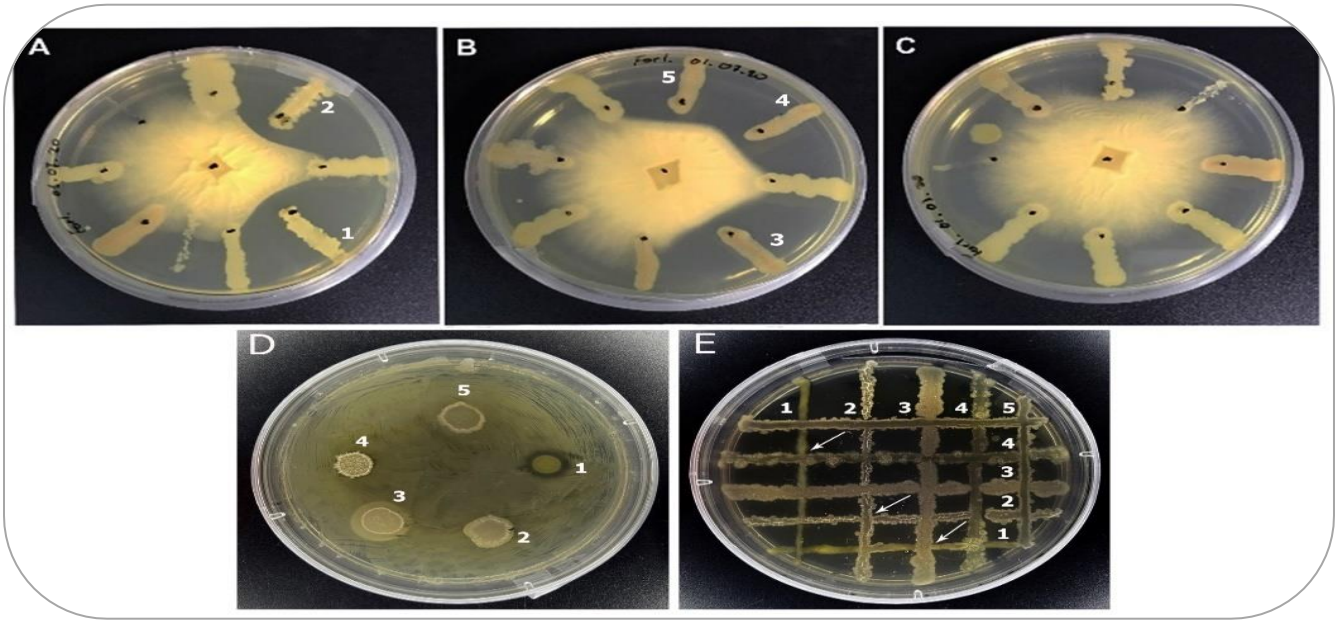


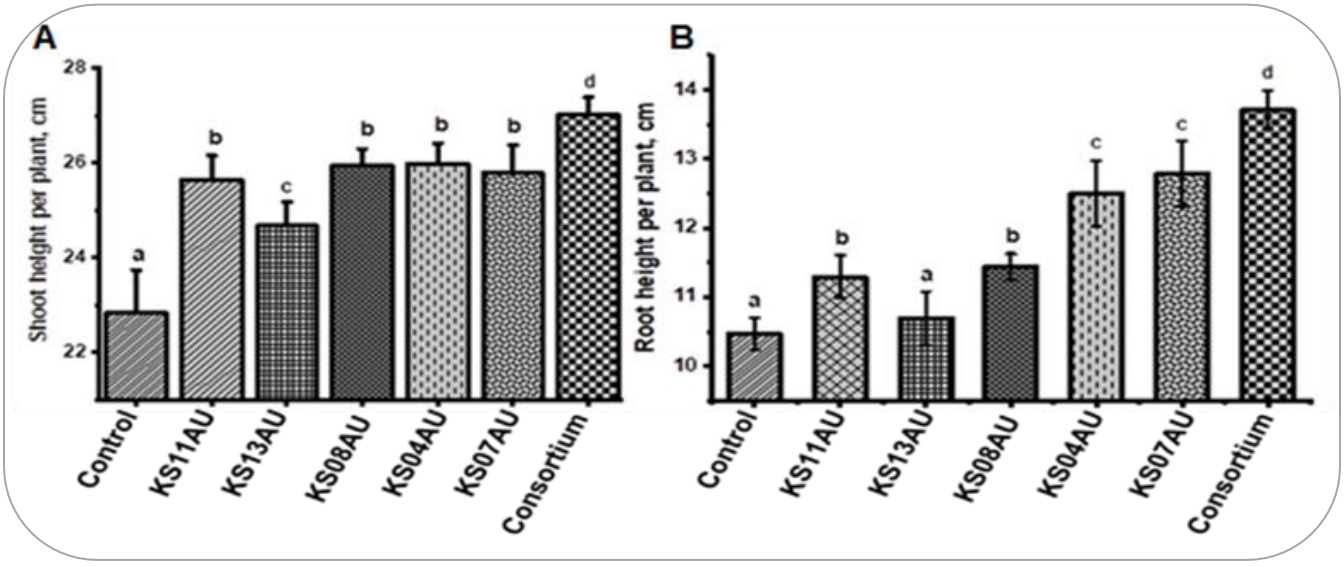
Figure 1. Antagonistic activity of bacteria isolated from the rhizosphere of *S. occidentalis* against *F. oxysporum* f.sp. *radicis lycopersici* ZUM2407 (A, B, and C) and *C. flaccumfaciens* MM16 (D). Compatibility test (E) between isolated bacteria with arrows indicating intersection zones. 1 – isolate KS04AU ; 2 – isolate KS11AU ; 3 – isolate KS13AU ; 4 – isolate KSO8AU ; 5 – isolate KS07AU.

After analyzing the sequenced chromatogram, the comparative analysis of the obtained 16S rDNA partial gene sequences with GenBank nucleotide revealed that four bacterial isolates, belong to the genus *Bacillus*, were identified as *B. subtilis*, *B. subtilis*, *B. megaterum*, and *B. velenzensis* (Table 1). The bacterial isolate KS13AU belonging to the genus *Actinomyces* was identified as *Streptomyces hydrogenans*. The 16S rDNA gene partial sequences were submitted to NCBI GenBank (Table 1).

Table 1. Identification of plant growth-promoting bacteria with antimicrobial activity against *F. oxysporum* f.sp. *radicis lycopersici* ZUM2407 isolated from weed *S. occidentalis* rhizosphere.

Bacterial strain	Identified as	NCBI accession numbers
KS04AU	*B. velezensis*	MW350014.1
KS07AU	*B. subtilis*	MW350012.1
KS08AU	*B. megaterium*	MW349992
KS11AU	*B. subtilis*	MW350010.1
KS13AU	*S. hydrogenans*	MW350038.1

To combine strains as a consortium, a compatibility test was done. The test demonstrated non-antagonistic activity between inoculants, as no bacterial growth suppressant effect was observed during *in vitro* co-culture in their intersection zones (Figure 1E).



The ability of the isolated bacteria to act as plant-growth promoters (single-strain inoculant or microbial consortium) on spring wheat showed that both inoculum preparations can promote the growth and plant development (Figure 2 and 3). When bacterial isolates *B. subtilis* KS11AU, *S. hydrogenans* KS13AU, *B. megaterium* KSO8AU, *B. velezensis,* and *B. subtilis* KS07AU were applied as a single strain inoculant, length of the shoots increased by 10.42%, 10.51%, 11.76%, 11.93%, and 11.07% respectively, as compared to the control, which shoots length was 23.21 cm (Figure 2A).

Figure 2. The effect of seed inoculation with bacterial suspension cultures on the growth of aboveground (A) and underground (B) parts of plants, spring wheat (*Triticum aestivum*). Significant differences between groups (Tukey test, $p < 0.05$) are indicated by different letters. Data are represented as the mean ± SE.

No statistical difference was found among bacterial isolates but was significant in comparison to the control (Figure 2A). The improvement induced by isolated strains was also accentuated by the growth of roots and plant dry mass. As shown in Figure 2B, roots length of plants treated with *B. subtilis* KS11AU, *S. hydrogenans* KS13AU, *B. megaterium* KSO8AU, *B. velezensis* KS04AU, and *B. subtilis* KS07AU were increased by 9.51 %, 10.84 %, 10.86%, 16.77%, and 16.77 %, respectively, as compared to the control, which length value was 10.31 cm. The shoot dry mass increment was by 14.90%, 7.51%, 15.09%, 20.61%, and 21.81% respectively, as compared to the control, in which the value was 0.016 g per plant (Figure 3A). Root dry mass was 6.55%, 4.11 %, 8.36%, 8.35%, and 7.78 % improved, compared to the

control, in which the value was 0.0122 g per plant (Figure 3B). However, the dry mass increments of plants treated with *S. hydrogenans* KS13AU statistically ($p < 0.05$) did not differ from the control.

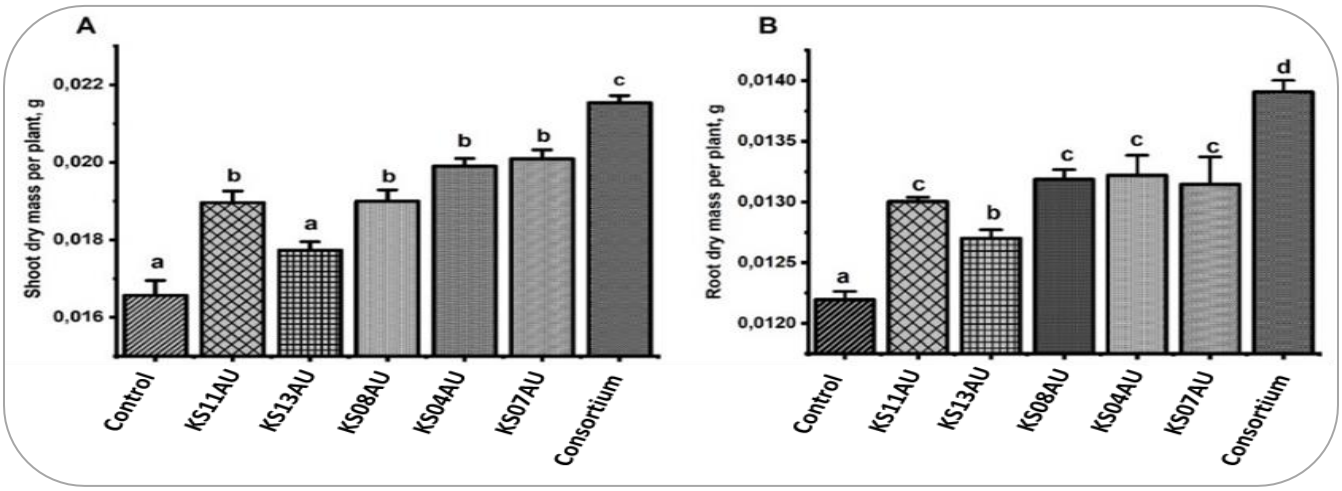


Figure 3. The effect of seed inoculation with bacterial suspension cultures on the accumulation of (A) aboveground and (B) underground parts of the dry mass of the plant. Significant differences between the groups (Tukey test, $p < 0.05$) are indicated by different letters. Data are represented as the mean ± SE.

On the other hand, when bacterial isolates were applied as a consortium, the result revealed a significant ($p < 0.05$) increment of biometric plant parameters as compared to the control and plant treated with a single-strain inoculant (Figure 2 and 3). Shoot and root length plants were respectively, 26.51% and 19.46% increased as compared to the control (Figure 2). The accumulation of dry shoot and root mass of the plants treated with consortium was 28.72% and 14.01% higher than the control (Figure 3).

DISCUSSION

Wheat represents one of the important crops infected by Fusarium spp. causing significant yield losses (Arias *et al.,* 2013). Due to adverse environmental impacts such as soil degradation, resistance to plant diseases caused by excessive and uncontrolled application of pesticides, the use of microorganisms in agriculture as biocontrol, bioremediation is a promising and environmentally friendly method for sustainable agriculture practices, and human safety index (Damalas and Eleftherohorinos, 2011; Gill and Garg ,2014). Therefore, there is a need for novel source of microorganisms with high antimicrobial, plant-growth-promoting, and bioremediation proprieties. Weed as a source of microbial biodiversity of novel plant growth-promoting with high antimicrobial activities for crop improvement has been reported by Sturz *et al*. (2001), Arun *et al*. (2012), and by Phi *et al*. (2021).

In this study, bacteria isolated from weed rhizosphere, *S. occidentalis*, demonstrated a high ability to suppress the growth of phytopathogenic *Forl* ZUM2407, a causal agent of tomato root rot diseases. A similar result was found by Phi *et al*. (2021), in which bacteria isolated from different parts of weed, *Echinochloa colonum*, presented antagonistic abilities against stem end rot pitaya diseases caused by *Alternaria alternata*. Furthermore, analysis of the 16S rDNA sequence revealed that all isolated microorganisms belong to the genera *Bacillus* and *Streptomyces.* Species that are well known as plant growth promoting bacteria and have been reported to be non-phytopathogenic. For example, depending on the type of plant tissue, plant genotype, type of microbial taxon, as well as biotic and abiotic environmental conditions, *B. velezensis*, closely related to *B. subtilis*, can act as a root, leaf, or seed endophyte (Hardoim, *et al.,* 2015; Palazzini *et al.,* 2016; Gao *et al.,* 2017; Cao *et al.,* 2018).

Most bacteria of genera such as *Bacillus* and *Streptomyces* with antagonistic properties against pathogenic plants may act as plant growth-promoting bacteria (Viaene *et al.,* 2016; Shafi *et al.,* 2017; Miljaković *et al.,* 2020). Mechanisms of action, which are more related to their ability to synthesize compounds involved in plant development such as auxin, cytokinins gibberellin, and in facilitating nutrients uptake from soil (Lugtenberg, 2015). Therefore, the ability of the bacterial isolates to act as a plant growth promoter was tested as a single-strain inoculant or microbial

consortium. When applied as a single-strain inoculum, an average increment of biometric plant parameters such as root and shoots heights plant was up to 18%, as compared to the control (Figure 2 and 3). A similar result was previously reported by Arun *et al*. (2012). In their study, plant growth-promoting bacteria isolated from *Cassia occidentalis* showed the potential to positively affect the growth of mung bean (*Vigna radiata)*. Similar results were obtained by Sturz *et al*. (2001). In their study, plant growth-promoting Rhizobacteria isolated from different weed species significantly increase potato (*Solanum tuberosum*) growth and development. The results of the plant growth promoting and antimicrobial effect of *B. subtilis*, *B. megaterium, B. velezensis*, and *S. hydrogenans* obtained in this study are consistent with previously reported research (Kaur and Manhas, 2014; Kaur *et al.,* 2014; Cao *et al.,* 2018), in which the properties of Bacillus species and *S. hydrogenans* as a plant protector and growth stimulator were evaluated.

The formulation of the microbial consortium depends on their mutual compatibility, as well as the ability to maintain their microbial potential (Niu *et al.,* 2020; Santoyo *et al.,* 2021). From this point of view, the selection of microorganisms and the preliminary compatibility testing are fundamental factors for creating effective consortiums that contribute to the protection and development of plants. In this study, during their in vitro co-culture, no antagonistic activity was observed (Figure 1B).

The use of microbial consortia for the restoration of functional and useful microorganisms associated with soil fertility represents a new ecological approach related to rhizosphere engineering (Ahkami *et al.,* 2017; Thomloudi *et al.,*2019). Microbial consortia of several bacterial strains with plant growth-promoting ability increase their inoculation effectiveness (Guetsky *et al.,* 2002; Bradáčová *et al.,* 2019a). Based on the fact that all bacterial isolates were isolated from the same habitat in which they were intended to interact, we hypothesized that the ability to promote plant growth of these isolated bacteria may be more effective when applied as a consortium. The results demonstrated that wheat seed treatment with a consortium of Bacillus plant growth promoting strains and *Streptomyces hydrogenans* significantly ($p < 0.05$) improved plant performance compared to single-strain inoculation (Figure 2 and 3). The obtained results are consistent with those previous studies (Bradáčová *et al.,* 2019b), in which the treatment with a microbial consortium of compatible microorganisms, isolated from the same ecological niche better promoted growth and plant development.

Despite the negative impact of weeds in agriculture, these plants can serve as sources of biodiversity of rare or beneficial microorganisms with high antimicrobial activity, which can positively affect microbial diversity and the sustainability of agricultural soils. In this study, an attempt was made to isolate from *S. occidentalis* rhizosphere microorganisms with inhibitory activity (antagonistic activity) against a most important soil-borne pathogen that causes severe yield loss, *Fusarium oxysporum*. The results obtained in this study indicate that isolated bacteria can potentially be used as biocontrol and growth-stimulating agents for agriculture and ecological sustainability. However, field tests are necessary to determine the effectiveness of these isolates in the natural environment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Roderic G. C. Diabankana	:	Conceived idea; Data analysis and results interpretation
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Daniel M. Afordoanyi	:	Designed experiments and conducted research