BACTERIAL ENDOPHYTES AS POTENTIAL BIOCONTROL AGENTS AGAINST SWEET POTATO PATHOGENS

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Abstract. Bacterial endophytes are plant-associated microorganisms colonizing internal tissues without causing any symptoms to their hosts. Their intimate relation with the plant can trigger several beneficial traits for their host, such as growth improvement and tolerance to some biotic and abiotic factors. Therefore, endophytic bacteria can be used as agro-inoculants in various crops suitable for organic production, such as sweet potato. The aim of this study is to reveal the potential use of bacterial endophytes as biocontrol agents against sweet potato pathogens. Selected endophytic bacterial strains revealed antifungal activity against important tuber, root and stem pathogens, belonging to *Fusarium*, *Botrytis, Curvularia* and *Trichothecium* genera.

Keywords: biocontrol, endophyte bacteria, sweet potato, fungal phytopathogens, plant diseases.

Rezumat. Endofiții bacterieni cu potențial de utilizare ca agenți de biocontrol împotriva patogenilor cartofului dulce. Bacteriile endofite sunt microorganisme asociate plantelor, capabile să colonizeze interiorul țesuturilor vegetale fără a produce simptome plantelor gazdă. Prin relația foarte strânsă pe care o au cu plantele pe care le colonizează, bacteriile endofite aduc plantei mai multe beneficii, precum stimularea creșterii și toleranță la unii factori biotici și abiotici. Din aceste considerente, unele bacterii de control biologic pot fi utilizate ca agro-inoculanți pentru acele culturi care se pretează la sistemul ecologic de producție, așa cum este cartoful dulce. Scopul acestei lucrări este de a evidenția potențialul bacteriilor endofite de a fi utilizate ca agenți de combatere biologică împotriva unor agenți dăunători cartofului dulce. Tulpinile selecționate de bacterii endofite au evidențiat activitate antifungică împotriva unor fitopatogeni importanți ce atacă tuberculii, rădăcinile și tulpinile plantelor de cartof dulce, cum ar fi patogeni din genurile *Fusarium*, *Botrytis, Curvularia* și *Trichothecium*.

Cuvinte cheie: combatere biologică, bacterii endofite, cartof dulce, fungi fitopatogeni, boli ale plantelor.

INTRODUCTION

Environmentally friendly agricultural practices are highly appreciated nowadays. The use of microbial inoculants can bring many benefits for agriculture and environmental protection. Various types of microorganisms can be applied to improve soil quality and fertility (MAHANTY et al., 2016), most of which ensure plant biostimulation (BHARDWAJ et al., 2014), while others are involved in bioconversion and bioremediation (WEYENS et al., 2009; MESA et al., 2017). Another important class of beneficial microorganisms are those involved in plant protection (KÖHL et al., 2019). Such microbial inoculants can reduce the risk of phytopathogenic attack (KHALAF & RAIZADA, 2018), prevent spoilage loses (GÁLVEZ et al., 2010), and protect harvests against contamination with microbial toxins or health detrimental germs (ABDELHAMID & EL-DOUGDOUG, 2020; ZADRAVEC et al., 2022).

Among biocontrol bacteria, those associated to plants are the most studied. Bacterial colonizers found on the surface of the roots are called rhizobacteria (BACKER et al., 2018). They are the first protection barrier against phytopathogenic attack, preventing plants from infections and contamination. Another category of plant-beneficial bacteria are endophytes, which are adapted to inhabit the inner plant tissues. Their way of living inside plants is not harmful to the host (MENGISTU, 2020). On the contrary, endophytism creates an intimate relationship between the associates, stimulating the endophyte to support the wellbeing of the plant, by helping the host plant growth under normal and stress conditions (LÓPEZ et al., 2018).

Using selected endophytes as agro-inoculants is a promising approach in sustainable agriculture (VYAS, 2018). Studies have showed that selected endophytes, applied as microbial inoculants, can also improve germination and growth parameters when applied to plants, other than their initial hosts (CUEVA-YESQUÉN et al., 2021). This is mainly due to the wide variety of beneficial traits revealed by such plant beneficial microorganisms (RYAN et al., 2008; TIAN et al., 2017; AFZAL et al., 2019).

Significant results were also obtained when using bacterial endophytes to suppress plant pathogens. A special attention was given to the biocontrol potential of endophytes against vascular wilt diseases, mostly as they are colonizing the same ecological niche (ELJOUNAIDI et al., 2016). Promising results were also observed when using endophytes against other types of fungal and oomycete pathogens (KHALAF & RAIZADA, 2018).

The present study is focused on the antifungal activity of three endophytic bacteria against various sweet potato pathogens causing crown rot, plant wilt, leaf spots, and tuber rot. The aim is to reveal the potential use of bacterial endophytes as biocontrol agents against sweet potato pathogens.

MATERIALS AND METHODS

Sweet potato pathogens. All tested pathogens, except one, were isolated from Romanian produced sweet potato, during the growing season of 2021. *Botrytis cinerea* is the only exception. This pathogen was previously

isolated from naturally contaminated tubers of KSP1 cultivar, formerly named Pumpkin cultivar from South Korea, expressing symptoms of grey mold (BOIU-SICUIA et al., 2016).

For pathogen isolation and identification, infected plant parts and tubers of sweet potato were collected from the experimental fields of SCDCPN Dăbuleni. Infected plant materials were transferred into RDIPP laboratory, where they were maintained in humid chambers to facilitate sporulation of the plant pathogenic microorganisms. The direct isolation method was then applied, and fungal spores were collected and transferred on Potato-Dextrose-Agar (PDA) supplemented with antibiotics. Repeated plating was than performed on PDA in order to obtain pure cultures. Plant pathogens were maintained on PDA in refreshed, pure cultures.

Bacteria isolation source. The seeding material produced on sandy soils at SCDCPN Dăbuleni, Dolj county was washed and surface-disinfected in two steps. First with 70% ethanol, and secondly with 5% sodium hypochlorite. After intensive rinsing with sterile distilled water supplemented with Tween 80 (SILVANI et al., 2008), the plant material was axenically grinded and infused for 20 minutes in sterile phosphate saline buffer. Volumes of 100 µl infusion were inoculated, by the spreading technique, on Plate Count Agar (PCA medium containing: 5 g/L tryptone, 2.5 g/L yeast extract, 1 g/L glucose, 18 g/L agar, pH 7.2 ± 0.2 at 25°C). The bacteria were then purified and maintained on Luria Bertani agar. Glycerol stocks were also prepared and stored at -20°C.

To confirm endophyte harvesting, the rinsing water was plated on PCA to reveal the lack of non-endophytic microbial contaminants on the seed surface.

Microbial identification. Isolated fungi were identified based on their microscopic features. Fungal characteristics were checked out before their isolation from sweet potato, as well as after *in vitro* purification (CLARK & MOYER, 1988). The phytopathogens were first observed under the magnifier, and then under the microscope, in freshly prepared microscopic slides.

Bacteria were identified from primary culture on non-selective agar, based on colonial appearance and, then, based on microscopic appearance in Gram stained slides (LOGAN & DE VOS, 2009).

Microbial antagonism. The double culture technique was performed to evaluate the antifungal activity of bacterial endophytes against sweet potato pathogenic fungi. Tests were performed *in vitro*, on PDA medium. Bacterial biomass was inoculated at 2.5 cm distance from the fungal inoculum, placed in the center of the plate. Tested fungi were calibrated as mycelial plugs of 6 mm diameter. Control plates were also prepared, for each of the tested fungi. Tests were performed in triplicate and incubated at 26°C.

Data collection and statistical analysis

The biocontrol potential of the isolated bacteria was evaluated within *in vitro* tests. Fungal growth in the test plates were periodically analyzed and compared to the control. Data were collected by biometric measurements. Results were subjected to One Way ANOVA to see if there were any significant differences between the bacterial inoculated variants and the uninoculated control. Antifungal activity was evaluated after 10 days of incubation, according to DINU et al. (2012).

RESULTS AND DISCUSSIONS

Phytopathogenic fungi associated with sweet potato. The visual and stereomicroscope analysis suggested fungal infections of sweet potato (Fig. 1).

Figure 1. Fungal infection of sweet potato
a. rotten tubers and stems, **b**. fungal sporulation on plant stems.

To confirm fungal infections, microscopic slides were either directly prepared from the contaminated plant tissue or the infected stems were first maintained in humid chamber and only afterwards were the microscopic slides prepared. Together with the slides, fungal isolations were performed and colony morphology was also analysed.

From the contaminated tubers and stems of different sweet potato breeding lines and variety, three strains of fusaria were isolated. These *Fusarium* sp. isolates were named DK19/1, DK 19/4, and KSP1 based on the sweet potato cultivar code. The identification was made based on microscopic characteristics. The pathogens revealed septate hyaline hyphae with acute angle branching. The macroconidia were mostly seen when pathogen sporulated on vegetal material. These macroconidia were banana shaped, with 3 to 5 septs (Fig. 2a). On agar slants, the micro- and mesoconidia were more abundant (Fig. 2b). Their shape was either oval, elliptical or kidney like. These conidia were formed on short monophyalides (Fig. 3c). Older cultures were able to form chlamydospores (Fig. 2d), mostly single or in pairs, either terminal or intercalary in the mycelia.

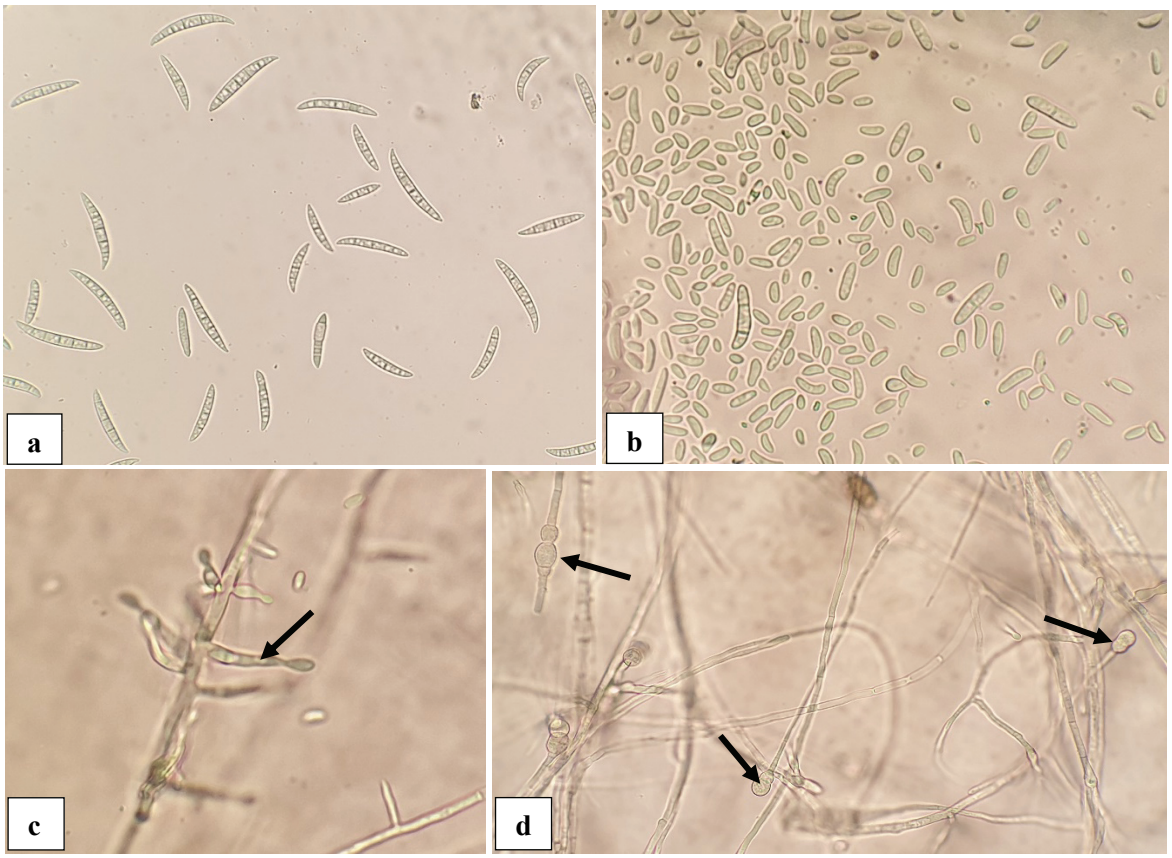

Figure 2. *Fusarium* sp. microscopic characteristics
a. Macroconidia, **b**. Micro- and mesoconidia, **c**. monophyalides (arrow), **d**. chlamydospores (arrows).

From the contaminated stems of sweet potato (Fig. 3a), one strain of *Trichothecium roseum* was isolated. On PDA colony were flat and powdery, from white to light salmon-orange in color with age (Fig. 3b). The conidiophores were unbranched, bearing basipetal zig-zag arranged conidia with retrogressive development. Conidia are two-celled, with obliquely pedunculated basal cell (Fig. 3c).

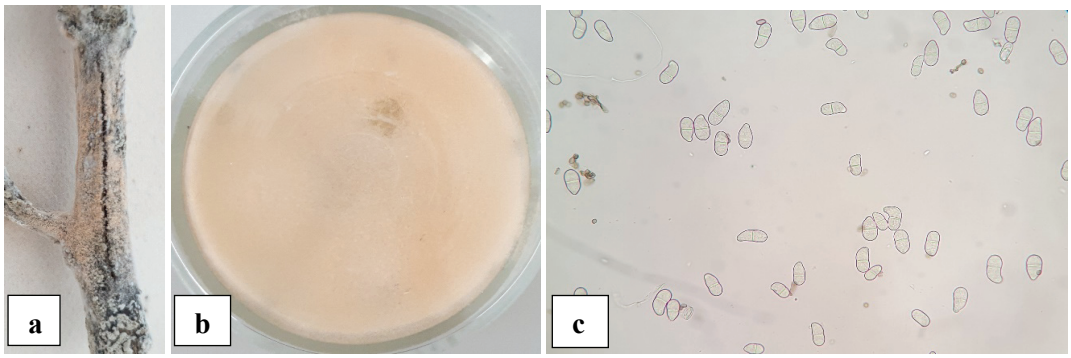

Figure 3. *Trichothecium roseum* characteristics **a**. Contaminated stems of sweet potato, **b**. upper face culture on PDA, **c**. conidia.

One strain of *Curvularia* sp. was isolated from contaminated leaves of sweet potato. On PDA, the colonies were fluffy-black. The mycelia revealed brown (Fig. 4a), septate hyphae, producing geniculate conidiophores (Fig. 4b). Conidia were slightly curved, transversely septate, with an expanded middle cell which gives the curvature of the conidium.

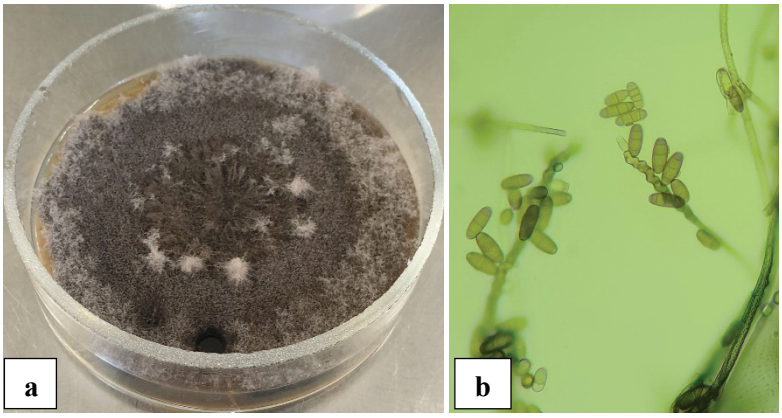

Figure 4. *Curvularia* sp. characteristics
a. Aspect of the upper face culture on PDA, **b**. geniculate conidiophores with conidia.

All these sweet potato phytopathogens, as well as the gray mold caused by *Botrytis cinerea*, are described in the Compendium of Sweet Potato Diseases (CLARK & MOYER, 1988), the only exception being *Trichothecium roseum.*

Isolated bacteria. Three strains of endophytic bacteria were isolated, named Bs1, Bs2 and Bs3, depending on the seed source. Microbial growth obtained on agar slants revealed *Bacillus* type colonies (LOGAN & DE VOS, 2009). On the Nutrient Agar medium supplemented with soluble starch, the colonies were opaque, with a rough aspect, and fuzzy white or slightly sandy in color, having irregular edges (Fig. 5).

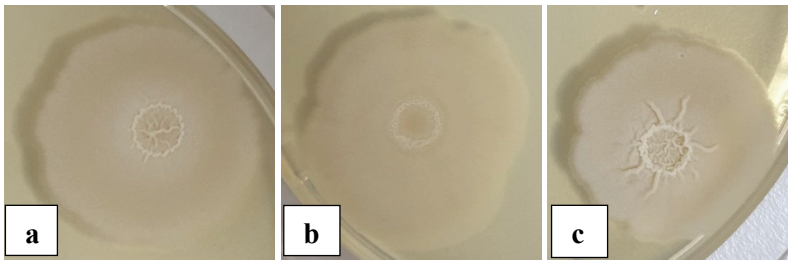

Figure 5. Endophyte bacteria obtained from various seeds.
Aspect of the upper face colony of Bs1 (**a**), Bs2 (**b**) and Bs3 strains (**c**).

Analysed by Gram staining, all isolates revealed rod shaped cells, single or arranged in pairs or chains. These bacteria were considered sporulated as all three strains survived after 1h incubation at 65°C.

Antifungal activity

The antifungal activity of the isolated endophytes was evaluated against six pathogens of sweet potato. A clear inhibition zone was measured after one week of incubation at 26°C. This biometric parameter reveals which beneficial strains are able to release, into the substrate, antimicrobial compounds in such concentrations able to inhibit the fungal growth. The diffusion zone containing inhibitory compounds was measured for each microbial interaction (Table 1).

Table 1**.** Antifungal activity of endophytic bacteria against sweet potato phytopathogens (after one week of co-cultivation at 26°C).

Bacterial endophytes / **Fungal phytopathogens**	**Bs1**	**Bs2**	**Bs3**
	Clear inhibition zone (cm) -arithmetic mean values-		
Fusarium sp. *DK19/1*	*0.6*	*0.4*	*0.5*
Fusarium sp. *DK19/4*	*0.3*	*0.4*	*0.3*
Fusarium sp. KSP1	*1.0*	*0.8*	*0.9*
Trichothecium roseum	*0.1*	*0.0*	*0.2*
Botrytis cinerea	*0.4*	*0.3*	*0.45*
Curvularia sp.	*0.9*	*0.7*	*0.8*

Although the clear inhibition zone is relevant for detecting the biocontrol potential of the tested bacteria based on their antifungal diffusible compounds, there are other mechanisms of actions also involved in microbial antagonism. Such mechanisms are the direct inhibition, competition, or hyperparasitism. All these are reducing the growth rate of the pathogens. Therefore, fungal growth inhibition in the presence of bacterial endophytes was also evaluated. This

parameter was determined based on the biometric measurements. Statistically significant differences ($p < 0.05$) were seen when the bacterial inoculated plates were compared with the uninoculated control (Table 2).

Table 2. One-way Anova to reveal statistically significant differences ($p<0.05$) on fungal growth within antimicrobial tests.

Biocontrol test against sweet potato pathogens	**Anova: Single Factor**						
	Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Fusarium sp. DK19/1	Between Groups	0.548	3	0.183	31.286	5.91E-06	3.490
	Within Groups	0.070	12	0.006			
	Total	0.618	15				
Fusarium sp. DK19/4	Between Groups	11.240	3	3.747	562.000	3.64E-13	3.490
	Within Groups	0.079	12	0.007			
	Total	11.320	15				
Fusarium sp. KSP1	Between Groups	2.673	3	0.891	237.556	6.00E-11	3.490
	Within Groups	0.045	12	0.004			
	Total	2.718	15				
Trichothecium roseum	Between Groups	9.992	3	3.331	940.412	1.69E-14	3.490
	Within Groups	0.043	12	0.004			
	Total	10.034	15				
Botrytis cinerea	Between Groups	11.337	3	3.778	671.815	1.26E-13	3.490
	Within Groups	0.068	12	0.006			
	Total	11.404	15				
Curvularia sp.	Between Groups	4.453	3	1.484	209.529	1.26E-10	3.490
	Within Groups	0.085	12	0.007			
	Total	4.538	15				

As the F values are greater than the F critical values, it can be said that there is a statistically different finding when comparing the biocontrol variants with the untreated control.

Antifungal efficacy was calculated based of the biometric measurements that compute fungal development in the presence of each tested strain, with the fungal growth in the control plates. The resulted fungal inhibition efficacy is revealed in Figure 6.

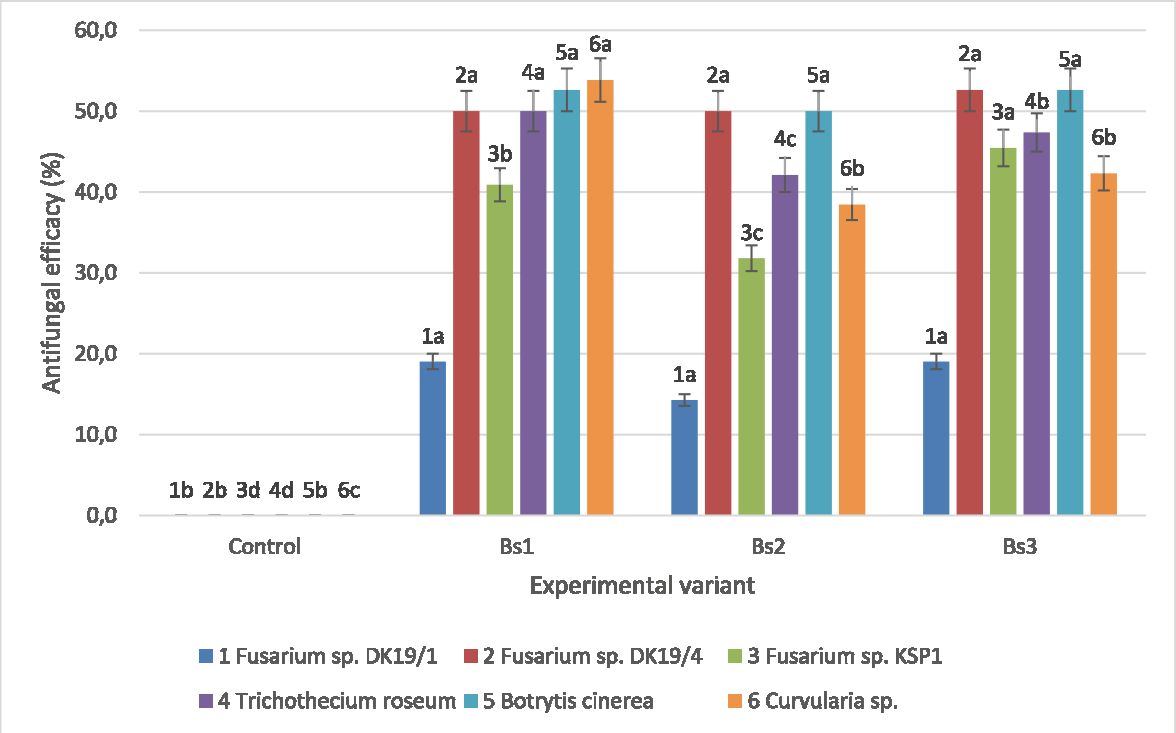

Figure 6. Fungal inhibition efficacy.
A number was affiliated to each antagonism test performed against a certain sweet potato pathogen. Different letters of the same number indicate a significant difference between the experimental variants regarding the fungal inhibitory activity.

The tested endophytic strains were able to alter the pathogens' growth and act as potential biocontrol agents. The best results for the *in vitro* control of sweet potato fungal pathogens were obtained with Bs1 endophytic strain, followed by Bs3, with no significant differences.

A reduced inhibition potential was seen against the *Fusarium* sp. isolate collected from DK 19/1 biologic line of sweet potato. This may be due to the pathogen virulence or, rather, to its tolerance to the mechanism of action expressed by the tested endophytes.

CONCLUSIONS

Various types of fungal pathogens were identified on sweet potato, producing tuber, root and stem infections. For these contaminations, pathogens belonging to *Botrytis, Curvularia, Fusarium* and *Trichothecium* genera were identified. *Bacillus* endophytic strains, Bs1, Bs2 and Bs3 were also isolated in this study. Among these strains Bs1 and Bs3 revealed better antagonistic effect against sweet potato fungal pathogens. However, one strain of *Fusarium* sp. was refractory to biological control.

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