CURRENT STATUS AND FUTURE PERSPECTIVES IN ROMANIA ON BIOLOGICAL CONTROL OF SWEET POTATO FUNGAL PATHOGEN

BOIU-SICUIA Oana-Alina, CONSTANTINESCU Florica, DIACONU Aurelia, DRĂGHICI Reta

Abstract. Sweet potato (*Ipomoea batatas* (L) Lam.) is a less grown vegetable in Romania, mostly due to its growth requirements. This crop demands for warm weather and well-drained soils for its best growth. In Romania, such growth conditions are found in the south area of the country, especially on the sandy soils of the Dolj County. At the Research-Development Station for Field Crops on Sandy Soils (RDSFCSS) – Dăbuleni, the sweet potato was successfully acclimatized and established in the crop rotation, mostly due to the pedo-climatic conditions of the area. These vegetables increased crop diversification and ensured a good productivity under the current climate change conditions. In countries were sweet potato is a traditional vegetable, a wide range of plant pathogens is mentioned for this crop. Until now, no major phytosanitary problems have been encountered in Romania, allowing this culture to be successfully introduced into the organic production system. However, some fungal pathogens, like *Alternaria* spp., *Fusarium* spp., *Botrytis cinerea, Pythium* spp., *Penicillum* spp. and *Rhizopus stolonifer*, were previously isolated from sweet potato plants and tubers cultivated in SCDCPN - Dăbuleni, against which several bacterial antagonists were previously tested. Based on these results, the aim of present study is to analyze the mechanisms by which different biocontrol strains suppress sweet potato pathogens in order to establish the best measures of biological control. The studies were performed *in vitro* both against pathogenic species encountered for this vegetable in Romania, and against pathogens that are present in our country without infecting sweet potato (*Macrophomina phaseolina, Rhizoctonia solani*), although they induce economic losses in other country conditions. Fungal disease suppression was found to be related with microbial competitors of plant beneficial bacteria, able to produce lytic enzymes and antibiotic compounds. The studied biocontrol bacterial strains were able to induce, *in vitro*, mycelial growth inhibition, fungal cells lysis and other alterations of the hyphae.

Keywords: *Ipomoea batatas*, biological control, microbial interactions.

Rezumat. Stadiul actual şi perspective privind controlul biologic al cartofului dulce cultivat în România. Cartoful dulce (*Ipomoea batatas* (L) Lam.) este o legumă mai puţin cultivată în România, în special datorită cerinţelor de creştere pe care le are. Această specie este iubitoare de căldură şi necesită soluri bine drenate. În România, astfel de condiţii de cultivare sunt întâlnite în zona de sud a ţării, în special pe solurile nisipoase din judeţul Dolj. La Staţiunea de Cercetare - Dezvoltare pentru Cultura Plantelor pe Nisipuri (RDSFCSS / SCDCPN) – Dăbuleni, cartoful dulce a fost aclimatizat şi introdus cu succes în asolament, în special datorită condiţiilor pedo-climatice din zonă. Ca urmare, această legumă a permis diversificarea spectrului de culturi, cu o bună productivitate în condiţiile actuale ale schimbărilor climatice. În ţările cu tradiţie pentru această cultură, este menţionat un spectru larg de fungi fitopatogeni. Cu toate acestea, în România nu au fost întâmpinate probleme fitosanitare grave. Acest lucru ar permite introducerea cu succes a acestei culturi în sistemul de producţie ecologică. Cu toate acestea, o serie de fungi fitopatogeni, precum: *Alternaria* spp., *Fusarium* spp., *Botrytis cinerea, Pythium* spp., *Penicillum* spp. şi *Rhizopus stolonifer*, au fost izolaţi de la plante sau de pe tuberculi de cartof dulce cultivaţi la SCDCPN – Dăbuleni, împotriva cărora au fost testate diferite izolate bacteriene autohtone. Din aceste considerente, scopul prezentei lucrări este de a analiza mecanismele prin care diferite tulpini de biocontrol acţionează asupra unor potenţiali patogeni ai cartofului dulce, în ideea de a stabili cele mai eficiente măsuri de combatere biologică. Astfel au fost realizate studii *in vitro* faţă de diferite specii microbiene fitopatogene. Spectrul de boli analizat, a cuprins atât specii patogene întâlnite în ţara noastră la această cultură, cât şi specii de patogeni care sunt prezenţi în România (*Macrophomina phaseolina, Rhizoctonia solani*) fără a fi întâlniţi la cultura de cartof dulce, dar care în ţări cu climat diferit produc pagube economice. Reducerea infecţiilor cu fungi fitopatogeni a fost corelată competiţiei microbiene cu bacterii benefice plantelor, capabile să producă enzime litice şi compuşi cu activitate antimicrobiană. Tulpinile bacteriene studiate au prezentat activitate de control biologic. *In vitro*, acestea au fost capabile să inhibe creşterile miceliene, să inducă liză celulară şi să modifice morfologia micelială a ciupercilor fitopatogene.

Cuvinte cheie: *Ipomoea batatas*, combatere biologică, interacţiuni microbiene.

INTRODUCTION

Sweet potato (*Ipomoea batatas* (L) Lam.) is among world's most important crops. Due to its productivity it is considered the seventh most important food crop worldwide (LIU, 2017). For its best growth, this crop demands for warm weather, with optimum temperatures between 21 and 29°C, and well-drained soils, such as the sandy loam (***. DAFF, 2011). In Romania, similar growth conditions are found in the south area of the country, especially on the sandy soils of Dolj County. At the RDSFCSS – Dăbuleni, the sweet potato was successfully acclimatized and established in the crop rotation, mostly due to the pedo-climatic conditions of the area. These vegetable increased crop diversification and ensured a good productivity (up to 53.3 t/ha in KSP1 cultivar) under the current climate change conditions (DINU & SOARE, 2015).

In countries where sweet potato is a traditional crop, a wide range of plant and tuber fungal pathogens is mentioned for this vegetable. The frequently mentioned plant diseases (AMES et al., 1997; CLARK et al., 2015; EKMAN & LOVATT, 2015) are listed in table 1.

Table 1. Worldwide plant diseases of sweet potato.

Plant disease	Plant pathogen
Alternaria leaf spot, or leaf, petiole and stem blight	*Alternaria* species (mainly *A.alternata, A.bataticola, and A.tenuissima)*
black rot	*Ceratocystis fimbriata*
Fusarium wilt	*F.oxysporum* f.sp. *batatas*
violet root rot	*Helicobasidium mompa*
leaf and stem scab	*Sphaceloma batatas* (syn. *Elsinoe batatas*)
sclerotial blight	*Sclerotium rolfsii*
Rhizoctonia stem canker	*Rhizoctonia solani*
Minor fungal pathogens	
chlorotic leaf distortions	*Fusarium lateritium*
leaves spots	*Phomopsis ipomoea-batatas* (syn. *Phyllosticta batatas), Cercospora* sp., *Septoria* sp., *Ascochyta* sp., *Curvularia* sp., *Colletotrichum* sp., and *Pestalotia batatae*
Storage fungal diseases	
foot rot	*Plenodomus destruens*
java black rot	*Lasiodiplodia theobromae* (syn. *Diplodia gossypina*)
charcoal rot	*Macrophomina phaseolina* (syn. *Sclerotium bataticola*)
grey mold rot	*Botrytis cinerea*
soft rot	*Rhizopus stolonifer* and *Mucor* sp.
blue mold	*Penicillium expansum* and other *Penicillium* species
dry rot	*Diaporthe phaseolorum* (syn. *Phomopsis phaseoli*)
Fusarium root rot	*F.solani* and *F.javanicum*

In the past few years, several pathogenic infections extended their areal of infections along the sweet potato growth regions. LEE et al. (2016) mentioned the first report of dry rot caused by *Diaporthe batatas* (formerly *D. phaseolorum*) in Korea, at 'Juwhangmi' cultivar of sweet potato. PAUL et al. (2017) mentioned the first report of *Rhizopus microsporus* causing Rhizopus soft rot of sweet potato, also in Korea. The same research group also mentioned the first report in South Korea, of Fusarium root rot caused by *F.solani* in sweet potato, during storage of 2016 and 2017 (YANG et al., 2018). Such notes are of great importance for Romania, since at the RDSFCSS - Dăbuleni, which is the biggest center for growing sweet potato in our country, almost all sweet potato cultivars are Korean varieties. This vegetal material exchange between Romania and South Korea is based on the collaboration protocol between the two countries.

Although, worldwide, the spectrum of sweet potato pathogens is relatively large, in our country only a few pathogens were noticed in the field conditions of RDSFCSS - Dăbuleni. No management problems or economical important losses were registered before harvest in the research and production plots of sweet potato in that region. However, it is expected that, due to the climatic changing conditions and the exchange of biological material, several pathogens that currently do not attack sweet potato, might find appropriate conditions to infect. It is also believed that when growers will extend this vegetable's culture, phytopathogenic attacks will be increased, and species of pathogens that are present in our country at different other crops will find appropriate conditions to infect also sweet potato. The plant diseases and tuber storage rot of which we consider to extend their spectrum of infection are *Pectobacterium carotovorum*, *Fusarium solani*, *Macrophomina phaseolina*, and *Rhizoctonia solani*.

Sweet potato pathogens, isolated during 2015 to 2017 were *Alternaria* spp., *Fusarium* spp., *Botrytis cinerea, Pythium* spp., *Penicillum* spp. and *Rhizopus stolonifer*, most of them from unappropriated storage conditions. Against these pathogens, we previously tested 52 bacterial antagonists (BOIU-SICUIA et al., 2016; 2017b). Based on these results, the aim of present study is to analyze the mechanisms by which different biological control strains suppress sweet potato pathogens in order to establish the best measures of biological diseases control. These studies were performed *in vitro* against pathogenic species encountered for this vegetable in Romania, and against pathogens that are present in our country but did not infected sweet potato until now (*Macrophomina phaseolina, Rhizoctonia solani*), although they induce economic losses in other country conditions.

MATERIAL AND METHODS

Plant pathogens. Several phytopathogenic fungi (Table 2) were used in this study in order to examine the direct interaction mechanisms involved in microbial biocontrol. All plant pathogenic fungi were grown and maintained on potato-dextrose-agar during the study.

Beneficial bacteria. The bacterial strains used in this study were: *Bacillus amyloliquefaciens* OS17, *B. endophyticus* 1T2, *B. atrophaeus / subtilis* 6T4, *B.subtilis* ssp. *subtilis* Dj3*, B. subtilis*/ *mojavensis* Dj6, and *Pseudomonas chlororaphis* Sal.c2. These strains are Romanian native, and were previously isolated and characterized at the RDIPP as potential biocontrol agents (DINU et al., 2012; SICUIA, 2013; BOIU-SICUIA et al., 2017a; b). Routinely, these bacteria were grown on Luria Bertani (LB) agar, at 28°C. However, for longer preservation, they were stored at -80°C, in LB broth with 30% glycerol.

Table 2. Filamentous fungal species.

Phytopathogenic fungi	Provenience
Alternaria sp.	USAMV Bucharest, Faculty of Biotechnology collection
Botrytis cinerea	Isolated from harvested sweet potato, of local production (RDIPP - Bucharest collection)
Fusarium oxysporum	RDIPP - Bucharest collection
Fusarium solani	RDIPP - Bucharest collection
Macrophomina phaseolina (syn. *Sclerotium bataticola*)	RDIPP - Bucharest collection
Rhizoctonia solani DSM 63002	DSMZ Collection, Germany

Study on microbial interactions. Direct interaction among the biocontrol and plant pathogenic microorganisms was analysed by optical microscopy in dual cultures, using MC1 microscope. Both microorganisms, the phytopathogenic fungi and the biocontrol bacteria, were inoculated simultaneously, in the same plates, at 2cm distance from each other. The fungal inoculum consisted of calibrated plugs (6mm in diameter) of 7 to 10 days old cultures. The beneficial strains were inoculated in spots, using two days old bacterial biomass. Microbial co-cultivation was performed on PDA medium. Plates were incubated at 28°C and analysed after 3 to 14 days.

RESULTS AND DISCUSSION

In order to understand the effect of bacterial inoculants during the biocontrol process, we studied the fungal growth and its morphology in the presence of beneficial bacteria. Fungal modification obtained in pathogenic growth due biocontrol treatment, was analysed by comparing fungal growth from the untreated control plates to the one developed in the presence of some plant beneficial bacteria. Some correlations were possible between fungal modifications and the biocontrol traits of the beneficial bacteria.

Alternaria sp. growth was inhibited by all tested bacterial strains. The pathogen increased its sporulation at 2÷3 millimetres from the colony edge exposed to the biocontrol bacterial (Fig. 1a). In the mentioned area, the sporulation process was rushed compared with the rest of the colony. In only three days of incubation, an intense brown line, correlated with conidia formation (Fig. 1b) could be seen near the edge of the colony at the interaction zone with any biocontrol bacteria tested.

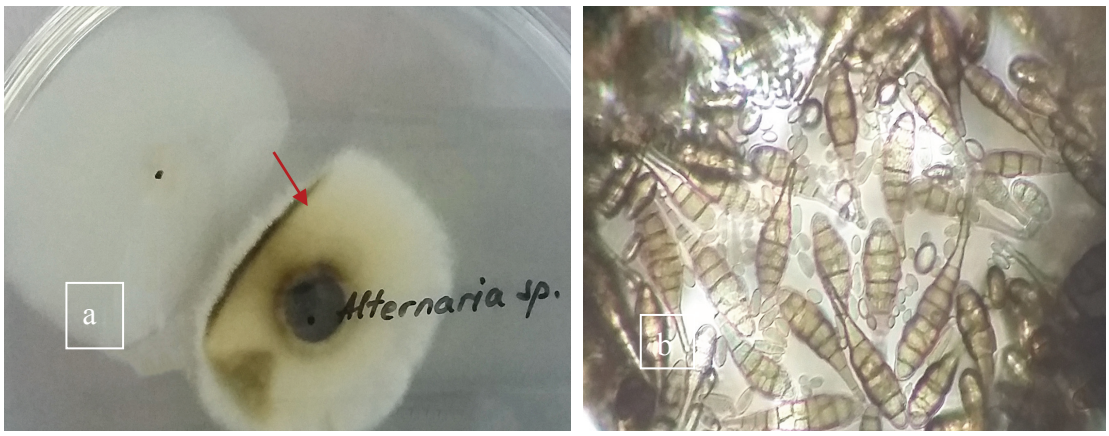

Figure 1**.** Co-cultivation of *Alternaria* sp. and biocontrol bacteria. **a.** Double culture technique of *Bacillus* sp. and *Alternaria* sp. (the arrow indicates the line with abundant conidia formation) **b.** *Alternaria* sp. conidia.

In the interaction zone with *Pseudomonas chlororaphis* Sal.c2, *Alternaria* sp. presented irregular cells along the mycelial growth (Fig. 2a). Although the tested *Bacillus* spp. strains showed *Alternaria* sp. growth inhibition (Fig. 2b), there were no clear mycelial modification at the microscopic level, only fungal colonisation of the hyphae by the bacterial strains, which express *in vitro* swimming and swarming motility (SICUIA, 2013).

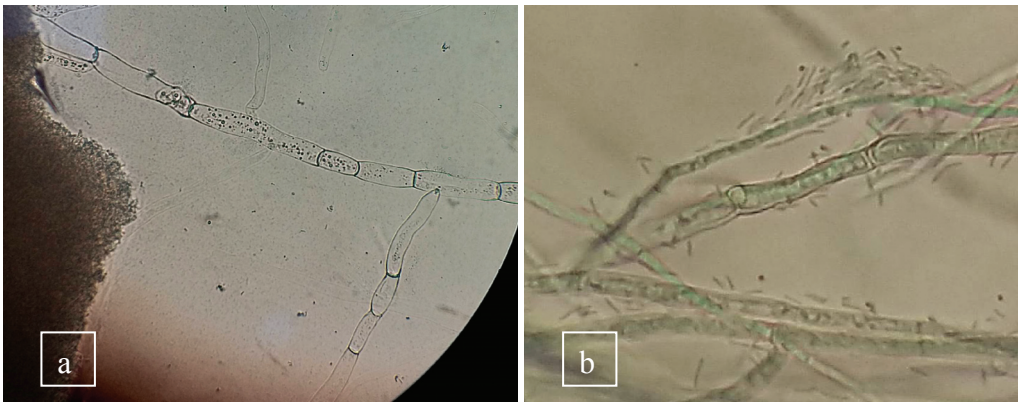

Figure 2. Microbial interaction between *Alternaria* sp. and biocontrol bacteria.
a. Irregular cells in *Alternaria* sp. growth near *Pseudomonas chlororaphis* Sal.c2 strain;
b. Hyphae colonized by *Bacillus amyloliquefaciens* OS17 bacterial cells.

In *Botrytis cinerea* interactions with the biocontrol bacteria, swelling of the apical fungal cells were seen, in the first days of co-cultivation. After 3 to 5 days of incubation, these fungal cells were lysed. The cellular coating was degraded and leakages of the cytoplasmic content were seen (Fig. 3). These could be due to bacteria lytic enzymes production, as the tested strains are known to produce chitinases, cellulases, proteases, and/or lipases (DINU et al., 2012; SICUIA, 2013; BOIU-SICUIA et al., 2017a; b).

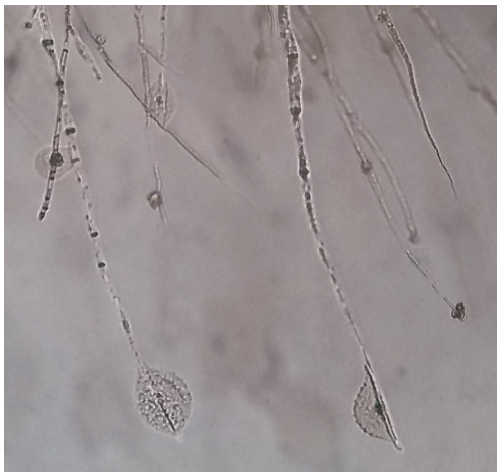

Figure 3. Fungal cell lysis and cytoplasm leaks in *Botrytis cinerea* biocontrol.

Swellings of the fungal cells (Fig. 4) were also noticed in *Fusarium oxysporum* and *F. solani*, when *B.subtilis* ssp. *subtilis* Dj3 was used as biocontrol strain. Similar aspects were also described in *F.oxysporum* biocontrol with strains of *Bacillus brevis* (BAPAT & SHAH, 2000) or *Paenibacillus polymyxa* (DIJKSTERHUIS et al., 1999). It is mentioned that anti-fungal compounds produced by the biocontrol bacteria are counteracted by magnesium ions (DIJKSTERHUIS et al., 1999). This suggests that biocontrol bacteria could induce osmotic stress, probably by nutrient competition. The presence of living bacteria increases fungal repression compared with cell-free bacterial supernatant (DIJKSTERHUIS et al., 1999; SICUIA, 2013).

In *F. oxysporum* and *F. solani*, some biocontrol bacteria induced an increased number of vacuoles. As these vacuoles increased their volume, the hyphae architecture was modified (Fig. 5). It is believed that these modifications were generated due to osmotic stress caused by the antifungal bacterial strains. As vacuoles are involved in maintaining cellular homeostasis (RICHARDS et al., 2012), the osmotic stress could be responsible for a higher accumulation of ions and molecules inside the vacuoles in order to maintain the integrity of the cell and avoid fungal cell lysis in the presence of biocontrol bacteria.

If we refer to *Rhizoctonia solani*, this pathogen is present in Romania, however it has not yet been reported to infect sweet potato grown in our country. As Rhizoctonia stem canker is mentioned to infect this crop in USA (CLARK et al., 2015) and China (LIFEI et al., 2016) we considered important to study the impact of autochthon biocontrol-bacteria treatment on such pathogen. Therefore, we compared the fungal growth morphology with and without the presence of biocontrol bacteria from *in vitro* cultures. Typical *Rhizoctonia solani* branches in right angles from the main hypha, having a septum near the branch origin (Fig. 6). In order to survive adverse conditions, Rhizoctonia produce specialized hyphae with compact monilioid cells, that fuse together in order to produce sclerotia (TREDWAY & BURPEE, 2001).

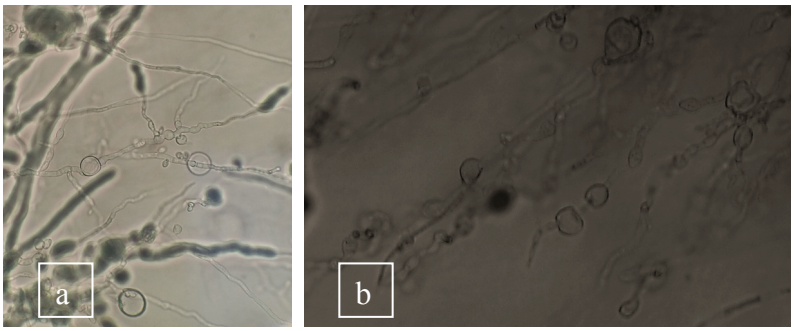

Figure 4. Fungal cells swelling in the presence of *B.subtilis* Dj3 biocontrol bacteria.
a. *Fusarium oxysporum*, **b.** *Fusarium solani*

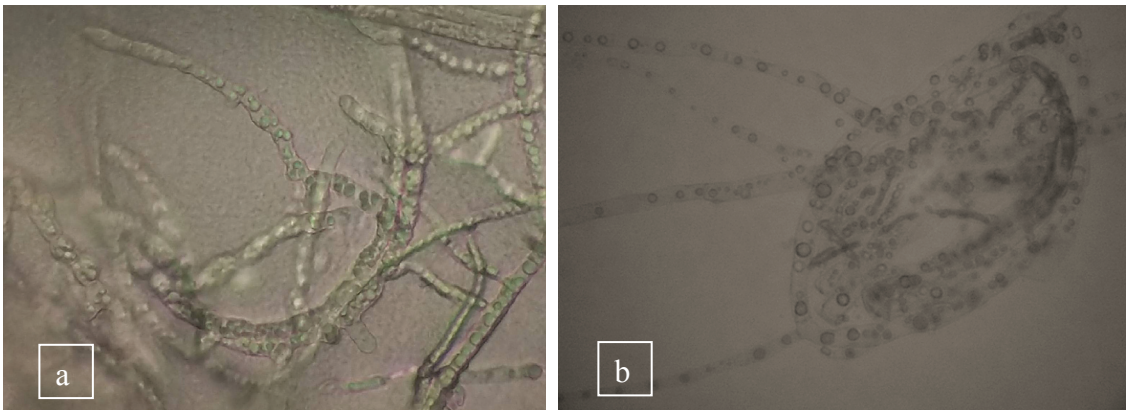

Figure 5. Increased number of vacuoles and vacuole size in *Fusarium oxysporum* cells in the presence of some biocontrol bacteria.
a. *Bacillus atrophaeus / subtilis* 6T4, **b.** *B.amyloliquefaciens* OS17

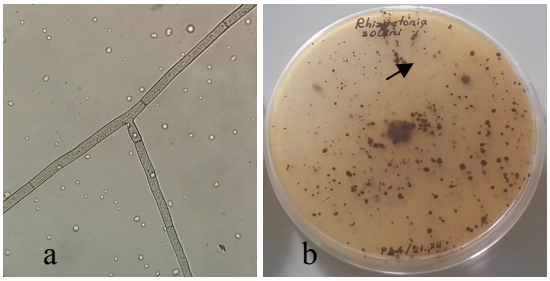

Figure 6. *Rhizoctonia solani* pathogen. **a.** mycelia branching in right angles from the main hypha,
b. six weeks old culture of *R.solani* on PDA medium (sclerotia are indicated by arrow).

Microbial interactions among *Rh. solani* and bacterial antagonists revealed different biocontrol mechanisms. In the presence of *Bacillus endophyticus* 1T2, hyphal morphology was changed. Fungal cells were shorter and swollen (Fig. 7A). Similar aspects were also seen in the presence of *B. amyloliquefaciens* OS17. Moreover, due to the high motility of this biocontrol bacteria strain, the fungal hyphae were colonized with bacterial cells (Fig. 7B). Hyphal growth deformation was induced by *B..subtilis* Dj3, consisting in mycelial curling (Fig. 7D). Likewise, in dual culture of *Rh. solani* with *B. atrophaeus / subtilis* 6T4 an increased number of vacuoles were observed in the fungal cells.

Dual culture technique of *Rh. solani* and *Pseudomonas chlororaphis* Sal.c2, revealed an increased number of vacuoles in the fungal cell, increased vacuole size, and cytoplasmic coagulation within the hyphae (Fig. 8). Similar changes induced by another *Ps. chlororaphis* biocontrol strain were also reported in *Rosellinia necatrix* (CALDERÓN et al., 2014). Moreover, different *Pseudomonas* species were mentioned to induce cytoplasmic coagulation not only in Rh. Solani, but also in other pathogens like *Botrytis cinerea*, *Macrophomina phaseolina* or *Phytophthora capsici* (BARKA et al., 2000; KUMAR et al., 2005; DIBY et al., 2005).

Macrophomina phaseolina (syn. *Sclerotium bataticola*) is a soil-borne plant pathogen with a broad spectrum of plant hosts, where it can express various disease symptoms. On sweet potato, it produces charcoal rot, and the disease evolves during storage. Although this pathogen is present in Romania, it has not been reported yet on locally produced sweet potatoes. Despite of this, we preventively analyze the possibility to biologically control this pathogen with plant beneficial bacteria. *In vitro* studies of microbial interactions showed that *Macrophomina phaseolina* could be severely affected by the biocontrol strains. Fungal growth was inhibited by all tested bacterial strains. The microscopic analysis of the interaction zone revealed fungal cell swelling, cell lysis and cytoplasm likings (Fig. 9).

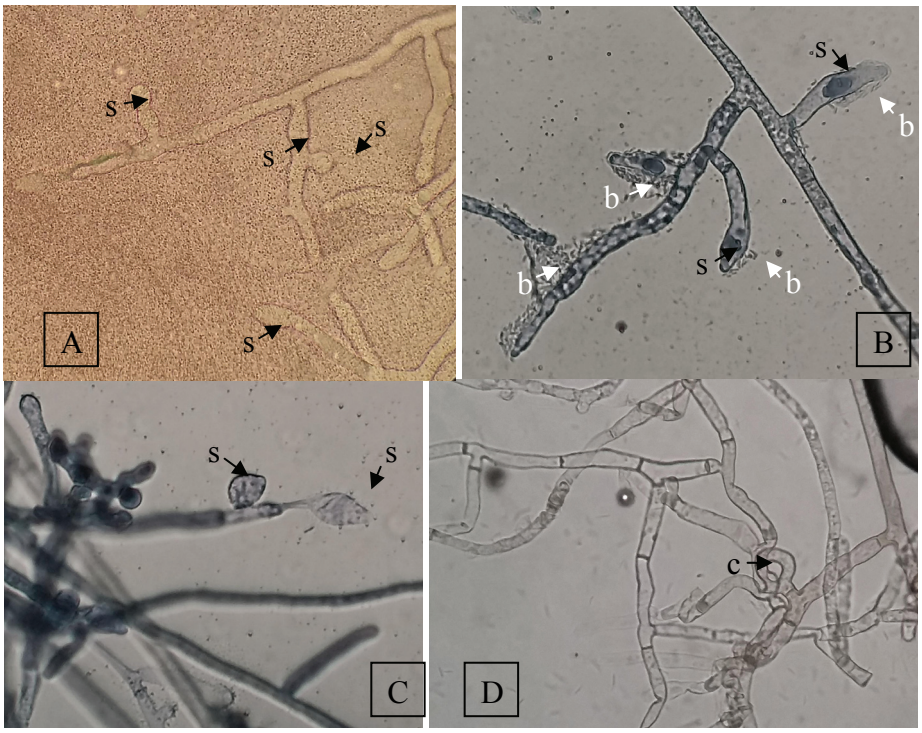

Figure 7. Fungal growth deformations of *Rhizoctonia solani* due to some biocontrol bacteria like *Bacillus endophyticus* 1T2 (**A**), *B.amyloliquefaciens* OS17 (**B**), *B. subtilis/ mojavensis* Dj6 (**C**), and *B.subtilis* Dj3 (**D**) c = mycelial curling, s = fungal cell swelling, b = bacterial cells colonizing the mycelia.

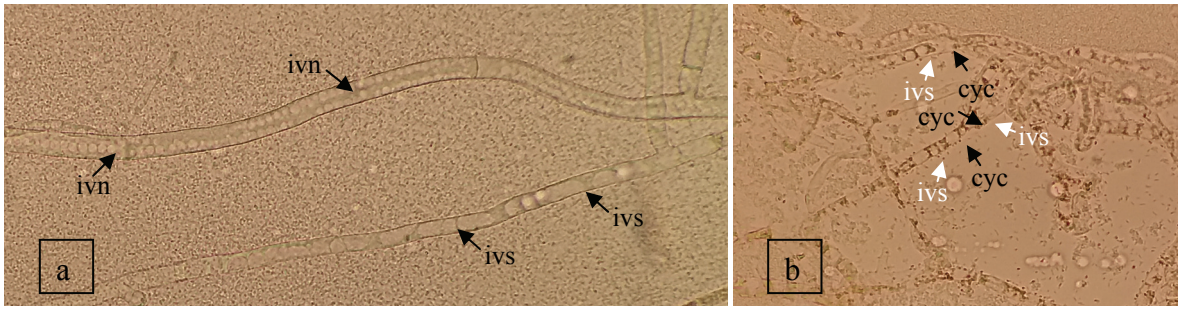

Figure 8. Bacterial interaction of *Pseudomonas chlororaphis* Sal.c2 with *Rhizoctonia solani* hyphae observed under the optical light microscope. Details in **a)** illustrate an increased number of vacuoles (ivn) in the fungal hyphae and an increased vacuole size (ivs), **b)** illustrate the fungal cells with cytoplasmic coagulation (cyc) within the hyphae and vacuoles with an increased size.

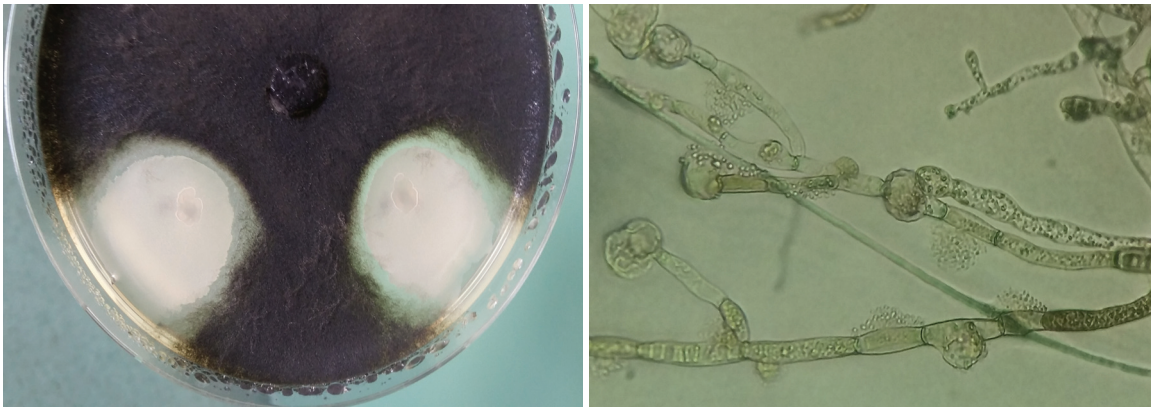

Figure 9**.** Microbial interaction between *Macrophomina phaseolina* and biocontrol bacteria.
a. Double culture technique of *Bacillus* sp. and *M. phaseolina*;
b. Fungal cell swelling, cell lysis and cytoplasm likings due to *Bacillus atrophaeus / subtilis* 6T4 biocontrol activity.

Biocontrol studies on *Macrophomina phaseolina* suggest that lytic enzymes, like chitinase and β-1,3-glucanase, produced by biocontrol bacteria are involved in hyphal degradation and cell wall digestion of *M. phaseolina* (SINGH et al., 2008).

CONCLUSIONS

Sweet potato is a relatively new crop for Romanian growers. This vegetable was successfully acclimatized and established in the crop rotation, mostly on the sandy soils of the Dolj county. No phytosanitary problems of economical important losses were registered on sweet potato, before harvest, in our country. However, it is expected that due to changing climate conditions and biological material transfer among states, several pathogens that currently do not attack sweet potato could find appropriate conditions to infect. It is also believed that when growers will expand this vegetable's culture, the incidence of diseases will be increased, and the spectrum of pathogens will be enlarged. We consider that fungal diseases like *Fusarium* spp., *Macrophomina phaseolina*, and *Rhizoctonia solani* will extend their spectrum of infection also to sweet potato.

Several fungal pathogens of sweet potato and pathogens mentioned worldwide to infect this vegetable were analyszed in dual culture, with several biocontrol agents (*Bacillus amyloliquefaciens* OS17, *B. endophyticus* 1T2, *B. atrophaeus / subtilis* 6T4, *B.subtilis* ssp. *subtilis* Dj3*, B. subtilis*/ *mojavensis* Dj6, and *Pseudomonas chlororaphis* Sal.c2). The purpose of these studies was to evaluate the potential of these Romanian native biocontrol strains to suppress pathogenic growth, and to evaluate the microbial interaction involved in the biological control process. Several mechanisms are supposed to be involved in the biocontrol process: the lytic enzyme production, antifungal compounds and competition with pathogens. The studied biocontrol strains were able to induced, *in vitro*, mycelial growth inhibition, fungal cells lysis and other alterations of the hyphae.

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Boiu-Sicuia Oana-Alina, Constantinescu Florica
Research and Development Institute for Plant Protection,
8 Ion Ionescu de la Brad Blvd., Bucharest, Romania.
E-mails: sicuia_oana@yahoo.com, cflori@yahoo.com

Boiu-Sicuia Oana-Alina
University of Agronomical Sciences and Veterinary Medicine Bucharest, Faculty of Biotechnologies,
59 Mărăşti Blvd, Bucharest, Romania.
E-mail: sicuia_oana@yahoo.com

Diaconu Aurelia, Drăghici Reta
Research-Development Station for Field Crops on Sandy Soils - Dăbuleni,
217 Petre Baniţă str., Călăraşi Village, PC 207220, Dolj County, Romania.
E-mails: aureliadiaconu@yahoo.com, retadraghici@yahoo.com

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