| Studii şi Cercetări | Martie 2019 | Biologie | 28/1 | 15-19 | Universitatea"Vasile Alecsandri" din Bacău |
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# ONE STRAIN OF ENDOPHYTIC *PREUSSIA*, A POTENTIAL BIOLOGICAL PARTNER OF TOMATO SEEDLINGS AGAINST ALTERNARIOSIS

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***Key words:*** *biological control, mutualist fungi, Alternaria, phytopathogens*

## INTRODUCTION

Endophytes are ubiquitous microorganisms (fungi or bacteria) that live inside plant tissues without causing any symptoms of disease. Endophytic microorganisms have been known to exist in symbiosis with hosts throughout their life cycle, starting from seed germination until fruit development (Yan et al., 2015). The failure to exploit endophytic fungi depends on our limited understanding of the evolutionary significance of these organisms and their dynamic interaction with their respective hosts. During the long period of co-evolution, a friendly relationship has been formed between each endophyte and its host plant. Fungal endophytes live mutualistically in plant tissues without inducing any symptoms of disease (Schulz & Boyle, 2005). Host plants provide a habitat and access to the nutrients for endophytes, whereas the endophytes confer fitness and defense-related benefits to host plants. The endophytic fungi induced growth-promoting benefits includes the increase in shoot and root biomass and resistance of host against abiotic stresses such as heat, drought, and salt and biotic stressors such as herbivores and pathogens (Redman et al., 2011).

The plant provides a protective sanctuary and access to nutrients, while in return, the endophyte produces bioactive secondary metabolites (Strobel, 2003), from various types of phenolics to hormone-like compounds (Correa et al., 2014) and enzymes (Kusari & Spiteller, 2012).

One endophytic strain of *Colletotrichum* sp., isolated from *Artemisia annua* was found to produce three new metabolites, 6-isoprenylindole-3-carboxylic acid, 3b,5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene, and 3b,5a-dihydroxy-6b-phenylacetyloxy-ergosta-7,22-diene (Lu et al., 2000). Various inhibitory activities were observed against *Candida albicans, Aspergillus niger* and phytopathogenic fungi *Gaeumannomyces graminis* pvar. *tritici*, *Rhizoctonia cerealis*, and *Helminthosporium sativum*, as well as against bacteria *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Pseudomonas* sp. A strain of *Colletotrichum gloeosporioides* from *Artemisia mongolica* produces a novel secondary metabolite named colletotric acid inhibiting growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus* (Zou et al., 2000). Three strains of *Aspergillus* spp. (i.e., SPS-02, SPS-04, and SPS-01) were isolated from *Artemisia annua* and were found to possess strong antimicrobial activities against the human pathogens *Escherichia coli*, *Staphylococcus aureus* and *Trichophyton rubrum*, and cytotoxic activities (Zhang et al., 2012). The authors also found an inhibitory effect on *Rhizoctonia cerealis* with a strain of *Mucor* sp. SPS-11, and the strongest antimicrobial activities observed against *Magnaporthe grisea* were exhibited by two strains of *Aspergillus fumigatus* (SPS-02) and *Cephalosporium* sp. (SPS-08). The same strain of *A. fumigatus* produced four ardeemin derivatives with various activities of reversing the multidrug-resistant phenotype in three cancer cell lines (Zhang et al., 2014).

The enzymes produced by fungal endophytes inside host tissues could help in supplementing the direct uptake of nutrients by microorganisms in the roots. Endophytes have the potential to secrete extracellular enzymes (cellulases, phosphatases and glucosidases) and plant-based hormones (such as auxin), which can help the host in harsh environmental conditions and also strengthen the symbiotic bond between the endophyte and the host (Khan et al., 2016). Many researchers use high-throughput *in vitro* methods to screen for potential biocontrol agents by isolating cultivatable organisms and test for their potential as BCAs.

Typically, *in vitro* confrontation assays (dual culture) are performed to look for the ability of the potential BCA to inhibit growth of the target pathogen directly, i.e. direct antimicrobial activity before moving on to in plant assays (Collinge et al., 2019).

The present study was designed to study the tripartite interaction between „tomato seedlings – endophyte fungus – fungal pathogen".

## MATERIALS AND METHODS

*Culture conditions of fungi*

The strains of *Preussia* sp. and *Alternaria* sp. were cultured for 14 days, on potatoes-dextrose-agar (PDA), at 25 ˚C, darkness. The endophytic fungus *Preussia* sp. was previously isolated from *Artemisia thuscula* (Cosoveanu et al., 2018) and *Alternaria* sp. was isolated from diseased tomatoes and kindly offered by prof. Beatrice Iacomi (USAMV Bucharest).

*Experimental design*

Tomato seeds were surface sterilized to supress epiphytic microorganisms (Cosoveanu et al., 2018). Subsequently, the seeds were dried on filter sterile paper under cabinet flow (approx. one hour). Tomato seeds were cultivated *in vitro* on Hoagland's nutrient solution in Petri plates (9 cm diameter). In each Petri plate three seeds were arranged in the middle, 3 circular inocula of endophytic fungus in the bottom and 3 circular inocula of pathogen (*Alternaria* sp.) in the top of plate, each with 0.4 cm diameter. Biological actors were placed equidistantly (Fig. 1) and interaction observations were made after 23 days. Five controls were maintained (C1 = EF + S, C2 = S + P, C3 = S, C4 = P and C5 = E; EF- endophytic fungi, S- seed, P- pathogen, C- control).

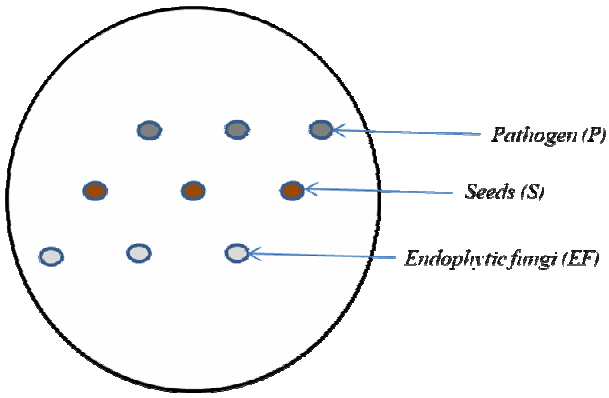


Fig. 1. The placement of parteners in the test

Plates were incubated vertically, under light : dark (16 : 8 h), relative humidity 65%, temperature 25 ˚C in a Sanyo versatile environmental test chamber. At 5 days interval, the plates were opened for oxygen requirement. Three biological repetitions were made for each variable.

The time sequence employed for the entrance of the biological actors in the setting was: moment 0 = endophyte, moment 1 = tomato seeds (after 2 days from M0), moment 2 = pathogen (after 14 days from M1) and moment 3 = data collection (after 7 days from M2).

*Interpretation of data*

Observations were made on germination, root and shoot weight, number of leaves and percentage of leaves pairs (1 – 3), index of development, index of necrosis and index of attack. For the germination percentage all seeds were taken into consideration, even if further did not develop. For the number of leaves value all leaves were taken into account including the cotyledons.

For the shoots and roots values, measurements were taken from the cotyledons to base and from the base to the top of the main root, respectively. Index of development, index of attack and index of necrosis were calculated (Cosoveanu et al., 2017). Statistical significance between two samples was performed with Mann Whitney test.

## RESULTS AND DISCUSSIONS

*Alternaria* sp. can cause serious problems for entire plant tomatoes crop. In some instances, annual economic yield losses due to early blight caused by several *Alternaria* species have been estimated at 79% (Adhikari et al., 2017). Severe attack can be observed especially on the green parts of the plant (leaves, shoots), but also on the roots and fruits, which can be identified by formation of dark brown cankers on stems and necrosis of leaf tissue between the veins (Witsenboer et al., 1991). Our study focused on tomato seedlings, up to 23 days-old with observations on the development of tomato seedlings under the influence of two parteners, the endophytic fungus *Preussia* sp. and the pathogen *Alternaria* sp. (Fig. 2).

Fig. 2. Tomato seedlings evolution under influence of endophytic fungus and pathogen

For the germination process (Fig. 3) a positive influence was observed when all three partners interacted, compared to all variables.

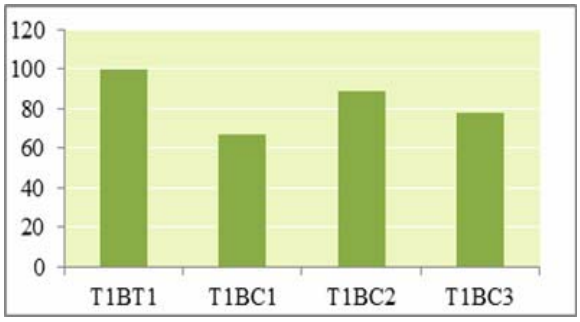


Fig. 3. The germination process under the influence of parteners

Registered germination values were slightly different among variables with all three parteners "T1" - 100% versus endophytic fungus and seed "C1"- 66.67%, pathogen and seed "C2" - 88.89 and seed alone "C3"- 77.78%.

Values of root/shoot index were higher for T1 and C3 compared to C2 ($p < 0.05$; Fig. 4). The presence of the endophytic fungus proved positive as the values for both root and shoot were higher than variables lacking *Preussia* strain (C2 and C3) with up to 30% increase of shoot (T1 versus C2). Yet, only shoot values were statistically significant (T1 vs C2 $p = 0.007$; T1 vs C3 $p = 0.004$; C1 vs C3 $p = 0.035$).

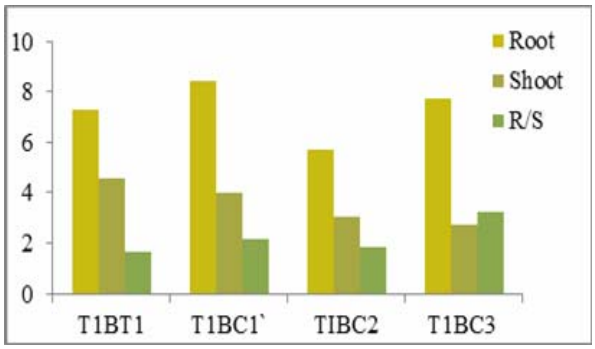


Fig. 4. Root, shoot and root/shoot values (cm)

The average number of pairs was similar among the variables (C2 - 1.5; C3 - 1.6; T1 and C1 - 1.7), which indicates that neither the endophytic fungus nor pathogen did have a significant effect. Index of development showed no differences among variables (Fig. 5). The influence of endophytic fungus on the evolution of tomato seedlings in terms of necrosis, yellow and brown spots was recorded as index of necrosis and index of attack.

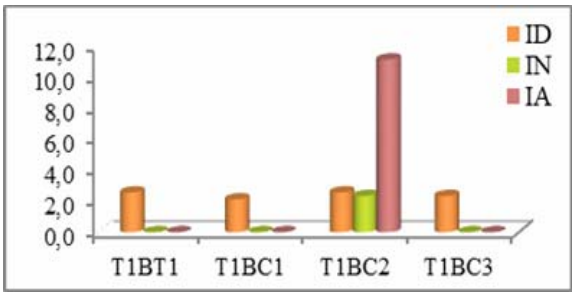


Fig. 5. Values for index of developement (ID), index of necrosis (IN) and index of attack (IA)

Both indices revealed zero values for T1 and C1 and maximum values for C2. Thus, we can consider that endophytic fungus *Preussia* interacted i) innocuously with tomato seedlings and ii) as potential protector of seedlings when in presence of pathogen.

The mechanisms contributing to biological control include antagonism, competition and induced resistance (Card et al., 2016; Collinge et al., 2019; Hardoim et al., 2015; Jensen et al., 2016; Muller & Krauss, 2005). Collinge et al (2019) opened a quizz on individual mechanisms which may contribute in concert, in a particular three-way interaction between plant, pathogen and antagonist. Also, timing of incubation both as single actor and interaction between actors (pairs or tripartite setup) can result in different observations due to the dynamics of bioactive compounds concentration resulted from the time spent on „solitude, pairing or antithesis" in biochemical communication (Cosoveanu, not published). Similarly, VOCs bioactivity was observed to increase when endophytic fungi were left to grow and develop in pure culture before adding the pathogen; with up to 35% inhibition compared to incubation at same time (Cosoveanu et al., 2016). Inside the tripartite interaction various factors may influence results besides the biochemical communication (i.e. generating unique compounds), including the accumulation of active substances (VOCs or $H_2O$ solubilized).

## CONCLUSIONS

The endophytic fungus showed a positive influence on growth and development of tomato seedlings and acted as protector of seeds as inside the tripartite interaction. Further analysis on biochemical communication are necessary to establish the potential use as biological control agent of HLP14.

## ABSTRACT

A fungal endophytic strain, isolated from *Artemisia* sp., identified as *Preussia sp.* was selected

for its potential of plant growth promotion and reduction of pathogen attack, *in vitro*.

The metaorganism tomato - endophytic fungus - fungal pathogen was observed in terms of germination, root and shoot weight, number of leaves and percentage of leaves pairs (1 – 3), index of development, index of necrosis and index of attack. The time sequence employed for the entrance of the partners in the setting was: moment 0 = endophyte, moment 1 = tomato seeds (after 2 days from M0), moment 2 = pathogen (after 14 days from M1) and moment 3 = data collection (after 7 days from M2). A positive influence on germination was observed when all three partners interacted, compared to all variables (all partners "T1" - 100% versus endophytic fungus and seed "C1"- 66.67%, pathogen and seed "C2" - 88.89, seed alone "C3"- 77.78%, respectively).

Root and shoot length was improved both when all partners "T1" (root – 7.30 cm, shoot – 4.58 cm) as well as when endophytic fungus and seed partners "C1" (root – 8.40 cm, shoot – 4 cm) were present, compared to seed alone "C3" (root – 7.70 cm and shoot – 2.73 cm) and seed and pathogen partners "C2" (root – 5.74 cm and shoot – 3.06 cm). Although the average number of pairs was similar among the variables (C2 - 1.5; C3 - 1.6; T1 and C1 - 1.7), the percentage of pairs of leaves differed significantly for the percentage of 1 pair of leaves: C3 = 42.9% versus T1 = 11.1%. An improvement was observed in the percentage of 2 pairs of leaves for the case of all partners as well as for the case of endophyte and seed: T1 = 77.78%, C1 = 85.7% versus C2 = 62.5% and C3 = 57.15. Yet, the index of development showed best case when seed was alone and no difference between pathogen or endophyte presence.

In terms of the indices of necrosis (IN) and attack (IA), the endophyte was innocuously interacting with seedling (IN and IA = 0) and also when both pathogen and endophyte (T1) were present the seedling presented no symptoms, compared to pathogen and seedling case (C2): IN - T1 = 0 versus C2 = 0.78 and IA – T1 = 0 versus C2 = 11.11.

## ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Research and Innovation, CCCDI UEFISCDI, project number PN-III-P1-1.2-PCCDI- 2017-03-01/28PCCDI/2018.

We thank Beatrice Iacomi (USAMV Bucharest) for useful tips in experimental design. We deeply appreciate Raimundo Cabrera (ULL, Tenerife, Spain) and Ana-Maria Andrei (ICDPP, Bucharest) for guidance and inspiration. Also, we are grateful to Luminita Dinu (ICDPP Bucharest) who helped with the laboratory preparatory.

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