AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Histological examination of endophytic *Chaetomium cochliodes* Palliser fungus localization in healthy tissues of agricultural crop roots

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Article Info

Article history:
Received 18 October 2020
Revised 13 July 2021
Accepted 20 July 2021
Available online 31 August 2021

Keywords:
Agricultural plants,
Chaetomium cochliodes,
Fungal endophytes,
Histological study,
Succinate dehydrogenase

Abstract

The ability was investigated of the *Chaetomium cochliodes* fungus to penetrate the healthy tissues of agricultural crop roots. It is well-known that endophytic fungi can exist in plant tissues for a long time without any sign of their presence and only become active under adverse environmental conditions. Localization of endophytic fungus mycelia within the macroorganism was revealed using histological methods. Seeds of wheat, barley, rye, triticale, buckwheat, maize, sunflower, soybean and flax were inoculated with the saprotrophic fungus *C. cochliodes* and grown in a vegetation experiment. Thin sections of the roots and plant root hairs were painted with an aniline blue lactic acid solution to visualize fungal mycelia and, in some cases, spores. Some small hyphae were found inside rhizodermal cells. The presence of fungal structures and their localization in healthy tissues of the roots of cereals, legumes and industrial crops indicated the endophytic ability of *C. cochliodes*. At the same time, an increase in the succinate dehydrogenase activity was observed in the roots of plants inoculated with the fungus for wheat (by 1.2 times), barley (by 3.2 times), rye (by 3.7 times), triticale (by 3.4 times), maize (by 3.2 times), sunflower (by 1.6 times), soybeans (by 2.9 times) and buckwheat (by 1.5 times). Additional evidence of the formation of active endophytic symbiotic systems was the increasing activity of succinate dehydrogenase with the simultaneous penetration of *C. cochliodes* into healthy plant roots. The studies conducted deepen the understanding of the biology of the relationship between a macroorganism and an endophytic fungus.

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https://doi.org/10.34044/j.anres.2021.55.4.01

Introduction

Endophytes are the microorganisms that penetrate inside the tissues of the root, stems or seeds, without causing damage to the microorganism (Spatafora et al., 2007). In recent decades, much research attention has been directed to the previously poorly studied ecological process of the endophytes of soil saprotrophic fungi on plant roots and it has been reported that metabolic and growth processes are activated and the immune status and resistance to stress factors are increased in plants infected with endophytic fungi (Arnold and Herre, 2000; Waller et al., 2005; Marquez et al., 2007). In turn, endophytic fungi localized in the roots gain direct access to the synthesized macroorganism nutrients and are protected from adverse environmental conditions. However, the formation of endophytic systems is not a prerequisite for plant development. (Spatafora et al., 2007).

Endophytes can stimulate plant growth in active or passive ways. Fungi are capable of producing growth-regulating substances or inducing the formation of phytohormones by plants, stimulating the accumulation of underground and aboveground biomass with the help of macrosymbionts (Kopilov et al., 2020). Passively, microsymbionts can increase the amount of mineral nutrient elements (phosphorus, potassium, zinc) supplied to the plants (Yadav et al., 2016; Yadav, 2017). In addition, the formation of an endophytic association activates the processes of plant photosynthesis (Selim et al., 2012).

Endophytic fungi play a significant role in the defense processes, inducing the immune system of the macroorganism, resulting in plant resistance to phytopathogenic diseases being increased and resistance to abiotic (drought, low temperature, salt stress) and biotic stress factors is developed (Arnold and Herre, 2000; Waller et al., 2005; Marquez et al., 2007; Qadir et al., 2020). In response to the phytopathogenic action, endophytic fungi use a variety of plant protection mechanisms such as: competition between endophyte and pathogen on the basis of the same resources; direct pathogen antagonism through antibiosis, parasitism or predation; changes in the hormonal pool of plants (synthesis of auxins, gibberellins, abscisic acid and ethylene); and the production of siderophores and essential plant vitamins (Bae et al., 2009).

Endophytic microorganisms penetrate the tissues of most plant species (wheat, rice, mustard, chili, sugar cane, citrus, potato, tomato, soybean, pea, bean, sunflower, cotton, chickpea, millet, strawberry) while forming symbiotic associations. (Verma et al., 2017; Yadav et al., 2018). There is only fragmentary information on the localization of endophytic microorganisms within the roots of plants such as intercellular mycelia, mycelia in the conductive vessels of secondary tissues, intercellular septic mycelia with some points of penetration into the cells (Verma et al., 2012).

Chaetomium is one of a large genus in the Chaetomiaceae family, having more than 100 species (Abdel-Azeem, 2020). Among the fungi of this genus, active biological control agents have been found that inhibit the growth of bacteria and fungi by direct competition, microparasitism or antibiosis (Sibounnavong et al., 2011). *Chaetomium cochliodes* has been shown to synthesize a variety of fatty acids, including arachidonic acid, which is a biogenic elicitor and induces a systemic plant immune response to pathogens' actions and adverse environmental factors (Kopylov, 2013). The fungus produces 2,4-epibrasinolide and ergosterol, which play an important role in the formation of plant resistance to disease pathogens (Dragovoz et al., 2018). In addition, C. *cochliodes* synthesizes a number of phytohormonal substances, including indolyl-3-acetic acid, that can be a mediating molecule in the formation of symbiotic systems with plants (Dragovoz et al., 2018). The fungus *C. cochliodes* 3250 was capable of synthesizing enzymes (exoglucanase, endoglucanase, β-glucosidase, polygalacturonase), which are very important for its penetration into the plant root (Kopilov et al., 2020).

Symbiotic-effective endophytic fungi and the study of their interaction features with plants are the subject of intensive research. Important aspects of applied microbiology in agricultural production include the study of diversity, ecological niches and the metabolic products of endophytic microorganisms and their usage to improve the growth and development of the plants and macroorganism protection from adverse environmental factors- (Verma et al. 2009, Verma et al., 2012). Despite the large number of fungi that can form mycorrhizal or endophytic symbiosis, many plants have not been studied regarding their ability to form associative systems with micromycetes. Fungi of the genus *Chaetomium* are actively used in agriculture as agents of biological control (Kopylov, 2013), but their role in plant growth stimulation remains poorly studied. Specifically relevant is studying the endophytic possibilities of fungi of this genus into the roots of plants and to enhance crop growth and development.

Furthermore, understanding the abilities of fungi to show endophytic activity with certain plant species and to form appropriate structures requires long-term microscopic studies. Therefore, it is important to find an effective marker that will help to determine the ability of the fungus to form endophytic associative systems with certain plants.

Materials and Methods

Fungi strain

Chaetomium cochliodes Palliser 3250 (a strain of the marsupial antagonistic fungus) was obtained from a collection of beneficial soil microorganisms at the Agricultural Microbiology and Agro-Industrial Production Institute of Agrarian Sciences National Academy of Ukraine (Volkogon et al., 2015).

Plant materials

The study of the ability of *C. cochliodes* 3250 to colonize the root zone of plants was performed under sterile conditions. Roots and root hairs were examined of the winter wheat (*Triticum aestivum*) of Smuglianka variety, triticale (*Triticosecale*) of the spring variety Kharkiv Oberig, barley (*Hordeum sativum*) of the spring variety Gosia, rye (*Secale cereal*) of the winter variety Synthetic 38, maize (*Zea mays*) of the Dniprovskyi hybrid 181 SV, sunflower (*Helianthus annuus*) of the variety Alfa, buckwheat (*Fagopyrum esculentum*) of the sowing varieties Antaria, soybean (*Glycine max*) of the Ustia variety and flax (*Linum usitatissimum*) of the Nadiynyi variety.

Plant seeds were sterilized beforehand with 0.1% $AgNO_3$ fluid for 1 min and subsequently were inoculated with *C. cochliodes* 3250 fungus (4×10^4 colony forming units per seed). Plants were grown for 21 d using the roll culture method (Volkogon, 2010) using a Knop fluid in glass vessels with a volume of 1 L for 22 d. The samples were removed from the vessels and prepared for histological examination.

Histological processing of samples and stain preparation

For microscopic examination of endophytic mycelia, the roots of wheat, barley, rye, triticale, buckwheat, maize, sunflower, soybean and flax were washed, dyed and fixed in advance. Thin root hairs were thinly cut by hand into small segments 5–7 mm long. For larger roots, a series of longitudinal sections hand cut using a thin blade were sampled to obtain blocks 5–7 mm long and 3–5 mm wide.

The obtained sections were dyed using the Kobel method for staining fungi in plant tissues (Bilay, 1982). Briefly, a mixture of the following composition was used as the dye: 0.1 g of blue aniline, 50 mL of lactic acid and 100 mL of water. Small root hairs, longitudinal and transverse sections of roots were immersed in an aniline blue lactic acid fluid for 5 min followed by washing the test sample with water. To differentiate the fungal structures, sections of roots and root hairs of the agricultural crops were heated in lactic acid droplets (Barykina et al., 2000). Afterward, the microproducts were studied using a Delta Optical Evolution Trino LED microscope (Poland) and a MICROmed camera (China) with 5 megapixels resolution.

Growth of plant materials

The study of the ability of *C. cochliodes* 3250 to form an associative system with agricultural plants was tested under vegetative conditions for 20 d, using plastic vessels with a volume of 2 L.

The soil used was a sod-medium, podzolic, dusty-sandy loam with a humus content of 1.02%; nitrogen content of 54.9 mg/kg, moving forms of phosphorus of 110–120 mg P_2O_5, exchangeable potassium of 120–130 mg K_2O per 1 kg of soil, pH_{salty} of 5,2, pH_{water} of 6,0, Ca at 5,8 mg equivalent per 100 g of soil and Mg-0 at 61 mg equivalent per 100 g of soil.

In the control treatment, the seeds of the studied plants were moistened with tap water (1% by weight). The experimental variant pre-sowing processing of *C. cochliodes* 3250 was carried out at the rate of 4×10^5 colony forming units per seed.

Determination of succinate dehydrogenase activity

The activity of succinate dehydrogenase (SDH) in the roots of the control and inoculated plants was investigated using potassium ferricyanide (Resyapkin et al., 2009). The activity determination was based on the ability of SDH to restore potassium ferricyanide ($K_3[Fe(CN)_6]$), which as a fluid has a yellow color, to colorless potassium ferrocyanide ($K_4[Fe(CN)_6]$), with enzyme activity proportional to the amount of reduced ferricyanide.

The corresponding plant tissues were homogenized beforehand in 0.1 M phosphate buffer at a ratio of 1:10. The rapid homogenization of the roots at low temperature and the use of a buffer fluid minimized the decrease in activity of the studied enzyme.

The incubation mixture added to the test tube samples consisted of 1 mL each of 0.1 M potassium phosphate buffer at pH 7.8, 0.1 M succinic acid at pH 7.8, 25 mM methylenediaminetetraacetic acid, 150 mM sodium azide and H_2O. After insertion of the homogenate tissue into the incubation mixture, the samples were incubated for 5 min at room temperature to inhibit cytochrome oxidase

with sodium azide. The reaction was started with the introduction of potassium ferricyanide for 15 min at 30°C and stopped with the introduction of a 20% trichloroacetic acid (TCO) fluid. Samples were centrifuged for 15 min at of 4,000 revolutions per minute and the SDH activity was determined in the supernatant.

In the control samples, the SDH was completely denatured prior to incubation resulting in no specific ferricyanide recovery occurring, with trichloroacetic acid being added to the control sample containing all components of the reaction mixture before making the tissue homogenate.

A mixture of 20% TCO and 0.1 M phosphate buffer in a 1:1 ratio was used as the optical control.

A calibration curve (from 100 to 1,000 µg of ferricyanide in 4 mL of test fluid) was constructed to determine the content of ferricyanide in the test samples.

The different amounts of ferricyanide recovered during incubation were calculated. As the reaction proceeds stoichiometrically, the SDH activity was expressed by the amount of oxidized succinate and measured in N mol succinate per milligram of proteins per minute. Three biological and three analytical replicates were used.

Statistical analysis

Data were expressed as mean±SD. The t-test was used to compare between mean of a control and treatment within species at a significance test level of $p < 0.05$.

Results and Discussion

Spatial interactions of C. cochliodes 3250 with agricultural crops

Colonization of plant roots by endophytic fungi can be a powerful factor in the intensification of plant growth, the activation of metabolic processes and consequently, the better development of the macroorganism as a whole. The current study tested the ability of the fungus *C. cochliodes* 3250 to form spatial relationships with various agricultural crops.

Microscopy of the root hairs and root cross-sections of the investigated plants established that the saprotrophic fungus *C. cochliodes* 3250 had actively developed in the root zone of the studied crops and formed fruiting bodies on the roots of wheat, rye, triticale, maize, sunflower, soybean and buckwheat (Fig. 1).

Fruit bodies of *C. cochliodes* formed mainly on the secondary and tertiary lateral roots of plants. Some small hyphae were found inside the rhizodermal cells, with larger hyphae observed both between the rhizodermal cells and in the spaces of the mesoderm parenchymal cells (Fig. 2). In addition, there was penetration of C. *cochliodes* 3250 hyphae into the root and the root hairs of the studied plants. Localization of fungal spores on the plant root hairs and on samples with sections of mesodermal parenchymal cells was considered as evidence of the formation of an endophytic associative system. Micromycetes of C. *cochliodes* 3250 had penetrated the rhizodermal cells. Notably, *C. cochliodes* 3250 did not inhabit the flax roots, with no fruit bodies on the roots of this culture, nor was there any hyphal penetration of the flax root hairs.

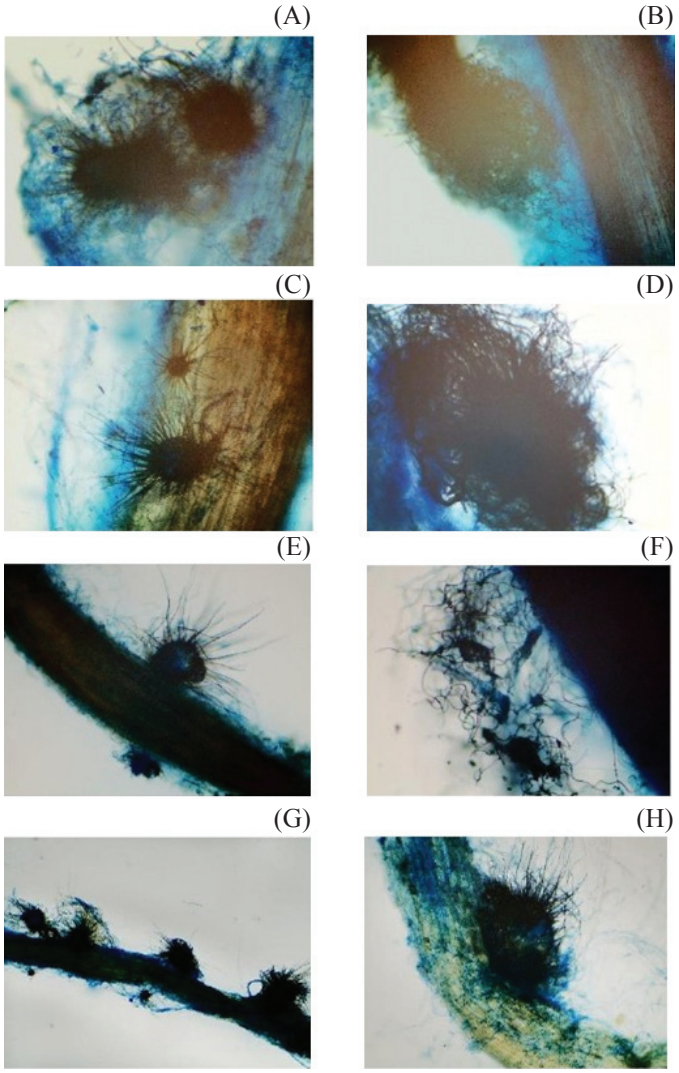

Fig. 1 Fruit bodies of *Chaetomium cochliodes* 3250 fungus on: (A) wheat roots of variety Smuglianka; (B) rye of variety Synthetic 38; (C) triticale of Kharkiv Oberig variety; (D) maize of Dniprovskyi hybrid 181 SV; (E) sunflower of variety Alpha; (F) soybeans of variety Ustia; (G & H) buckwheat of variety Antaria, where magnification is 100× for A–F & H and 40× for G

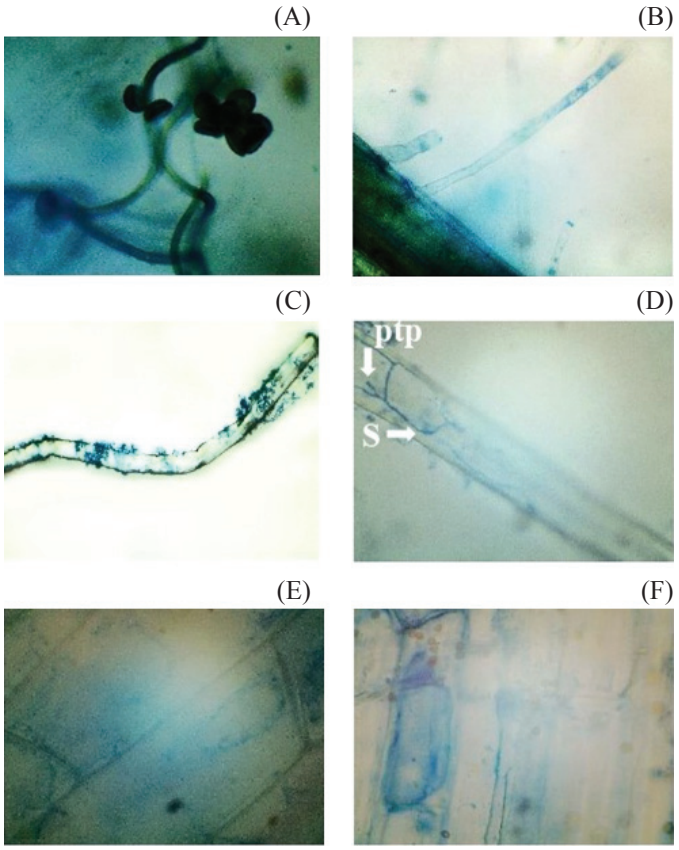

Fig. 2 Fungus *Chaetomium cochliodes* 3250: (A) spores on soybean root hairs of Ustia variety; (B) penetration of hyphae in sowed buckwheat root hairs of Antaria variety; (C) barley root hair of Gosia variety braided with hyphae; (D) barley root hair of Gosia variety with fungal mycelia showing secondary mycelia (ptp) and septation (S); (E) mycelia on sowed buckwheat rhizodermal cells of Antaria variety; (F) spores with wheat parenchymal cells, where magnification is 600× for A & E, 400× for B, D & F and 100× for C

The presence of the fungal structures and their localization in the healthy tissues of the roots of the cereals, legumes and industrial crops indicated the ability of the saprotrophic fungus *C. cochliodes* to act as an endophyte. However, *C. cochliodes* 3250 did not form any endophytic associations with the flax plant.

Activity of succinate dehydrogenase

The fungal penetration in the plant roots and the creation of certain mycorrhizal materials are indicated by the increased SDH enzyme activity that is widely used as a cytochemical marker to create classic mycorrhiza (Macdonald and Lewis, 1978; Ocampo and Barea, 1985; Sylvia, 1988; Hamel et al., 1990; Vierheilig and Ocampo, 1991; Van Aarle et al., 2005).

SDH is a multifunctional enzyme complex that participates in catabolic and anabolic processes, where the enzyme catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and also the recovery of fumarate to succinate in higher organisms (Eprintsev et al., 2007). The current results suggested that it is closely linked to the inner membrane of the mitochondria, as well being a component of the Krebs cycle and the electron transport chain. Thus, its regulation is related to the function of two simultaneous processes of oxidative cell metabolism (Affourtita et al., 2001).

The interdependence of symbiotic and SDH activities has been confirmed by histochemical staining of external (Hamel et al., 1990; Vierheilig and Ocampo, 1991) and internal (Macdonald and Lewis, 1978; Ocampo and Barea, 1985; Saito et al., 1993; Tawaraya et al., 1994) hyphae of arbuscular fungi for wheat, soybean and onion plants.

Table 1 indites that grain plants such as wheat, triticale, barley and rye responded to the pre-sowing processing of *C. cochliodes* 3250 with increases in SDH activity, indicating the requirement for higher metabolic activity in their root zones. Similar results were obtained for the legumes, cereals and industrial crops in the current study.

The SDH activity in wheat roots increased by 22% due to saprotrophic fungus actions. As shown in Fig. 1A, *C. cochliodes* 3250 penetrated the root hairs of winter wheat and formed fruiting bodies on its roots.

Other members of the cereal family appeared to be more sensitive to *C. cochliodes* 3250. Thus, pre-sowing processing with fungus resulted in an increase in succinate dehydrogenase activity in the triticale roots by 3.4 times, in barley by 3.2 times and in rye by 3.7 times. Figs. 2C–2D indicate that the fungus *C. cochliodes* 3250 could establish on the roots of barley and on the roots of rye (Fig. 1B) and formed fruiting bodies on the triticale root hairs (Fig. 1C).

The results obtained complemented the list of fungi that asymptomatically penetrate into the internal tissues of plants and have a positive effect on macroorganisms in general and which were isolated by different groups of researchers from the roots of wheat, barley and rye (Nevo, 2007; Witcombe et al., 2008; Lakew et al., 2011; Lens et al., 2016; Yokoya et al., 2017).

The activity of SDH maize plants by the action of the soil fungus *C. cochliodes* 3250 increased by 3.2 times (Table 1). The microscopic inspection revealed the formation of an associative system involving the saprotrophic fungus C. *cochliodes* 3250 and maize plants (Fig. 1D). The dominant fungal endophytes species of maize belong to the genera

Fusarium, Sarocladium, Aspergillus and *Penicillium* (Potshangbam et al., 2017). Currently, worldwide studies on the interaction of maize and endophytic microorganisms have been focused on the isolation and identification of root isolates with a positive effect on the plant (Orole and Adejumo, 2009; Amin, 2013).

In the roots of sunflower in the current study, there was an increase in the SDH activity by 1.6 times (Table 1) with the evidence of the fungus C. *cochliodes* 3250 forming an endophytic association with this agricultural crop shown in Fig. 1E. Waqas et al. (2015) reported that sunflower plants treated with endophytic fungi *Penicillium citrinum* and *Aspergillus terreus* showed resistance to a number of diseases, increased harvest and oil yields.

Endophytes of soybean plants have been reported from the genera *Fusarium, Aspergillus, Cladosporium, Alternaria, Diaporthe, Epicoccu* and *Schaetium* (Harrison, 2005; Kopylov and Nadkernichnyi, 2008). Kopylov and Nadkernichnyi (2008) reported that *C. cochliodes* 3250 may be a component of triple symbiosis (*Glycinemax-Bradyrhizobium japonicum* 2490-C. *cochliodes* 3250). The combined processing of *C. cochliodes* 3250 and *B. japonicum* 2490 helped to increase the number of brood buds on the plant, while at the same time, the activity of symbiotic nitrogen fixation increased compared to both the variant without inoculation and to the use of only tuber bacteria (Kopylov and Nadkernichnyi, 2008). In the current study, pre-sowing processing of soybean seeds of the Ustia variety with the micromycetes increased the SDH activity by 2.9 times and the microscopic studies showed corresponding structures of *C. cochliodes* 3250 on plant roots (Fig. 1F and Fig. 2A).

Buckwheat is an important cereal crop as it used not only as a foodstuff, but also as a food supplement, due to its high content of flavonoids, minerals, vitamins and balanced amino acid composition (Gaberscik et al., 2002). Thus, in addition to scientific interest, improving the root nutrition of buckwheat plants through the formation of associative systems with endophytic fungi is important ecologically and economically.

Despite the substantial value of buckwheat as an agricultural crop, there is only limited information regarding its ability to form endophytic symbiotic systems with micromycetes (Likar et al., 2008; Impullitti and Malvick, 2013). Therefore, the formation issue of the endophytic or mycorrhizal symbiosis by the buckwheat has been controversial and not fully explored.

In the current vegetation experiment with buckwheat, there was an increase in the activity of the enzyme SDH by 1.5 times. *C. cochliodes* 3250 could not only actively develop on the roots of crops but could also penetrate the root hairs and cells of the rhizoderm (Fig. 1G–H, Fig. 2B, 2E).

Thus, the fungus *C. cochliodes* 3250 formed fruiting bodies on the roots and could penetrate the root cells of wheat, barley, rye, triticale, buckwheat, corn, sunflower and soybeans. Accordingly, the localization of fungal structures in healthy root tissues of cereals, legumes and industrial crops indicated the ability of the saprotrophic fungus to form endophytic symbiotic systems. All the studied plants, with the exception of flax, responded to the processing with the saprotrophic fungus *C. cochliodes* 3250 with increased SDH activity in the root zone; the simultaneous penetration of *C. cochliodes* into healthy plant roots was additional evidence of the formation of active endophytic symbiotic systems.

Table 1 Succinate dehydrogenase (SDH) activity in mycorrhized agricultural crop roots (growing experiment for 20 d)

Agricultural plant	SDH activity (N mol of succinate/mg proteins/min)	
	Control (processing with tap water)	Pre-sowing processing *C. cochliodes* 3250
Wheat of Smuglianka variety	1.31±0.04	1.60±0.05*
Triticale of Kharkiv Oberig variety	0.69±0.04	2.33±0.08*
Barley of Gosia variety	0.49±0.02	1.56±0.04*
Rye of Synthetic 38 variety	0.93±0.03	3.42±0.20*
Maize of Dniprovskyi hybrid 181SV	2.60±0.04	8.26±0.17*
Sunflower of Alpha variety	4.73±0.18	7.80±0.05*
Soybean of Ustia variety	1.98±0.02	5.84±0.08*
Flax of Nadiynyi variety	4.00±0.48	4.31±0.57
Buckwheat of Antaria variety	3.06±0.09	4.52±0.21*

* indicates significant ($p < 0.05$) difference as compared to the respective control

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The research was performed as part of basic research No. 0116U003068.

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