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Review

EDIBLE MUSHROOMS COULD TAKE ADVANTAGE OF THE GROWTH-PROMOTING AND BIOCONTROL POTENTIAL OF *AZOSPIRILLUM*

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Investigations of biologically active substances sourced from xylotrophic macromycetes have shown their fundamental and practical significance. These studies have promoted the development of biotechnologies by obtaining the valuable products from the mycelial biomass and cultivation liquid and using these fungal species in "white chemistry". Research into environmentally safe biological methods of stimulating the growth of medicinal and edible mushrooms can assist the development of scientific foundations of culture technologies. From the viewpoint of their biotechnological potential, selected factors of biological origin that promote the growth of wide-scale cultivated basidiomycetes are of interest. Improving mushroom resistance against negative environmental impacts seems to be feasible when the mushroom grows in combination with growth-promoting bacteria. The soil bacteria from the Azospirillum *genus have attracted attention, as microorganisms are capable of actively influencing the growth and development of agricultural crops. Thus, revealing and exploring the growth-promoting properties of different species of* Azospirillum *with respect to edible and/or medicinal mushrooms, as well as the properties promoting the suppression of contaminants in double culture, are believed to be feasible and to reveal undoubted novelty. Cultivation jointly with the beneficial bacteria is a more effective biotechnological method for obtaining this valuable functional food faster, with a greater and much better and maintained mushroom yield. Future work involves addressing the problems of mushroom growth promoting bacterial interactions with mushrooms and implementing these synthetic microbial associations into agriculture.*

Keywords: *xylotrophic basidiomycetes, PGPR, fungal-bacterial coculture, MGPB.*

INTRODUCTION

Mushrooms (macromycetes) play a significant role as food and organisms capable of producing many physiologically active compounds. A considerable amount of literature has been published on the ecology, physiology, genetics, and biotechnology of mushrooms. Edible mushrooms are readily available at any food market owing to their commercial cultivation (Kim *et al.*, 2015). These mushrooms do not merely constitute a highly nutritious source of food. The extremely important biologically active extracts and compounds derived from cultivable mushrooms exert pharmacological effects. The discovery that many mushrooms produce a broad range of medicinally useful metabolites (Ashraf *et al.*, 2013) was followed by intense development of the biomedicinal area of fungi exploitation (Rathore *et al.*, 2019). For instance, various chemical agents in the composition of mycelia exhibit antiviral activity against many viruses that are pathogenic in humans (Teplyakova and Kosogova, 2016) and that currently present a great pharmaceutical challenge. Thus, from the viewpoint of biotechnological potential, selected factors of biological origin that promote the growth of wide-scale cultivated basidiomycetes are of interest.

One of the preferred research approaches in current mushroom science is decoding the methods of inducing the biochemical processes of basidiomycete ontogenesis. Complex

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physiological and biochemical processes occur during the growth and development of fungal organisms that are determined primarily by the biotic factors in the environment, which must be studied in greater detail. There is a necessity for studying their joint cultivation with bacteria in relation to the processes of cytodifferentiation and morphogenesis of mushrooms and in revealing the role of these factors in the vital activity of fungal cultures. Research of this kind is useful for directed selection of effective inducers to enhance the stress-resistance of edible cultivable mushrooms, as is also true for plants. Understanding the mechanisms and peculiarities of associative interactions of the bacterial-fungal communities could assist in the development of more efficient and productive methods of growing cultivable edible and medicinal mushrooms.

Hence, a challenging problem is the development of ecologically safe ways of applying growth-promoting bacteria and bioactive substances of bacterial origin to technologies enhancing the resistance of cultivable basidiomycetes against pathogenic bacterial and lower fungal contamination and to technologies for controlling the abundance of the contaminating microflora representatives in industrial mushroom growth practices.

Analysing the current literature with respect to works on xylotrophic basidiomycetes and bacteria co-cultivation showed absolute insufficiency of research into these problems, in spite of their high potential for fundamental and applied research in this area. Bacteria from the genus *Azospirillum* are rhizospheric microorganisms that enhance plant growth and development via nitrogen fixation and hormone production (Steenhoudt and Vanderleyden, 2000). The bactericidal and fungicidal activity of azospirilla against several bacteria and lower micromycetes has been shown (Red'kina, 1990; Bashan and De-Bashan, 2002). Systemic research into the joint culture of basidiomycetes with bacteria from the genus *Azospirillum* under artificial conditions was lacking in the literature until studies were initiated at the Laboratory of Microbiology of IBPPM RAS in relation to the double culture of *Lentinula edodes* (shiitake mushrooms) strain F-249 and *Azospirillum brasilense* Sp7 (Nikitina *et al.*, 2006; Tsivileva *et al.*, 2010). The effect of *A. brasilense* Sp7 on the growth and morphological peculiarities of *L. ådodes* F-249 was determined (Loshchinina *et al.*, 2006; 2012), but the reasons for enhanced development of the mycelium remain unclear. Currently, a search for sufficiently effective strains of azospirilla with respect to mushrooms from different systematic groups is a necessity.

PGPR INTERACTIONS WITH MYCORRHIZAL FUNGI

The bacteria involved in the associative and symbiotic relations with plant roots are known to contribute positively to plant metabolism and growth parameters. Such bacteria are called "Plant growth-promoting rhizobacteria" (PGPR), i.e., rhizobacteria facilitating plant growth (Bhat *et al.*, 2019; Kumar *et al.*, 2019; Siyar *et al.*, 2019; Goswami and Deka, 2020). The phytostimulating properties of PGPR representatives such as *Azospirillum* have been well studied (e.g., Fukami *et al.*, 2018).

PGPR capable of positively influencing the development of mycorrhizal structures or mycorrhizal fungi during the formation of mycorrhizal symbiosis constitute the "Mycorrhization helper bacteria" (MHB) group (Labutova, 2009; Wang *et al.*, 2015; Fu *et al.*, 2016). The intense citation of works that have dealt with mycorrhizal fungi is out of the scope of this review. We will give a few representative examples. *Pseudomonas fluorescens* can exert a positive effect on the mycelial development of the ectomycorrhizal fungus *Laccaria bicolor in vitro* by means of the production of thiamine assimilated by the fungus. At the same time, *L. bicolor* is capable of accumulating trehalose in hyphae, which promotes the growth of *P. fluorescens* colonies. Both organisms demonstrate synergetic interactions important for their vital activity (Deveau *et al.*, 2010). Endophytic bacteria *Pseudomonas aeruginosa* and *Burkholderia cepacia* form associations with the mycorrhizal fungi *Glomus intraradices* and *Glomus clarum* and are capable of positively influencing the mycelium of these fungi (Sundram *et al.*, 2011). Even in the absence of a host plant, the mutual positive effects of mycorrhizal fungi and MHB are manifested (Frey-Klett *et al.*, 2007). MHB can be non-specific and promote mycorrhizal formation by various fungi (Aspray *et al.*, 2006).

The reasons for studying different aspects of favourable MHB action on mycorrhizal basidiomycetes are clear, but the mechanisms responsible for the favourable effect of soil bacteria on the mycorrhizal fungi are not clear (Obase, 2019; Zhang *et al.*, 2019).

PGPR INTERACTIONS WITH CULTIVABLE MUSHROOMS

Putting into practice the biological techniques of mycelium growth stimulation and its protection from interfering microbiota would allow one to improve the growing technology via a less prolonged cultivation period and concurrent suppression of the contaminating microbiota. Research into microbial populations isolated from the surface of mycelium during the formation of *Pleurotus ostreatus* fruiting bodies was performed. Dominating the bacterial community, *Pseudomonas* spp. facilitated fruiting when used as the inoculum for pure cultures of the basidiomycete *in vitro* (Cho *et al*., 2003). In double culture of the same basidiomycete with *Ps. fluorescens*, the formation of bacterial biofilms was observed. The biofilm was successfully applied by the authors (Jayasinghearachchi and Seneviratae, 2010) in their experiments with tomato plants. During the joint cultivation of *P. ostreatus* with a strain of the diazotroph *Bradyrhizobium elkanii*, not only dense colonisation of mycelium by bacteria but also an active nitrogen fixation within the biofilm on hyphae was observed (Jayasinghearachchi and Seneviratae, 2010). More recently, the same mushroom attracted the attention of Febriansyah and co-authors (Febriansyah *et al.*, 2018), who studied the

effect of a bacterial cell-free extract on *P. ostreatus* mycelium development. The nineteen bacteria strains that were tested led to an increase in mycelial growth, but without statistically significant influence. The indole-3-acetic acid content was assayed for three chosen bacterial isolates, *Bacillus cereus*, *Bacillus aryabhattai*, and *Acinetobacter pittii* (Febriansyah *et al.*, 2018), and of these, the highest content was produced by *Bacillus aryabhattai* strain B8W22.

Agaricus bisporus is an edible mushroom cultivated for food worldwide. In a study of *A. bisporus* vegetative growth on compost, the presence of bacteria in the casing layer strongly influenced the fruiting body initiation of this mushroom (Ebadi *et al.*, 2012). In a later study, a greenhouse experiment was conducted using numerous bacterial isolates, where sixty rhizobia and thirty pseudomonades strains were tested to estimate the effect of these plant growth-promoting bacteria on mushroom fruiting body formation. The rhizobial bacteria were *Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *phaseoli*, and the pseudomonades were *Pseudomonas fluorescens* species. Provided that the growth-promoting bacteria were implemented in *A. bisporus* cultivation on compost, increased yield and quality of this edible mushroom fruit bodies were obtained. Thus, PGPR was beneficial in the production of healthy food (Ebadi *et al.*, 2012).

Zarenejad and coauthors (Zarenejad *et al.*, 2012) evaluated the assumed mushroom-growth-promoting bacteria (MGPB) from soil samples of *A. bisporus* mushroom farms. Two *Pseudomonas putida* strains, which were isolated from the casing layers of *A. bisporus,* were reported to be potent MGPB (Zarenejad *et al.*, 2012) and showed positive effects on *A. bisporus* production.

It seems clear that bacteria inhabiting the casing soil and compost may colonise the fungal hyphae and then enter the fruiting body of *Agaricus* species mushrooms. That is why the microbes in the mushroom fruiting bodies have received some attention. Xiang and co-authors reported (Xiang *et al.*, 2017) on the diversity of bacterial isolates from the fruiting body of *A. bisporus*. Seven bacterial families and 55 bacterial strains were isolated. *Bacillus* spp. was the dominant genus in the mushrooms. However, only additional studies can clarify whether these isolates are capable of serving as a growth-promoting factor for *A. bisporus*.

Fifty-six bacterial isolates were collected from the casing soil of another species of *Agaricus*, *A. blazei*, in order to estimate their effect on mushroom mycelial growth and productivity (Young *et al.*, 2013). A few isolates were selected and shortlisted to test their effects on *A. blazei* productivity: *Advenella incenata*, *Agaricicola taiwanensis*, *Curtobacterium citreum*, *Gordonia hydrophobica*, *Microbacterium humi*, and *Streptomyces violaceorubidus*. Several of the tested species promoted *A. blazei* fruiting, which efficiently reduced the required time for harvesting and increased the total fresh matter yield to different levels. In the sterilised casing soil, inoculation with *Exiguobacterium* sp. appeared to be capable of shortening the period of time until harvest, and compared to the non-inoculated control, it increased both the fresh fungal biomass and total polysaccharide content in this mushroom (Young *et al.*, 2012).

Taking into consideration the conducted studies on the PGPR interactions with cultivable mushrooms, we should recognise that the exact role of PGPR in increasing the growth and yield of cultivable mushrooms has not been clearly elucidated. The number of mushrooms studied has been very limited and restricted to the most common *Agaricus bisporus*, *A. blazei*, and *Pleurotus ostreatus,* and the same is true for the presumably beneficial bacteria, which has been restricted to rhizobia and pseudomonades. Such an approach is partially substantiated by previous studies, e.g., it is known that fluorescent *Pseudomonas* spp. accounts for 14–41% of the overall bacteria found in the casing layer for *A. bisporus* growth (Zarenejad *et al.*, 2012). Frequently, in this type of study, a substantial number of bacterial isolates are studied as potent MGPBs. However, these studies tend to start from a total analogy with plant benefits gained from PGPR, where phytohormone production, phosphate solubilisation, siderophore secretion, antipathogenic properties, nitrogen-fixation capability, or the secretion of cellulase cause the improvements in plant development and yield. Unfortunately, the aforementioned biochemical properties fail to make these bacteria potent MGPB. Despite previous assessment (Xiang *et al.*, 2017), the screening of MGPB for mushroom growth remains limited, and thus the issue of feasible bacterial inoculants ready to be applied to the mushroom production process is still unresolved.

FUNGAL-*AZOSPIRILLUM* INTERACTIONS

Azospirilla as **MHB.** Mycorrhization helper bacteria also include representatives of the *Azospirillum* genus (Bianciotto *et al.*, 2001; Alarcon *et al.*, 2002; Russo *et al.*, 2008; Choudhary *et al.*, 2016; 2017). For example, in a series of experiments on crops, the inoculums of the fungus *Glomus mosseae* and bacteria *Azospirillum brasilense* were introduced in the soil for selected plants. In the presence of one of the inoculums, an appreciable increase in plant biomass was observed; however, with the introduction of both inoculums, the plant biomass was much greater (Sridevi and Ramakrishnan, 2010; Bama and Ramakrishnan, 2010). Studies have confirmed that nitrogen-fixing bacteria are associated with mycorrhizal fungi, spores, and fruiting bodies (Levy *et al.*, 2003; Duponnois and Lesueur, 2004).

Inhabiting the root zone of the soil, bacteria enter into interactions with mycorrhizal fungi, but the group of higher fungi that do not form mycorrhiza is also large. Many medicinal and edible mushrooms are xylotrophs. Their associations with bacteria have been poorly studied in comparison to the mycorrhizal components.

Our contribution to the area. The feasibility of submerged joint cultivation of the basidiomycetes *Flammulina*

velutipes, *Ganoderma lucidum*, and *Pleurotus ostreatus* with the bacteria *Azospirillum brasilense* was evaluated, and the appropriate conditions were screened and selected (Shaternikov *et al.*, 2018; Tsivileva *et al.*, 2020a; 2020b). The choice of mushroom objects was based on the best properties of these cultivable basidiomycetes. Winter mushroom *Flammulina velutipes* is a source of valuable nutritional food ingredients (Su *et al.*, 2019; Nie *et al.*, 2019), and it provides a natural basis for developing high-performance pharmaceuticals (Huang *et al.*, 2019) and veterinary and medicinal preparations (Mahfuz *et al.*, 2019; Zhao *et al.*, 2019). The extremely important biologically active extracts and compounds from lacquered polypore *Ganoderma lucidum* exert pharmacological effects on tumour cells. Extensive research over the last 10 years has provided evidence of the anticancer activities of both the triterpenoids isolated from *Ganoderma* (Wu *et al.*, 2013) and the carbohydrate-enriched crude extracts from this fungi (Osińska-Jaroszuk *et al.*, 2014). *Ganoderma* species exhibit a broad spectrum of antibacterial, antiviral (Gao *et al.*, 2003), immunostimulatory (Osińska-Jaroszuk *et al.*, 2014), cytotoxic (De S. Pereira-Jr *et al.*, 2013), and antifungal (Sridhar *et al.*, 2011) activities. The studies of the antibacterial action of *Ganoderma* extracts have not been restricted to *G. lucidum* (Prasad and Wesely, 2008), and other species were also tested (Osińska-Jaroszuk *et al.*, 2014; De S. Pereira-Jr *et al.*, 2013; Ofodile *et al.*, 2012).

The xylotrophic basidiomycete *Pleurotus ostreatus* (oyster mushroom) has long been known for its excellent edible and medicinal properties (Roy and Prasad, 2013). Artificial growth of oyster mushrooms is of great importance owing primarily to the high productivity and valuable nutritional properties of this culture. Mycelium of *P. ostreatus* contain a large proteinaceous component (Majesty *et al.*, 2019; Mutukwa *et al.*, 2019). Therefore, fruiting bodies can be obtained using solid substrates unlikely to be applicable to any other purposes (non-food agricultural and industrial wastes) (Nam *et al.*, 2018; Phan *et al.*, 2019; Sangeetha *et al.*, 2019). Contemporary studies on the growth of the basidiomycete *P. ostreatus* in liquid media are targeted to the development of submerged culture technology to yield mycelial biomass for use as food supplements, fodders, various physiologically active preparations (Vetvicka *et al.*, 2019; Zhu *et al.*, 2019), and biotechnologically valuable products (Pozdnyakova *et al.*, 2018; Karthikeyan *et al.*, 2019; Noman *et al.*, 2019). The submerged culture technique is commonly recognised as a rapid and efficient method for seeding mycelium production for the wide-scale growth of mushrooms (Bamigboye *et al.*, 2019; Zhang *et al.*, 2019).

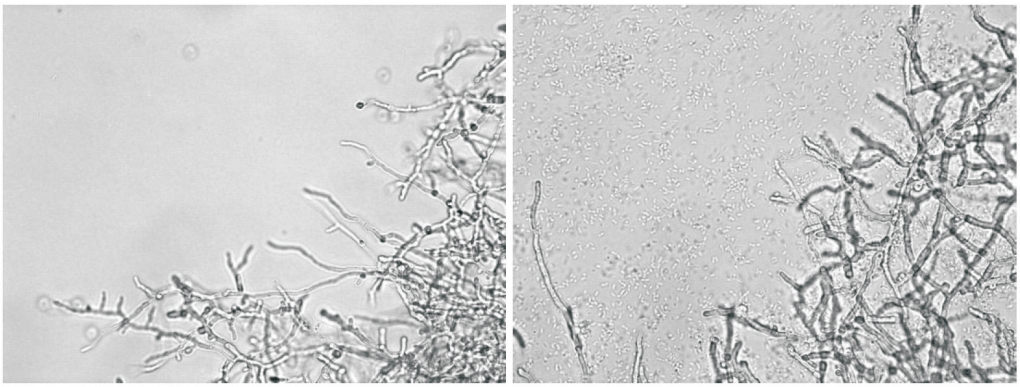
When choosing bacteria applicable for joint cultivation with mushrooms, it seems reasonable to consider first those possessing the ability of favourably influencing plants. Such microorganisms include bacteria from the *Azospirillum* genus. Azospirilla as a representative of the rhizospheric bacterial group that has properties of promoting the growth and development of plants to different extent. The bactericidal and fungicidal activity of azospirilla against several (phyto)pathogenic bacteria and microscopic fungi has been demonstrated (Red'kina, 1990; Bashan and De-Bashan, 2002), which substantiates the choice of these bacterial strains (obtained from the Collection of Rhizosphere Microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms (IBPPM) of the Russian Academy of Sciences (RAS), WFCC No. 975, WDCM No. 1021). Lignin-containing substrate decay by bacteria is catalysed by enzymes analogous to those involved in lignin transformation by higher fungi (Janusz *et al.*, 2017). For the dual-mode culture with xylotrophic basidiomycetes, we chose two strains: a model object for an endophytic symbiosis study — *A. brasilense* Sp245, and the epiphytic strain SR80, both of which show the ability to degrade lignin (Kupryashina *et al.*, 2015).

The parameters for growing the mushrooms with *A. brasilense* strains SR80 or Sp245 were selected. Temperature of cultivation was about 28 °C. The optimal age of liquid seeding co-culture was found to be seven days for an inoculum of *A. brasilense* (Shaternikov *et al.*, 2018). The different nutrient media we tested for co-culture included diluted barley wort; monosaccharide-Asn media without salts (**I**) or with salts (**II**); modified malate–salt medium (Day and Döbereiner, 1976) (**III**); and several formulations based on yeast extract, including high-Glc medium (**IV**), low-Glc medium with peptone (**V**), and the medium (**V**) or concentrated medium (**VI**). The most favourable nutrient media for growing both cultures appeared to be (**I**) and (**II**), and to a lesser extent (**IV**). Other media based on yeast extract — (**V**) and (**VI**) — supported normal growth of both bacterial strains, but (**VI**) was somewhat worse than (**V**). Barley wort appeared to be efficiently applicable for mycelium culture on its own, and the liquid malate formulation (**III**) was used to obtain seeding bacterial suspensions (Shaternikov *et al.*, 2018).

Under optimised conditions of liquid co-culture, the studied azospirilla strains exhibited active motility and were in tight contact with the fungal hyphae. The dry weight of *P. ostreatus* mycelium increased by up to 54% compared to the pure culture of the mushroom (Tsivileva *et al.*, 2020a).

The cell concentration of the bacterial strains, given under specific experimental conditions significantly influenced the binary culture growth. *F. velutipes* cultivated jointly with *A. brasilense* Sp245 at the optimal concentration of bacterial cells in the inoculum allowed us to obtain a dry-biomass of 235% relative to the control. The intensive growth of a mushroom dual culture with azospirilla strains was on medium based on Glc and Fru at a mass proportion of 1:1 and Asn (Tsivileva *et al.*, 2020b).

A problem in cultivation of edible/medicinal basidiomycetes under artificial conditions is the low resistance of the seeding mycelium vs the interfering microflora. Oyster mushroom cultivation in dual culture with growth-promoting bacteria appeared to be an effective biotechnological method for obtaining faster-growing seeding mycelium re-



a b

Fig. 1. Light microscopy of *Pleurotus ostreatus* HK352 grown on wheat powder medium for 21 days in monoculture (a), incl. 14 days in co-culture with *Azospirillum brasilense* SR80 (b).

sistant to contaminants, which in turn allowed the mushroom yield to be greater, and the process of fruiting bodies formation to be less prolonged (Tsivileva *et al.*, 2020a). The characteristics of *P. ostreatus* fruiting body development and decrease of contamination caused by the implemented azospirilla were estimated. Mixed bacterial-fungal cultures of *P. ostreatus* with *A. brasilense* SR80 were used as the seeding material (publication in preparation). *P. ostreatus* HK352 grown on wheat powder medium in co-culture with *A. brasilense* SR80 (Fig. 1 b) exhibited long and densely branched hyphae, even better-developed compared to those in monoculture (Fig.1 a), and appeared to possess the highest efficiency in parameters such as submerged mycelial biomass accumulation, the rate of grain substrate colonisation, and the abundance of fruiting-body formation (publication in preparation).

Implementation of plant growth-promoting bacteria in the biocontrol of diseases of plants, but not of mushrooms, has been previously discussed in comprehensive reviews, e.g., Compant *et al.* (2005). Assays on stimulating of growth of *P. ostreatus* in double culture with *A. brasilense* allowed us to demonstrate the explicit advantages of these dual cultures (over pure cultures of oyster mushrooms) with respect to the enhanced suppression of contaminating microflora (Tsivileva *et al.*, 2020a). The data gained are indicative of the great potential of applying binary mushroom-bacterial cultures for efficiently obtaining mycelial biomass and fruiting of basidiomycetes.

CONCLUSIONS

Understanding the mechanisms and features of associative interactions of bacterial-fungal communities could assist in developing more effective and productive methods of growing agricultural crops, including mushrooms. Edible mushroom cultivation jointly with growth-promoting bacteria appears to be an effective biotechnological method for obtaining faster-growing seeding mycelium resistant to contaminants, that allows the mushroom yield to be greater and better maintained. However, the investigations on xylotrophic basidiomycetes are limited to a few isolated papers. The principal lines of future research into the mushroom-bacterial interactions should be aimed at the following issues: (1) development of the fundamental basis and methods of enhancing xylotrophic basidiomycete resistance against environmental stresses by means of culture in conjunction with growth-promoting bacteria; (2) determining the feasibility and biochemical prerequisites of lowering the biotic influence of interfering microflora on mycelium under the conditions of dual culture of the basidiomycetes with growth-promoting bacteria; and (3) characterising the antimicrobial potentiality of the extracellular compounds in the mushroom cultures influenced by the growth-promoting bacteria. In general, the content of future work should substantiate the prerequisites of applying a mixed culture of valuable mushrooms with MGPB as a kind of seeding material with high protective properties owing to the increased rate of mycelium expansion on a solid nutrient substrate. The plant growth-promoting bacteria, PGPB, have been studied in detail, yet relatively little is known about the mushroom growth-promoting bacteria, MGPB, which are useful for exploiting the cultivable mushroom resources.

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ĒDAMO SĒŅU KULTIVĒŠANU VAR UZLABOT, PATEICOTIES BAKTĒRIJU *AZOSPIRILLUM* AUGŠANAS STIMULĀCIJAI UN BIOKONTROLES POTENCIĀLAM

Apskata raksts, kurā parādīts, ka *Azospirillum* ģints augsnes baktēriju kultivēšana kopā ar ēdamo un ārstniecisko sēņu micēlija biomasu veicina ātrāku un kvalitatīvāku produktu iegūšanu.