

Article

The Impact of Forest Fungi on Promoting Growth and Development of *Brassica napus* L.

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Abstract: Inoculation of plants with fungi has been shown to increase yields by improving germination, seedling vigor, plant growth, root morphogenesis, photosynthesis, and flowering through direct or indirect mechanisms. These mechanisms include solubilization and mineralization of nutrients, facilitating their uptake by plants, regulation of hormone balance, production of volatile organic compounds and microbial enzymes, suppression of plant pathogens, and mitigation of abiotic stresses. In the presented experiments, the effect of selected forest soil fungi on the growth and development of *Brassica napus* L. seedlings was investigated. Inoculation was carried out in vivo and in pot experiments with ectomycorrhizal and saprophytic fungi typical of forest soils: *Collybia tuberosa*, *Clitocybe* sp., *Laccaria laccata*, *Hebeloma mesophaeum*, and *Cyathus olla*. It was shown that all analyzed fungi produced IAA. In the in vitro experiment, *B. napus* inoculated with *L. laccata* showed stimulated root growth and greater number of leaves compared to control plants. A similar stimulatory effect on lateral root formation was observed in cuttings grown in pots in the presence of the *C. olla* fungus. In the pot experiment, the seedlings inoculated with the *L. laccata* fungus also showed increased growth of shoots and biomass. The effect of inoculation with the tested fungal strains, especially *C. olla*, on the growth and development of oilseed rape was probably indirect, as it also contributed to an increase in the number of microorganisms, especially soil bacteria. The expression of the metallothioneins in *B. napus* (*BnMT1-BnMT3*) varied depending on the fungal species. The presence of *C. olla* significantly increased *BnMT2* expression in oilseed rape. It was found that *BnMT1* expression increased and *BnMT3* transcripts decreased in plants growing in the presence of *L. laccata*. This indicates the involvement of *BnMT* in the adaptation of oilseed rape to growth in fungi presence.

Keywords: agroforestry; *Cyathus olla*; *Laccaria laccata*; metallothioneins; rapeseed



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1. Introduction

The close proximity of two areas of completely different land use, and hence soil quality and species richness (in both plants and microorganisms), means that the proximity of forest and cropland creates an area of mutual influence. These relationships can be both harmful and beneficial to both parties. The positive effect of agroforestry can be seen in the production of plant biomass, especially in light soils (and sand in particular) with low water capacity, high permeability, and considerable susceptibility to wind erosion, especially in years when extreme weather situations prevail, e.g., severe drought [1]. It has been shown that habitat improvement through contact with tree stands can increase

yields of spring cereals by 12%, winter cereals by 18%, and root crops and legumes by 5%, especially in dry years [1]. Soil microorganisms interact with each other and with plants, ensuring the maintenance of the ecological balance in the soil. The interactions between plants and microorganisms are very important as they affect the bioavailability of nutrients and thus the yield of plants. As a result, these interactions affect soil welfare and plant survival, especially under unfavorable conditions [2]. Microorganisms, including fungi, have a positive effect on plant vegetation by supporting their growth both directly and indirectly [3,4]. The presence of fungi has a positive effect on soil parameters and plant microbiome, and indirectly on plant growth and resistance [5–7]. Fungi are involved in all major ecological processes, either directly or indirectly. As saprophytes, they control the carbon cycle by preventing excessive accumulation of organic matter through decomposition into compounds that can be used by other organisms [2]. They increase water uptake and mineral supply to plant, and protect them from pathogens and environmental stress. The commonly reported effects are increases in biomass production, flowering, root hair development, and yield [8,9]. In arable soils, symbiotic endomycorrhizal fungi dominate, colonizing the roots of nearly 90% of herbaceous plants, but we find virtually no trace of the presence or activity of ectomycorrhizal fungi and saprotrophic fungi [10], which mostly form symbiotic relationships with forest trees. Saprotrophic fungi have a positive effect on plants, for example, by producing enzymes that facilitate the transformation of humic substances and the formation and mineralization of humus [10]. The production of enzymes enables plants to utilize organic N and P forms that would otherwise be largely unavailable to roots [11]. The production of organic acids leads to chemical leaching of mineral surfaces and mobilizes nutrients such as P, K, Ca, and Mg that can be transferred to host plants [12–15]. However, the role of fungi is not limited to stimulating plant nutrition, but can also be associated with increasing root absorptive surface area and increasing plant stress tolerance, among other effects [16].

Basidiomycete fungi are dominant decomposers and mycorrhizal mutualists in soils [17]. They produce a variety of enzymes that can degrade major components of plant cell walls [18]. In soil they form an extensive network of hyphae, and they secrete metabolites composed of glucolipids, polysaccharides, and glycoproteins. It is known that these products have antimicrobial activities, and play a role in soil aggregation and stabilization [19,20]. Under the influence of fungal inoculation, the gene expression profile changes. Studies have confirmed that among genes overexpressed during fungal-plant interaction are those encoding for metallothioneins (MTs).

Metallothioneins have been identified in angiosperm species, both monocotyledonous and dicotyledonous, and in some gymnosperms, ferns, and bryophytes [21–23]. MTs are low molecular weight proteins (from 5 to 10 kDa). Cysteine residues account for between 15% and 30% of the total amino acid pool, giving MTs the ability to coordinate the binding of various metals from groups 11 and 12 of the periodic table. MTs are involved in the maintenance of micronutrient homeostasis, especially of Zn and Cu, and furthermore in the detoxification of toxic metals (Cd, Pb) [24–26]. The induction of MTs expression depends on many biotic and abiotic factors. *Cis*-elements responsible for the response to the presence of fungal elicitors are present in the MT genes promoters [27]. In higher plants, individual types of metallothioneins show organ- and tissue-specific expression, but expression can differ greatly between species [28–30]. In *Brassica napus*, there are four types of MT (BnMT1–BnMT4), of which type 4 (BnMT4) is expressed only in seeds and early stages of seedling growth [30,31].

Plants in the *Brassicaceae* family are non-mycorrhizal organisms. Glenn et al. [32] showed that the arbuscular mycorrhizal fungus *Glomus mosseae* penetrate *Brassica* roots, but mycelial hyphae, which only penetrate the dead cells of the root cortex, were unable to establish functional cooperation with the plant. However, Tommerup [33] demonstrated the presence of reduced arbuscules in *B. napus*, so that a positive effect of mycorrhizal fungi and other soil fungi on the growth of oilseed rape cannot be excluded. Also of interest are reports of some soil fungi that have the ability to function near plants containing

isothiocyanates, including plants of the *Brassicaceae* family [34]. Studies have shown that basidiomycete fungi, *Piriformospora indica*, promoted the growth and development of sweet potato. Moreover *P. indica* also increased the resistance of potato to biotic and abiotic stresses [35].

The effects of fungi on non-mycorrhizal plants and on stress-tolerance mechanisms are still poorly understood. An example is a study of Zhang et al. [36], in which 97 endophytic fungi were isolated from the roots, stems, and leaves of *B. napus*. The authors identified 40 fungal species, 80% of which belong to the *Ascomycota*. Some of the fungi, i.e., *Aspergillus flavipes*, *Chaetomium globosum*, *Clonostachys rosea*, and *Leptosphaeria biglobosa*, showed antagonism to the oilseed rape pathogen *Sclerotinia sclerotiorum*. Furthermore, *Alternaria alternata* CanL-18, *Fusarium tricinctum* CanR-70 and CanR-71r, and *Leptosphaeria biglobosa* CanS-51 showed a growth-promoting effect on *B. napus*. Studies by Znajewska et al. [7] showed the stimulatory effect of *Trichoderma* sp. fungi on germination and early growth stages of oilseed rape. A similar positive effect on the growth of this crop was found for the presence of *Acaulospora longula*, *Glomus geosporum*, *Glomus mossae*, and *Scutellospora calospora* [37].

The aim of the study was to assess the impact of inoculation with fungi typical of forest soils (*Clitocybe* sp., *Collybia tuberosa*, *Cyathus olla*, *Hebeloma mesophaeum*, and *Laccaria laccata*) on the growth and development of *B. napus* seedlings in vitro and in pot experiments, and to determine changes in bacteria and fungi populations in soil after pot experiments. MT promoters in *Arabidopsis thaliana* and *Oryza sativa* contain regulatory sequences of response to fungal elicitors [38], and we suppose that these genes may be involved in plants' adaptation to changes in environmental conditions. We also studied changes in MT gene expression in rapeseed (*Brassica napus* L.) in response to inoculation with saprofitic fungi.

2. Materials and Methods

The study material comprised seeds and plants of the *B. napus* variety Clipper (Agro-Bras, Wagrowiec, Poland). Seeds previously sterilized in 70% ethyl alcohol for 1 min were used for the in vitro experiment. Basic sterilization was performed with a 30% sodium hypochlorite solution (Domestos, Unilever, Warszawa, Poland) for 30 min. Sterile seeds were also used for the pot experiment. The fungi used in the study were: *Laccaria laccata* (Scop.) Cooke, *Clitocybe* sp., *Collybia tuberosa* (Bull.) P. Kumm., *Hebeloma mesophaeum* (Pers.) Quel, and *Cyathus olla* (Batsch) Pers. which were provided by Prof. Katarzyna Hryniewicz from the Department of Microbiology, Nicolaus Copernicus University in Toruń.

2.1. Preparation of Inoculum from Fungi

Fungal strains were propagated on microbiological PDA medium (Potato Dextrose Agar) (Difco Laboratories, Detroit MI, USA) prepared according to the manufacturer's instructions. Fragments of individual mycelium (5.0 × 5.0 mm) were excised with a scalpel and transferred to fresh PDA medium. The dishes were incubated at 25 °C. Cultivation continued until the dish was overgrown by the fungal strain under study, which was then used to inoculate *B. napus* seedlings in the in vitro and pot experiments.

2.2. In Vitro Experiment

Sterile seeds (20–30 items) were placed in 150 mm diameter Petri dishes containing MS medium without growth regulators (MS0, Murashige and Skoog [30]) and placed in a culture chamber (continuous light, temperature 25 ± 1 °C). Three to four days after sowing, four rapeseed seedlings with similar root length (1–2 cm) were transferred into sterile dishes containing MS0 medium. Two fragments of different strains of mycelium (5.0 × 5.0 mm) were introduced into the root zone between 2 seedlings at the same distance.

Seedlings that were not inoculated with mycelial fragments served as control material. Plates containing the co-culture were wrapped in parafilm and placed vertically. The co-culture was carried out for 10 days under continuous light and at 25 ± 1 °C. The length of

roots and shoots and the number of lateral roots and leaves were analyzed. The experiment was carried out in three replicates.

2.3. Pot Experiment

Seeds of *B. napus* were placed in 2200 mL pots with soil mixed with vermiculite in a ratio of 2:1. The soil had the following parameters: 8.02% organic matter (OM); 3.71% organic carbon (Corg); 0.303% nitrogen (Nt); 1/2 ratio of carbon to nitrogen (C/N); 1.29% CaCO₃; pH 7.27. After germination, four seedlings were left in each pot. On day 28 of the experiment, plants were inoculated with individual fungi so that each plant had a 5.0 × 5.0 mm fragment of mycelium placed in its rhizosphere zone at a depth of 2–3 cm. Inoculation was repeated on day 56 of cultivation. Plants that were not inoculated with fungi served as controls. The experiment was conducted in three replicates. The experiment was conducted for 16 weeks in a culture chamber under continuous light and at a temperature of 25 ± 1 °C. On day 84 of the experiment, chlorophyll content of leaves, shoot length, number of leaves, and fresh and dry biomass were analyzed. Chlorophyll content was determined using a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences, Hudson, NH, USA). Measurements were taken on three leaves of each plant per variant and the values averaged.

2.4. Analysis of Microorganism Abundance in Soil

Averaged soil samples (10 g) of each variant were suspended in 90 mL sterile water and shaken for 10 min. The serial dilution method yielded suspensions at 10^{−5} (for bacteria) and 10^{−3} (for fungi) dilutions. The soil suspensions were inoculated by pour plating. To analyze the abundance of fungi in the soil, 1 mL of the soil suspension was mixed with 20 mL of PDA medium containing tetracycline (0.0001 M). The number of bacteria was determined by pour plating on R2A medium (Reasoner's 2A Agar) with nystatin at a concentration of 0.0001 M. The plates were incubated in a thermostat at 25 °C. Bacterial colonies and fungi were counted after seven days of incubation.

2.5. Analysis of IAA Production by Fungi

IAA and IAA-related compounds were produced according to Gravel et al. [39] with minor adjustments. Liquid cultures of fungi were prepared in 100 mL flasks containing 20 mL TSB medium supplemented with 200 µg/mL L-tryptophan or not (control). The culture medium was inoculated with fungal cultures using two PDA disks. The samples were incubated for two weeks at RT on a rotary shaker. Two mL of the supernatant was collected and mixed with Salkowski reagent (0.5M FeCl₃:H₂O:70% HClO₄, 2:49:49). After 20 min incubation at RT, absorbance was measured at 535 nm. The concentration of IAA and IAA-related compounds was determined by comparison with a standard curve prepared using serial dilutions of IAA solution (Sigma-Aldrich, Darmstadt, Germany) in TSB.

2.6. Isolation of Total RNA and Reverse Transcription Reaction

Total RNA was isolated from the leaves of the plants after three months of rapeseed cultivation using TRI Reagent (Sigma-Aldrich®) according to the procedure of Chomczyński and Sacchi [40]. The quantity and quality of nucleic acids were evaluated based on the results of spectrophotometric measurement using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoretic separation in 1% agarose gels. High-quality RNA preparations were used for further analysis, where the A₂₆₀/A₂₈₀ ratio was approximately 2.0 and the electropherogram of the tested RNA preparations was normal.

The 1.5 micrograms of RNA were treated with 200 U DNase I (Promega GmbH, Mannheim, Germany) and incubated at 37 °C for 30 min. The enzyme was inactivated by the addition of 0.025 mM EDTA at 65 °C for 10 min. The whole extract was used for reverse transcription reactions with MMLV-RT (Novozymes, Bagsværd, Denmark) according to the protocol described by Dąbrowska et al. [41].

2.7. Semi-Quantitative RT-PCR (sqRT-PCR)

Rapeseed metallothionein gene sequences from GenBank NCBI (*BnMT1*: accession no. JX035784.1, *BnMT2*: JX103200.1, *BnMT3*: JX103201.1) were used for expression analysis. Semi-quantitative RT-PCR (sqRT-PCR) was used to evaluate the effects of ectomycorrhizal fungi on mRNA levels of *MT* genes in leaves: *BnMT1*, *BnMT2*, *BnMT3*. PCRs were optimized for analysis of the genes in this work. The level of the actin gene (*Bn5S*) was used as an internal standard. The PCR mixture included cDNA as template, 0.4 µL of 10 µM of each primer (Table 1), 0.4 µL of 10 mM dNTPs, 2 µL of 10 × buffer, and 1.25 U of OptiTaQ DNA polymerase (EURx, Gdańsk, Poland) in a total volume of 20 µL. The thermal cycling conditions were: 95 °C for 30 s; 52 °C (*BnMT1*, *BnMT2*) or 55 °C (*Bn5S*) or 56 °C (*BnMT3*) for 45 s; and 72 °C for 35 s for 26 cycles (*BnMT3*); 30 × (*BnMT1*, *BnMT2*, *BnAC*). PCR products were separated in 1.5% agarose gels stained with EtBr in TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA) for 40 min at 80 V. After electrophoresis, the gels were visualized under UV, and their images were used to quantify the amount of PCR product by densitometric examination. ImageGauge 3.46. software (FujiFilm, Tokyo, Japan) was used for signal quantification. Each reaction was repeated three times, and the error bars represent the standard deviation of the mean. Oligonucleotides (Table 1) were synthesized, and the sqRT-PCR products were sequenced in the DNA Synthesis and Sequencing Laboratory of IBB PAN in Warsaw (Poland) to confirm that the amplification had been performed correctly.

Table 1. Oligonucleotides used to determine the expression of *BnMT1*–*BnMT3* in rapeseed leaves.

Primer Name	Sequence 5'–3'	Application
<i>BnMT1_for</i> <i>BnMT1_rev</i>	TGGCAGGTTCTAACTGTGGA CAAATGAAAACATTATACACCACACA	expression of <i>BnMT1</i> gene
<i>BnMT2_for</i> <i>BnMT2_rev</i>	TCAATTTGATTAACATTCTCTGCT AAGCCTGCAGCCATTATTACA	expression of <i>BnMT2</i> gene
<i>BnMT3_for</i> <i>BnMT3_rev</i>	GCAAAACAACAAAACACACACA CCATTACATCACACACCATGC	expression of <i>BnMT3</i> gene
<i>Bn5S_for</i> <i>Bn5S_rev</i>	AGTCGCACAAATCGTGTCTG TCCATGCTCTCAGCATCAAC	expression of <i>Bn5S</i> reference gene

2.8. Bioinformatic Analysis of *B. napus* Metallothionein Gene Promoters

Promoter sequence of the 17 analyzed genes (714–1500 bp long) were derived from GenBank NCBI (National Center for Biotechnology Information) database (Table S1).

The promoter sequences were used for searching the databases PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 26 November 2021) [42] and PlantPAN 2.0 (an2.itps.ncku.edu.tw) to identify *cis*-elements.

2.9. Statistical Analysis

Statistical analysis was performed using the program PAST (Paleontological Statistics) [42]. The statistical significance of the results of the physiological parameters of the *in vitro* and *in vivo* experiments was determined by a one-way test ANOVA supported by a Kruskal–Wallis test. The Tukey test was used for the results of the soil microbial cultures from the pot experiments. In both cases, the significance threshold was set at ≤ 0.05 .

3. Results

3.1. Analysis of the Effect of Ectomycorrhizal Fungi on the Growth of Rapeseed Seedlings *In Vitro*

The presence of *L. laccata*, *C. tuberosa*, and *C. olla* fungi stimulated root growth compared to non-inoculated seedlings (the control), although the differences were not statistically significant. Conversely, inoculation with *C. rivulosa* fungi significantly inhibited their growth (Table 2). No statistically significant stimulatory effect on lateral root formation was observed in any of the variants, although intense lateral root development was observed in

seedlings growing in the presence of *C. olla* and *C. tuberosa* fungi (Figure 1). Shoot growth was also stimulated by the fungi *C. olla* and *C. tuberosa*, although the differences between the inoculated plants and the control were not statistically significant. No statistically significant differences were found in the number of leaves of rapeseed seedlings inoculated with fungi, although *L. laccata* and *C. olla* had a stimulatory effect (Table 2). Formation of four or even five leaves was observed in plants inoculated with these fungi, which was not observed in any of the other variants.

Table 2. Biometric parameters of rapeseed on day 10 of in vitro culture (mean value $\pm$ standard deviation) after inoculation with *L. laccata*, *C. tuberosa*, *Clitocybe* sp., *H. mesophaeum*, *C. olla*. Values marked with different letters differ significantly at $p \leq 0.05$.

Variants of Experience	Length of Roots (mm)	Number of Lateral Roots	Length of Shoots (mm)	Number of Leaves
control	114.6 $\pm$ 16.9 ^a	40.2 $\pm$ 7.3 ^{ab}	54.0 $\pm$ 10.7 ^{ab}	3.0 $\pm$ 1.1 ^{ab}
<i>L. laccata</i>	131.4 $\pm$ 25.0 ^a	35.0 $\pm$ 19.6 ^{ab}	58.6 $\pm$ 18.9 ^{ab}	3.6 $\pm$ 0.9 ^a
<i>C. tuberosa</i>	127.2 $\pm$ 12.6 ^a	47.7 $\pm$ 9.3 ^{ab}	62.8 $\pm$ 10.7 ^a	3.1 $\pm$ 0.3 ^{ab}
<i>Clitocybe</i> sp.	81.5 $\pm$ 7.4 ^b	43.1 $\pm$ 7.8 ^b	54.3 $\pm$ 13.6 ^{ab}	3.0 $\pm$ 0.0 ^{ab}
<i>H. mesophaeum</i>	112.5 $\pm$ 34.1 ^a	38.7 $\pm$ 7.7 ^b	59.8 $\pm$ 12.2 ^{ab}	3.0 $\pm$ 0.5 ^{ab}
<i>C. olla</i>	117.0 $\pm$ 32.3 ^a	61.0 $\pm$ 26.6 ^a	63.4 $\pm$ 13.3 ^a	3.6 $\pm$ 0.8 ^a

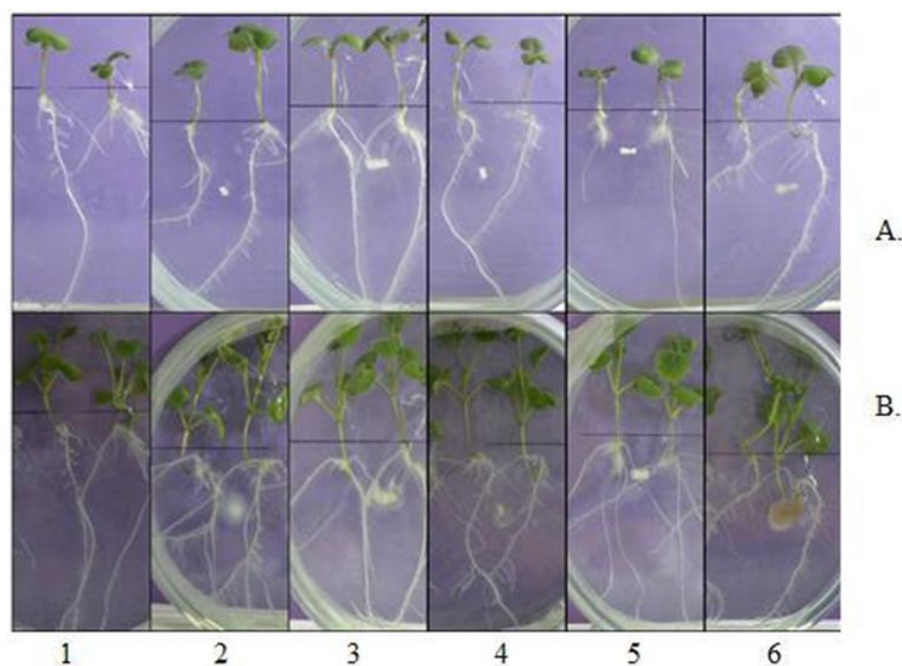


Figure 1. Rapeseed seedlings on day 4 in vitro (A) and day 10 in vitro (B). Order of variants: control (1), *L. laccata* (2), *C. tuberosa* (3), *Clitocybe* sp. (4), *H. mesophaeum* (5), *C. olla* (6).

3.2. Analysis of the Effect of Fungi on the Growth and Development of Rapeseed Shoots in a Pot Experiment

The highest chlorophyll levels were found in the leaves of non-inoculated plants and of plants inoculated with *L. laccata*, *H. mesophaeum*, and *C. olla*. Conversely, statistically significantly lower chlorophyll content was found in the leaves of plants inoculated with *C. tuberosa* and *Clitocybe* sp., which were 2 and 1.4 times lower, respectively, compared to control plants (Table 3). Shoot growth was also inhibited after fungal inoculation—most severely in *C. tuberosa*, *C. olla*, and *Clitocybe* sp.—and the differences were statistically significant. However, this was due to the shortening of internodes rather than their number, as indicated by the higher number of leaves in plants inoculated with the fungus *C. olla*. Only in plants inoculated with the *H. mesophaeum* was a lower number observed. The analysis of fresh and dry biomass of the above ground part of the plants showed that

the plants inoculated with *L. laccata* fungus had the highest biomass, whereas the lowest was recorded in the variants inoculated with *C. tuberosa*, which correlated with the lowest chlorophyll content.

Table 3. Effect of ectomycorrhizal fungus inoculation on leaf chlorophyll content and biometric parameters of *B. napus* L. on day 84 of cultivation in vivo (mean value $\pm$ standard deviation). Values marked with different letters differ significantly at $p \leq 0.05$.

Variants of Experience	Chlorophyll (mg cm ³)	Length of Shoots (mm)	Number of Leaves	Fresh Biomass (g)	Dry Biomass (g)
control	0.815 $\pm$ 0.412 ^a	665.3 $\pm$ 121.9 ^a	7.3 $\pm$ 1.4 ^{bc}	209.40 $\pm$ 1.77 ^b	17.54 $\pm$ 0.67 ^b
<i>L. laccata</i>	0.718 $\pm$ 0.412 ^{abc}	609.2 $\pm$ 169.4 ^{ab}	8.4 $\pm$ 3.5 ^{abc}	264.26 $\pm$ 11.87 ^a	21.58 $\pm$ 1.23 ^a
<i>C. tuberosa</i>	0.387 $\pm$ 0.222 ^d	473.8 $\pm$ 91.2 ^{bcd}	8.4 $\pm$ 2.6 ^{abc}	46.69 $\pm$ 1.57 ^d	3.68 $\pm$ 0.55 ^e
<i>Clitocybe</i> sp.	0.579 $\pm$ 0.237 ^c	530.0 $\pm$ 122.1 ^{bcd}	9.1 $\pm$ 1.9 ^{ab}	172.56 $\pm$ 3.42 ^c	11.29 $\pm$ 1.13 ^d
<i>H. mesophaeum</i>	0.624 $\pm$ 0.290 ^{abc}	646.60 $\pm$ 137.53 ^{abc}	6.8 $\pm$ 1.3 ^c	166.75 $\pm$ 3.98 ^c	11.64 $\pm$ 0.96 ^d
<i>C. olla</i>	0.636 $\pm$ 0.307 ^{abc}	507.00 $\pm$ 126.67 ^{bcd}	9.8 $\pm$ 2.0 ^a	195.42 $\pm$ 5.06 ^b	14.54 $\pm$ 1.42 ^c

3.3. Analysis of the Microorganism Populations in the Soil after the Pot Experiment

The effect of selected soil fungi on the abundance of microorganisms (bacterial colonies and fungi) in the inoculated soil from the pot experiment was analyzed. The highest number of bacterial colonies was observed in the soil of the variants inoculated with the *C. olla*, *H. mesophaeum*, and *Clitocybe* sp. (Figure 2A). In all soil samples tested that were inoculated with fungi, the number of these microorganisms was higher than in the control sample that contained only native fungal strains. This indicates the presence of the strains used as inoculum in the soil. The highest abundance of fungi was found in the variant containing the soil suspension inoculated with *L. laccata* fungi (Figure 2B). The difference in abundance of the studied microorganisms in the presence of *Clitocybe* sp. was relative to the control variant.

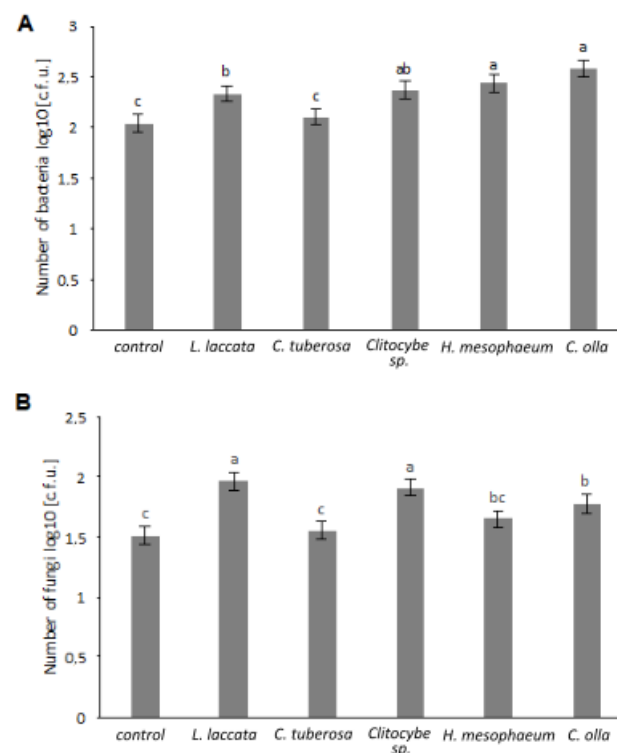


Figure 2. Abundance of bacterial (A) and fungal (B) colonies in soil after a pot experiment after inoculation with *L. laccata*, *C. tuberosa*, *Clitocybe* sp., *H. mesophaeum*, and *C. olla*. Values marked with different letters are significantly different at $p \leq 0.05$.

3.4. Analysis of IAA Production by Fungi

It was found that all the fungi studied produced IAA. In the presence of tryptophan, an inducer of IAA synthesis, *C. olla* and *C. tuberosa* produced the highest amounts of this phytohormone. The strains of *L. laccata* and *H. mesophaeum* showed a two-fold lower activity of IAA synthesis (Figure 3).

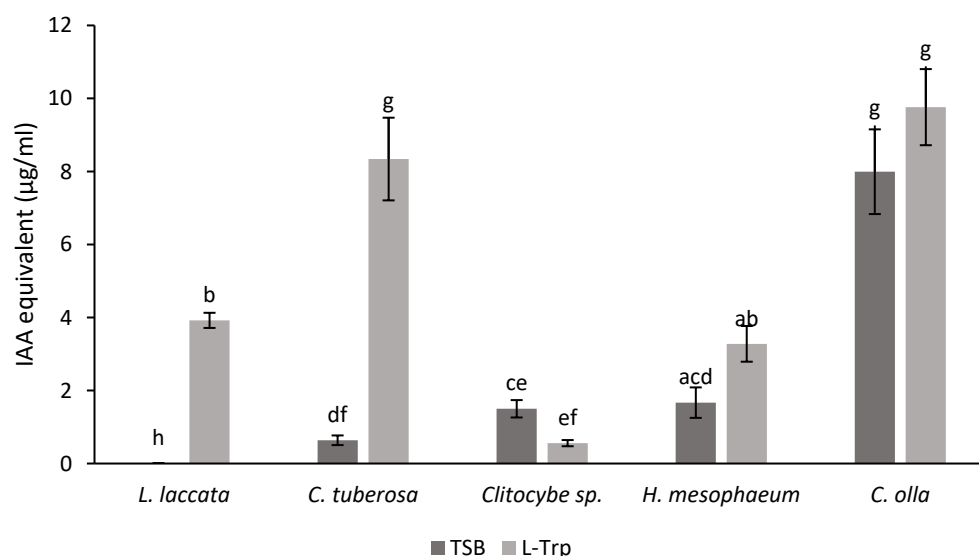


Figure 3. IAA production by studied fungi strains in TBS medium without and with L-tryptophan. Values marked with different letters are significantly different at $p \leq 0.05$.

3.5. In Silico Analysis of BnMT1-BnMT3 Sequences and Expression of These Genes in the Presence of Ectomycorrhizal Fungi

A search of the two databases of promoter regulatory elements, PlantCARE and PlantPan, showed that the *BnMT2* and *BnMT3* gene promoters contain the 5'TTGACC3' sequence of the W-box motif. This is a regulatory *cis*-element involved in the response to fungus elicitors (Table S2) [43]. Additionally, a second regulatory element of response to fungus elicitors was identified in the *BnMT2* promoter sequence—an AT-rich sequence (5'TAAAATACT3', 5'TAAAATAT3') [44]. The expression of *BnMT1-BnMT3* genes of rapeseed non-inoculated and inoculated with fungi was shown to change. The level of *BnMT1* gene transcripts in each of the variants differed from the control. In the case of rapeseed inoculation with fungi such as *L. laccata*, *C. tuberosa*, *H. mesophaeum*, and *C. olla*, the mRNA level of this gene increased relative to the control (Figure 4A). Only for inoculation with *Clitocybe sp.* mycelium were there no significant differences from the control. *BnMT2* gene transcripts were at a similar level in all leaves except for plants inoculated with *C. olla* fungus. The amount of mRNA in the leaves of these plants was significantly higher than in other variants (Figure 4B). Expression of the *BnMT3* gene was significantly lower in the leaves of plants inoculated with *L. laccata* and *C. tuberosa* than in control plants (Figure 4C). Inoculation with the remaining fungi did not significantly change the amounts of transcripts of this gene in rapeseed leaves (Figure 4A–C).

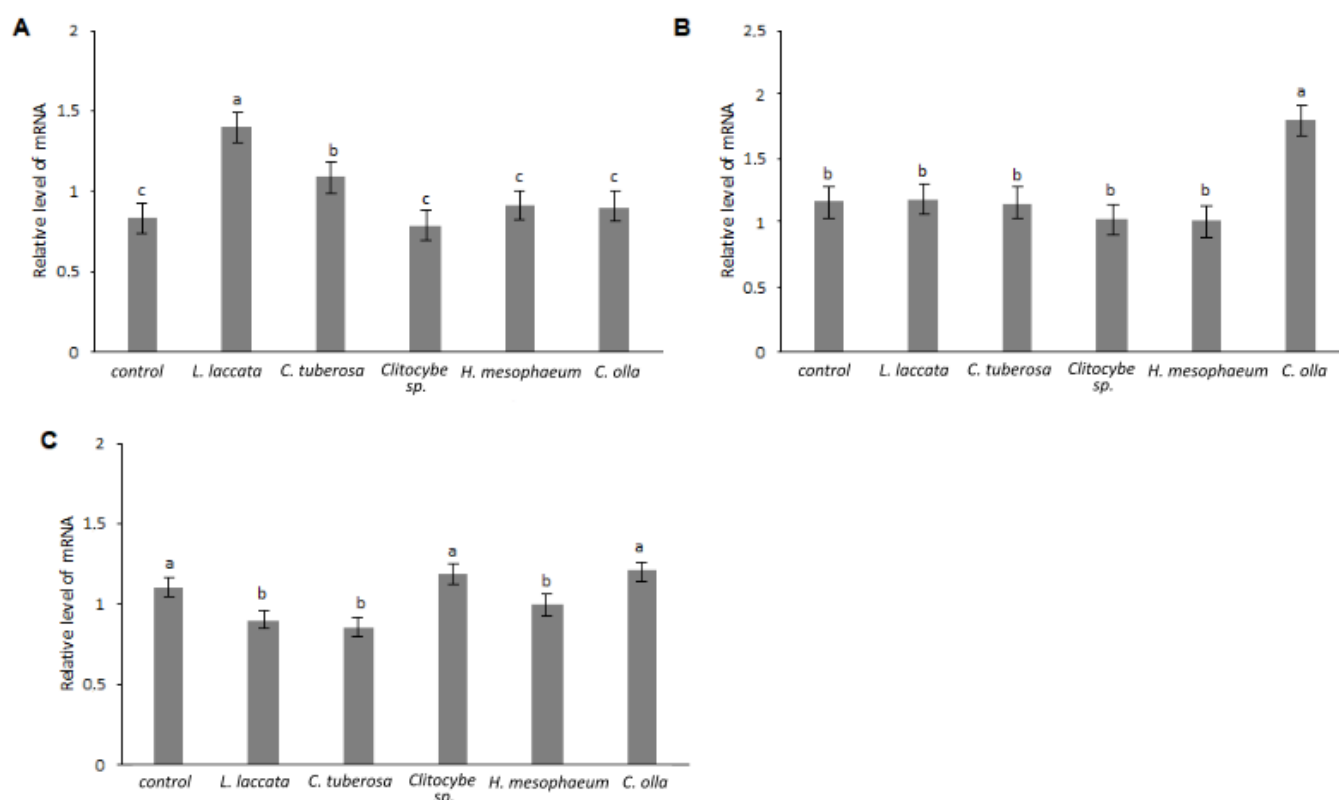


Figure 4. Relative mRNA level of genes (A) *BnMT1*, (B) *BnMT2*, and (C) *BnMT3* in *B. napus* leaves non-inoculated (control) or inoculated with fungi. The graphs show the relative level of *BnMT1*–*BnMT3* gene transcripts expressed in the ratio of the amount of *sqRT*-PCR product for the *BnMT1*–*BnMT3* genes to the amount of *Bn5S* gene product. Different lower case letter means statistical significance at $p \leq 0.05$.

4. Discussion

The neighborhood of forest and arable land favors soil-forming processes and natural colonization by fungi. The presented experiments investigated the influence of soil fungi living in forest areas on an arable crop. However, because not all crops form symbioses, it seemed important to test the hypothesis that the presence of soil fungi can positively influence microbiome and indirectly affect the growth of a non-symbiotic crop such as rapeseed.

The fungi *L. laccata* used in this study have the potential to form ectotrophic mycorrhizae [45]. In an in vitro experiment, rapeseed inoculated with *L. laccata* had a beneficial effect on root length and number of leaves. *C. olla* had a positive effect on lateral roots, shoot length and leaves. Inoculation of plants with *L. laccata* stimulated biomass accumulation, which was probably related to the greater number of leaves compared to control plants, although chlorophyll content was at a comparable level or photosynthesis was more efficient in plants inoculated with this fungus and control plants. This can be confirmed by the studies of Gao and Wu [46], who documented the positive effect of the fungus *Laccaria aemthystea* on photosystem function II in *Pinus* seedlings. A greater biomass of plants inoculated with *L. laccata* was also observed by Mortier et al. [47], who studied the effect of inoculation with this microorganism on the growth of *Pseudotsuga menziesii* (Douglas fir). The stimulatory effect of *L. laccata* is probably also related to its positive effect on soil microflora and macro- and microelement availability. Indirectly, this is evident from the marked difference in the abundance of soil fungi and bacteria observed in cultures of soil suspensions inoculated with this fungus. In previous studies, Dąbrowska et al. [37] observed an indirect effect of arbuscular mycorrhizal fungi such as *A. longula*, *G. geosporum*, *G. mosseae*, and *S. calospora* on the growth of *B. napus*. Previous research showed that the above mycorrhizal fungi stimulated shoot elongation in a sterile medium, although the fresh biomass of the aboveground part of the rapeseed was lower than that of the

control plants. Plants growing in the presence of AMF (arbuscular mycorrhizal fungi) in a non-sterile medium were characterized by greater fresh biomass, shoot length, and number of internodes than plants growing in a sterile medium, probably due to the positive effect of naturally occurring microorganisms in the soil, e.g., rhizosphere bacteria [37].

In the present study, the use of the ectomycorrhizal fungus *H. mesophaeum* as inoculum did not result in significant visible changes in biometric parameters. However, plants inoculated with this fungus in the in vivo experiment had about 20% lower biomass compared to control plants. Studies by Hryniewicz et al. [48] revealed that the presence of the fungus *H. mesophaeum* did not affect the growth of *Salix viminalis*. Only co-inoculation of willow with *H. mesophaeum* and *Bacillus cereus* bacteria promoted biomass production of willow growing in soil contaminated with heavy metals. Inoculated *S. viminalis* plants showed an increase in nitrogen, potassium, phosphorus, and zinc content in shoots. Inoculations with strains of the genera *Hebeloma* and *Laccaria* are available and recommended for forest nurseries, which maintain plant condition and health regardless of changes in environmental conditions [49]. The average number of lateral roots and stimulation of shoot growth and development (stem length and number of leaves) was highest in seedlings inoculated with the fungus *C. olla* after 84 days of growing plants in soil. The growth-promoting effect of this mycelial species is not sufficiently visible due to its slow growth as observed under laboratory conditions (Figure 1). Possibly, the development of the above-ground part of the plants treated with the *C. olla* fungus was related to the increased number of autochthonous (resident) bacteria, which were most numerous in the soil inoculated with the *C. olla* fungus.

It seems that, of the fungi studied, the presence of *L. laccata* and *C. olla* had a beneficial effect on rapeseed and the soil microbiome. Therefore, field trials to confirm the protective role of this fungus in relation to rapeseed are warranted. Several interesting studies have pointed out the role of auxin as plant signaling hormones in plant responses to plant growth-promoting fungi (PGPF) and, in particular, described their involvement in the control of shoot and root development [50]. The inoculated plants showed expression of auxin-regulated genes. As expected, mutations in genes involved in auxin transport or auxin signaling, *AUX1*, *BIG*, *EIR1*, and *AXR1*, reduced the growth-promoting and root development-promoting effects of *T. virens* inoculation in *Arabidopsis*. These results suggest that the promotion of plant growth by *T. virens* occurs through the classical auxin response pathway [8]. Similarly, *Piriformosporia indica*-induced expression of auxin-regulated genes was reported in barley [51] and in Chinese cabbage [9], and its induction was crucial for the strong growth-promoting effect of the fungus. It is suggested that microbial auxin may play a role in altering auxin biosynthesis or signal transduction in the host [52]. Sirrenberg et al. [53] previously found that the phenotype resulting from the interaction of *Arabidopsis* with *Pi. indica* is mimicked by an external application of IAA at a lower concentration than that of the fungus, suggesting a role for exogenous auxin. Similarly, Contreras-Cornejo et al. [8] showed that treatment with IAA and indole-3-acetaldehyde rescues the root hair defect phenotype of the *rhid6* mutant. This result may suggest that microbial auxin is involved in the suppression of root hair formation of *rhid6*. Thus, auxin may act as a reciprocal signaling molecule in the interaction between plants and microbes. Microorganisms that form various symbiotic interactions with plants play an important role in increasing the efficiency of plant production and in enabling plant colonization and survival in natural environments. Synthesis of phytohormones by microorganisms is necessary to form lasting associations with plant roots. Plant hormones cause cell division and differentiation in the meristematic root tissue so that the root elongates and forms more hairs and branches [54].

Several mycorrhizal fungi that may be present in forest soils were selected for the study. Because regulatory sequences responsible for plant responses to fungal elicitors have been identified in the promoters of the *MT A. thaliana* and *O. sativa* genes [27], we suppose that these may be involved in plants' adaptation to changes in environmental conditions. The change in the expression of plant metallothionein genes is related to the action of various

factors, both endogenous and exogenous, on the body [38]. It seems that the functions of MTs in plants are not limited to heavy metals, but that these proteins may act as stress proteins, protecting the body from various adverse environmental factors and enabling adaptation to changing conditions [21,23,38]. The expression profile of metallothionein plant genes can change under the influence of viruses, bacteria, and fungi. In silico analyses of the *BnMT1-BnMT3* sequence revealed the presence of regulatory elements for the response to fungal elicitors (W-box, AT-rich element). Similarly, in previous studies, Dąbrowska et al. [27] demonstrated the presence of a W-box motif in five metallothionein promoters, including four in *Arabidopsis* (*AtMT1B*, *AtMT2A*, *AtMT2B*, and *AtEC*) and *O. sativa* *OsMT2B*. The effect that infection with pathogenic fungi has on altering the expression of plant MTs is described in detail in the literature. Type 2 and 3 MTs in *Abutilon theophrasti* were expressed at higher levels on the first day of infection with *Colletotrichum coccodes* than in control plants [55]. Miles et al. [56] also compared the expression level of MT genes in response to *Colletotrichum acutatum* in two blueberry cultivars. Studies have confirmed that one of the genes overexpressed during pathogen–plant interaction is MT. Moreover, the increase in the expression of this gene was greater in a cultivar resistant to the fungal pathogen. Infection of transformed *A. thaliana* plants with the pathogenic fungus *Pernospora parasitica* results in different expression of the Gus reporter gene of the type 1 promoter of *B. napus* MT than in non-inoculated plants. In control plants, expression of the reporter gene was restricted only to the vascular bundles after seven days, whereas in infected plants it covered the entire cotyledon [29]. The expression of MTs genes is also changed in plants inoculated with bacteria that promote their growth, which is confirmed by the research of Hryniewicz et al. [48]. In contrast, in oil palm (*Elaeis guineensis*), the expression of type-3 MT increased in roots and leaves as a result of inoculation with the pathogenic fungus *Ganoderma bininense* and the symbiotic fungus *Trichoderma harzianum* [30]. Research by Voiblet et al. [57] showed that MT expression was downregulated in *Eucalyptus globus* in the presence of the fungus *Pisolithus tinctorius*. *PnMTA* transcripts were found by Rivera-Bicerell et al. [58] in the roots of peas inoculated with *Glomus intraradices* compared to plants not inoculated with AMF (arbuscular mycorrhizal fungi). By contrast, Dąbrowska et al. [59] did not find a change in the level of transcripts of the *Brassica napus* type-2 metallothionein gene (*BnMT2*) in roots in the presence of AM fungi spores, but found an increased number of transcripts in plant leaves after AM inoculation in soil that had been cleared of other microorganisms. High levels of *LcMT2* were also observed in *Lycopersicon esculentum* growing in soil containing heavy metals, but the presence of AMF caused a decrease in expression of this gene [60]. By contrast, gene expression in *Populus alba* (*PaMT1-3*) varied depending on the presence of AMF and soil type [61]. Changes in the level of MT expression are also influenced by such factors as: phytohormones [9], physiological drought [62,63], osmotic stress [64], temperature fluctuations [65], and excessively high or low light intensity [66]. Mechanical injuries to plants, which contribute to the formation of reactive oxygen species (ROS) [67], also induce the expression of MT genes. One example is the induction of expression by *MT1*, *MT2*, and *MT3* in *B. rapa* [68]. Research by Mierek-Adamska et al. [30] showed that *BnMT1-BnMT4* in rapeseed perform an antioxidant function when they are over-expressed in bacteria. Under stress conditions, plants activate additional processes to allow survival, with genes encoding oxidative enzymes and MTs being expressed [69].

5. Conclusions

Brassica napus, a non-mycorrhizal plant, interacts with selected fungi. Positive effects were demonstrated for *L. laccata* and *C. olla*. The fungi stimulated the growth of the roots and the above-ground part of the plants. The presence of the tested fungi, especially *C. olla*, increased the number of microorganisms in the soil, especially bacteria. This may indicate an indirect positive effect of this fungus on the growth and development of rapeseed.

The results of the in silico analyses concerning the promoter sequences of metallothionein genes were confirmed experimentally. The presence of regulatory sequences for

responses to fungal elicitors suggests the involvement of metallothionein interaction between plant and microorganism (fungus). It seems to us that MTs are necessary, firstly, to remove reactive oxygen species (ROS) during the stimulation of plant growth by fungi, when there is increased uptake of water and other nutrients, resulting in more intense cell division, where ROS occurs. Then the plants also have an increased need for metal ions such as Cu and Zn, whose reservoir is precisely metallothioneins.

We intend to continue research to characterize microorganisms in detail. The research will focus on checking the protective role of fungi, especially *L. laccata* and *C. olla*, against rape pathogens. The long-term goal is to develop a biopreparation that would support the growth of oilseed rape under conditions of dehydration stress and pathogen attack.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11122475/s1>, Table S1. Access numbers, length, and chromosomal location of the analysed rapeseed MT promoter sequences obtained from the NCBI database, Table S2. Selected *cis*-regulatory elements found in the rapeseed MT promoter sequences.

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