



# Biotization of highbush blueberry with ericoid mycorrhizal and endophytic fungi improves plant growth and vitality

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## Abstract

Ecological methods are becoming increasingly popular. One of these methods is plant biotization. In our paper, we focus on selection of *Vaccinium corymbosum* hairy root-inhabiting fungi for plant growth promotion in a single microorganism inoculation setup and then composed a multiorganismal inoculum enriched with a representative of another group of fungi, leaf endophytes. The hairy roots of *V. corymbosum* hosted 13 fungal taxa. In single inoculation of the plant with fungal strains, the most beneficial for plant growth were *Oidiodendron maius* and *Phialocephala fortinii*. Additional inoculation of the plants with three root symbiotic fungi (*O. maius*, *Hymenoscyphus* sp. and *P. fortinii*) and with the endophytic fungus *Xylaria* sp. increased plant height in laboratory experiments. On a semi-industrial scale, inoculation improved plant biomass and vitality. Therefore, the amendment of root-associated fungal communities with a mixture of ericoid mycorrhizal and endophytic fungi may represent an alternative to conventional fertilization and pesticide application in large-scale blueberry production.

## Key points

- *O. maius* and *P. fortinii* significantly stimulated *V. corymbosum* growth in a single inoculation.
- Multimicroorganismal inoculum increased plant biomass and vitality.
- Blueberry biotization with ericoid and endophytic fungi is recommended.

**Keywords** Ericoid fungi · Endophytes · *Vaccinium corymbosum* · *Oidiodendron* · *Xylaria*

## Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a plant species native to North America. In recent years, the production of *V. corymbosum* has increased significantly in many regions of the world, including the USA, South America and Europe (Strik and Yarborough 2005, [www.cbi.eu](http://www.cbi.eu)), and reached over 820,000 tons per year in 2019 (FAOSTAT 2019, [www.fao.org](http://www.fao.org)). The global blueberry market is predicted to reach 4.5 billion USD by 2024 ([www.reportlinker.com](http://www.reportlinker.com)).

To gain market advantage on the market, farmers seek new and verified, existing plant production methods to reach higher crop yields and improved fruit quality. Ecological methods are becoming increasingly popular, particularly due to restricting legislation that limits traditional fertilization and pest control utilization. An environmentally friendly alternative method is plant biotization, a new biotechnological approach that improves plant production. It relies on inoculating plants with appropriately selected symbiotic microorganisms, which promote plant growth and stress tolerance (Gianinazzi et al. 2003; Gollotte et al. 2009).

In intensive berry production, plant nutrition and protection are provided mainly by fertilization and pesticides, respectively. These agrotechnical treatments may lead to disturbances in plant symbioses with their native microbiota and deleterious effects on the environment. It has been shown that plant fertilization with excess N and P can decrease mycorrhizal fungal growth (Treseder 2004; Wang et al. 2009) and antioxidant concentrations in fruits, resulting in decreased nutritional value of crops. Blueberries are one of the richest sources of antioxidants among fresh fruits

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and vegetables (Prior et al. 1998); therefore, agrotechnical procedures in the cultivation of blueberries should be carried out with special caution. To provide the highest antioxidant capacity in berry fruits, pesticide and fertilizer utilization should be minimized. Furthermore, new, more ecological methods in field management are needed. The key factor in management programs is to consider plant-microorganism interactions (Gianinazzi et al. 2002; Jeffries et al. 2003).

Blueberries, which are ericoid plants, are associated with specialized mycorrhizas known as ericoid mycorrhizas. Fungal species involved in this type of symbiosis include *Rhizoscyphus ericae* aggregate (Fehrer et al. 2019) (= *Hymenoscyphus ericae* aggregate (anamorph *Scytalidium vaccinii*) (Pearson and Read 1973)), *Oidiodendron* spp. (teleomorph *Myxotrichum* and *Bysoascus*) (Jansa and Vosátka 2000) and *Cadophora* sp. (Bizabani and Dames 2015). Recently, it has been shown that roots, leaves and fruits of highbush blueberry are inhabited by representatives of another group, endophytic fungi, belonging to the following taxa: *Anteaglonium* sp. (Wu et al. 2021), *Penicillium* sp., *Fusarium oxysporum* (Hamim et al. 2017) and *Angustimassarina*, *Dothidea*, *Fellozyma*, *Pseudohyphozyma*, *Hannaella coprosmae* and *Oberwinklerozyma straminea* (Nguyen et al. 2021). Among endophytic bacteria, the most abundant genera hosted by blueberry plants are *Pseudomonas*, *Pantoea*, *Burkholderia* and *Bacillus* (Ortiz-Galeana et al. 2018). In addition, the above-enumerated taxa of endophytic fungi and bacteria and rhizosphere microorganisms can provide many benefits to the plant (Gaskins et al. 1985). Rhizosphere-inhabiting bacteria and fungi, *Pseudomonas fluorescens*, *Pseudomonas corrugata*, *Bacillus pumilus*, *Gliocladium virens* and *Trichoderma harzianum* have been studied as candidates with potential highbush blueberry growth-promoting properties (De Silva et al. 2000).

The majority of available reports concerning plant-fungal symbiosis and plant biotization focus on plant inoculation with a single microorganism, yielding very ambiguous results. In nature, almost all, if not all, plants are colonized not with one microorganism but with communities of microorganisms. These microorganisms play many diverse and important roles in host fitness and drive the functioning of microbial consortia introduced into the rhizosphere (Hu et al. 2016). The effect of multimicroorganismal inocula on plant growth has rarely been studied, but multimicroorganismal inocula usually provide many benefits for the plant (Remans et al. 2008; Liu et al. 2012; Ważny et al. 2018; Yu et al. 2020).

The following hypotheses were verified in this study: (i) fungi isolated from *V. corymbosum* hairy roots applied to plants in culture will improve rooting, growth rate and vitality; (ii) to optimize the plant growth-promoting effect, an appropriate combination of symbiotic microorganisms needs to be applied. Multiorganismal inocula will more effectively biotize plants than inoculation with single

microorganisms. (iii) Efficient biotization with appropriately selected microorganisms can be performed not only in the early stages of plant development but also later, in older plants. Here, we applied the treatment to plants growing in pots for 2 years.

## Materials and methods

### Experimental design

Preparation of highbush blueberry biotization technology was carried out in three steps (Fig. 1). In the first step, ericoid fungi were isolated from the hairy roots of plants and identified. In the second step, a series of experiments in plant growth chambers were carried out to select fungal components for the combined inoculum to improve blueberry growth. In the last step, the efficiency of the selected inoculum was verified on a semi-industrial scale of blueberry production in a greenhouse (polytunnel). In the semi-industrial experiment, we tested the growth parameters of plants inoculated at the moment of transferring them from in vitro to peat substrate (4-week-old plants) and at the moment of transferring (2-year-old) plants cultivated in peat substrate from small to larger pots. In the second case, we wanted to answer a question very frequently asked by blueberry producers: can we effectively biotize older plants?

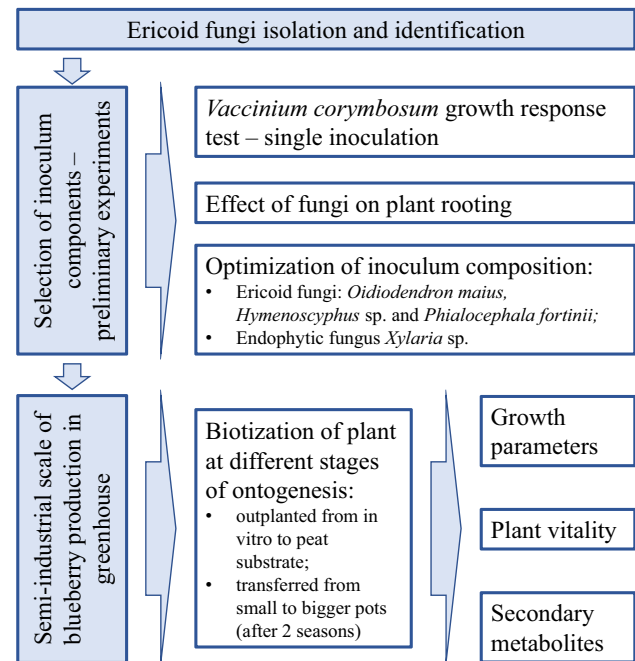


Fig. 1 Experimental design

## Fungal isolation and identification

Fungi were isolated from the hairy roots of the five plants derived from blueberry plantations where very good plant growth without any symptoms of pathogens was observed. Hairy roots were observed using light microscopy and fragments with visible mycelium coils in root cells were selected for fungal isolation. Selected hairy roots were surface sterilized for 50 s in 75% ethanol, followed by thorough washing in sterile distilled water (Turnau et al. 2007). The roots were cut into 3-mm pieces and placed on potato dextrose agar (PDA) medium. Samples were incubated in the dark at 26 °C and inspected every 1–2 days for 4 weeks. Cultures of emerging fungi were transferred to new PDA and incubated in the dark at 26 °C.

Pure cultures of fungi were identified based on morphological and anatomical features and using molecular methods (polymerase chain reaction (PCR) and sequencing of the internal transcribed spacer (ITS) rDNA sequence). DNA was extracted with the Genomic Mini AX Plant (A&A Biotechnology, Gdańsk, PL). The ITS rDNA region was amplified with ITS1F (Gardes and Bruns 1993) and ITS4 primers (White et al. 1990). PCR was performed with 10 ng DNA template, 1 µL of each of the primers at 10 pmol concentration for each sample and Maxima Hot Start Green PCR Master Mix (Thermo Scientific, Waltham, USA) according to the manufacturer's protocol. Isopropanol and 0.3 M sodium acetate precipitation was used for PCR purification (<http://openwetware.org>). Sanger sequencing was performed by the Macrogen Laboratory (Amsterdam, NL). The ITS4 primer was used for reading sequences. The sequences were edited with Chromas software ([www.techneleysium.com.au](http://www.techneleysium.com.au)) and compared with sequences available in NCBI (National Centre for Biotechnology Information; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using the BLASTn algorithm. Sequence data were deposited in the NCBI database under accession numbers OM729672–OM729684. Pure cultures of the four fungal species selected for *V. corymbosum* biotization technology were deposited in the culture collection of the Institute of Agricultural and Food Biotechnology (IAFB) in Poland belonging to the World Data Centre for Microorganisms (WDCM) under accession numbers KKP2073p (*Xylaria* sp.), KKP2075p (*Phialocephala fortinii*), KKP2076p (*Hymenoscyphus* sp.) and KKP2077p (*Oidiiodendron maius*).

## Substrate

Peat (Hollas, Pasłęk, PL) was used as a substrate for the pot experiments. Available P (Colwell 1963) and Kjeldahl N concentrations in the substrates were measured according to Wilke (2005). Briefly, to determine the concentration of N, the samples were dried at 40 °C for 12 h, transferred to glass tubes and digested with 98% H<sub>2</sub>SO<sub>4</sub> at a temperature

of 300 °C in the presence of sodium thiosulfate and the Kjeldahl catalyst. The solutions were distilled with water vapour. The concentration of nitrogen was determined using titration with 0.01 M HCl. To determine the concentration of P, a colorimetric method using a UV–Vis spectrophotometer (Bio Tek Epoch 2; BioTek, Winooski, VT, USA) with the external standard calibration method was used. To determine the potassium concentration, first, the water content in the samples (at 80 °C) was determined using a moisture analyser (Radwag MAC 50/1, Radom, PL), and then the concentration of K was measured using atomic absorption spectrometry (flame atomic absorption spectrometry) in emission mode, with a CSX 260 autosampler (Thermo Scientific, iCE 3000; Thermo Scientific, Waltham, MA, USA). Briefly, the samples were digested in 65% nitric acid (5 mL) for 2 h (room temperature—1 h, boiling point—1 h). After cooling, 1.65 mL of 30% H<sub>2</sub>O<sub>2</sub> was added, and the suspension was heated until boiling. The suspension was centrifuged for 15 min at 3000 rpm, and the supernatant was transferred to a graduated flask. The external calibration method was used as an analytical procedure. The chemical characteristics of the substrate used in the experiments are shown in Supplemental Table S1.

## Plant growth response tests

### Selection of inoculum components—preliminary experiments

To select candidates for a multimicroorganismal inoculum, one representative strain of 9 identified species (*O. maius*, *Oidiiodendron griseum*, *P. fortinii*, *Hymenoscyphus* sp., *Meliniomyces variabilis*, *Helotiales* sp., *Cadophora finlandica*, *Cadophora* sp. and *Leptodontium orchidicola*) was used for *V. corymbosum* inoculation in single inoculation experiment. *Cylindrocarpon pauciseptatum*, *Sporothrix inflata*, *Sporothrix variecibatus* and *T. harzianum* were not used due to difficulties in maintaining fungal cultures. Micropropagated rooted plants were transferred into pots with sterile substrate composed of peat and perlite (5:1; v:v) and inoculated with 4 mL of liquid inoculum. Twenty-four plants per treatment were prepared. Plants were cultivated in Sunbags (Sigma Aldrich, St. Louis, MO, USA) in greenhouse (16-h photoperiod, under 90 mmol·m<sup>-2</sup>·s<sup>-1</sup> of light intensity, 21/17 °C day/night temperature and 70% humidity) and irrigated twice a week (once with 2 mL sterile water and once with 2 mL Long Ashton (Hewitt 1966)). Three-month-old plants were harvested for analysis.

We selected the three ericoid mycorrhizal fungal species that enhanced plant growth to prepare inoculum for *V. corymbosum*: *O. maius*, *Hymenoscyphus* sp. and *P. fortinii*. In this experiment, *Xylaria* sp. as a representative of endophytic fungi was used to coinoculate *V. corymbosum*.

*Xylaria* sp. has been previously shown to enhance mycorrhiza development in *Verbascum* plants (Weżowicz et al. 2017). Plants were inoculated with 2 mL of solid inoculum of ericoid fungi and 3 mL of liquid inoculum of *Xylaria* sp., cultivated in a greenhouse (16-h photoperiod, 21/17 °C day/night temperature) and irrigated twice a week with sterile water or Long Ashton solution. Three-month-old plants were harvested for analysis.

### Semi-industrial scale blueberry production in greenhouses

To verify the ability of the inoculum to affect the growth of plants at different stages of ontogenesis, we used 4-week-old plants while transferring them from in vitro to the peat substrate, and the 2-year-old plants that were cultivated in peat substrate in pots. For clarity, plants inoculated during transfer from in vitro will be referred to as young, and plants inoculated during transfer to 1-L pots will be referred to as older. Inoculums composed of selected fungal species were investigated in greenhouse blueberry production scale. Plants transferred from in vitro to peat substrate were inoculated by adding 2 mL of solid inoculum of ericoid fungi and 3 mL of liquid inoculum of *Xylaria* sp. to the planting hole just before planting them out. In the case of the older plants, inoculum was mixed with peat in a concrete mixer (5 mL of solid ericoid inoculum and 1.5 mL of liquid *Xylaria* sp. inoculum per 1 L of peat), which was used as a new substrate in 1-L pots, into which the plants from small pots were transferred. Plants were cultivated in a greenhouse under natural day/night conditions and were watered with tap water every 2 days. Plants were inoculated in May (250 seedlings per treatment) and harvested after the growing season in September.

### Inoculum preparation

For the first experiment, the inoculum was prepared in a liquid medium. Fungi were cultured on 2% malt extract at 26 °C, in an orbital shaker at 130 rpm·min<sup>-1</sup> for 7 days. Fungal liquid cultures were centrifuged at 7000×g for 5 min and washed twice in sterile deionized water. Mycelium was homogenized with a blade and suspended in 50 mL sterile, deionized water. The plants were inoculated just before planting by adding 3 mL of inoculum to the substrate.

Selected for the semi-industrial scale experiment ericoid fungi were cultured on a carrier composed of wheat bran and diatomaceous earth. Carrier components were mixed (4:1, v:v), placed in jars (50 mL carrier/200-mL jar) and sterilized (121 °C, 20 min). Subsequently, 3 plugs of the mycelium (grown on PDA medium in the dark at 26 °C for 7 days) were placed on the carrier. The inoculum was incubated in the dark at 26 °C for 30 days, separately for each fungal species. To inoculate the plant, inocula of different species were

mixed in equal volumes and 2 mL of inoculum was placed in each planting hole just before planting.

### Photosynthetic efficiency

Chlorophyll fluorescence measurements were performed with a Handy Pea fluorimeter (Hansatech Instruments, King's Lynn, UK). One mature leaf from each plant (10 replicates) was dark-adapted for 20 min in a special clips before the measurement. Data were processed with BIOLYZER software (Laboratory of Bioenergetics, Geneva, Switzerland). Each fluorescent transient was calculated according to the OJIP test (Tsimilli-Michael and Strasser 2008). The method of OJIP fluorescence relies on the rapid rise of in vivo chlorophyll fluorescence quantum yield from PSII after illumination with light. In dark adapted leaves, exposition to light induces a fluorescent transient that rises from a minimum level (O level) to a maximum (P level) via two intermediate stages termed J and I. The kinetics of this transient can be used to assess the plants performance (Stirbet and Govindjee 2011). The following multiparametric indices were used to assess plant vitality (Strasser et al. 2000):

$PI_{ABS}$  is the performance of the photosynthesis apparatus expressed in relation to absorption:

$$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi_{P0}}{1 - \varphi_{P0}} \cdot \frac{\psi_0}{1 - \psi_0}$$

where  $RC/ABS$  is a measure of the fraction of reaction centre chlorophyll ( $Chl_{RC}$ ) per chlorophyll of the antennae ( $Chl_{Antenna}$ ).  $\varphi_{P0}/(1 - \varphi_{P0})$  indicates the contribution of the light reactions to primary photochemistry according to the JIP-test. Electron transport beyond Qa (primary quinone acceptor) is quantified as  $\psi_0/(1 - \psi_0)$ .

Performance index ( $PI_{total}$ ): where  $RE/ABS$  indicates the contribution of the reduction of end equivalents.

$$PI_{total} = PI_{ABS} \cdot \frac{RE}{ABS}$$

### Phenolic compounds analysis

The total phenolic, tartaric ester and flavonol concentrations were estimated using the spectrophotometric method described by Fukumoto and Mazza (2000). Four replicates of 100 mg of frozen leaf tissue were homogenized in 5 mL of 80% methanol and centrifuged at 4800×g at 4 °C for 15 min. Leaf extract (0.25 mL) was mixed with 0.25 mL 0.1% HCl in 96% ethanol and 4.50 mL 2% HCl in H<sub>2</sub>O and incubated at dark at room temperature for 15 min. Absorbance was measured at wavelengths of 280, 320, 360 and 520 nm. Calibration



curves for calculating total phenolics, tartaric esters and flavonols were made using the following standards: chlorogenic acid, caffeic acid and quercetin, respectively. Calculation of phenolic compounds was expressed as mg of standard equivalents per 1 g of leaf dry weight.

## Statistical analysis

Statistical analysis was performed using Statistica 13 (Tibco, Palo Alto, USA) and was considered significant at  $p \leq 0.05$ . Data normal distribution and variance homogeneity were assessed with Shapiro–Wilk’s and Levene’s tests, respectively. Differences were tested using  $t$ -test or analysis of variance (ANOVA) followed by Tuckey’s or Dunnett post hoc tests.

## Results

### Selection of inoculum components

#### Fungi isolated from blueberry hairy roots

Thirty-nine pure cultures of fungi were isolated from 273 *V. corymbosum* hairy root fragments. According to the sequences of the ITS rRNA region, a total of 13 endophytic fungal taxa inhabited plant hairy roots (Supplemental Table S2, Supplemental Fig. S1). There were as follows: *C. finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew, *Cadophora* sp. Lagerb. & Melin, *C. pauciseptatum* Schroers & Crous, *Helotiales* sp. Nannf., *Hymenoscyphus* sp. Gray, *L. orchidicola* Sigler et Currah, *M. variabilis* Hambl. & Sigler, *O. griseum* Robak, *O. maius* G.L. Barron, *P. fortinii* C.J.K. Wang & H.E. Wilcox, *S. inflata* de Hoog, *S. variecibatus* Roets, Z.W. de Beer & Crous and *T. harzianum* Rifai. All the fungi that we isolated belonged to the phylum *Ascomycota*. The most frequently isolated taxa were *Hymenoscyphus* sp. (11 isolates) and *O. maius* (5 isolates).

#### Screening for plant growth promoting fungi and inoculum testing

*O. maius* had a positive effect on most of the investigated parameters in comparison to control plants: plant height was improved by 40% (from 12.1 to 16.9 cm), plant fresh biomass by 47% (from 0.54 to 0.80 g) and dry biomass by 41% (from 0.12 to 0.17 g), leaf area by 45% (from 1.02 to 1.48 cm<sup>2</sup>) and root length by 59% (from 188 to 298 cm) (Fig. 2). *P. fortinii* increased only plant dry weight by 21% (from 0.12 to 0.15 g; Fig. 2). *L. orchidicola* negatively affected plant fresh weight; plants yielded 40% less dry weight than those not inoculated (from 0.12 to 0.07 g; Fig. 2). All of the other fungi showed a neutral effect on plant biometric parameters; plant height ranged from 9.0 (*L. orchidicola*)

to 13.9 cm (*Helotiales* sp.), plant fresh weight—from 0.27 (*L. orchidicola*) to 0.58 g (*O. griseum*), plant dry weight—from 0.07 (*L. orchidicola*) to 0.13 g (*O. griseum*) (Fig. 2). *Hymenoscyphus* sp. accelerated the process of plant rooting 10 days after inoculation (Fig. 3).

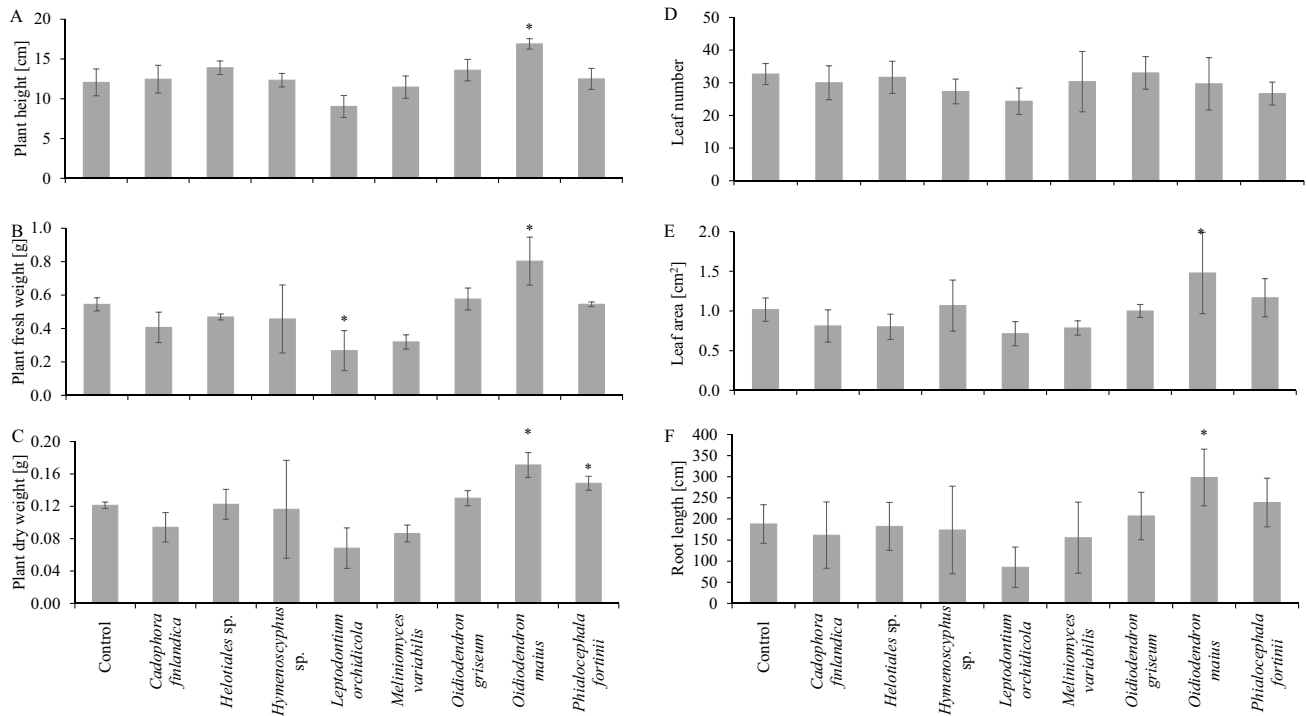
*O. maius*, *P. fortinii* and *Hymenoscyphus* sp. were selected for the final composition of fungi used for induction of blueberry growth. Additionally, to test whether the endophytic fungus *Xylaria* sp. can enhance the plant growth-promoting effect, combinations of different inocula supplemented with *Xylaria* sp. were tested. We tested *O. maius*, *P. fortinii* and *Hymenoscyphus* sp. in different combinations to find the optimal composition of inoculum used for blueberry growth promotion. Inoculation with a single strain of *O. maius*, with a combination of *O. maius* and *Hymenoscyphus* sp. and with *O. maius*, *Hymenoscyphus* sp. and *P. fortinii* increased plant height 7 weeks after inoculation up to 32%, 17% and 34%, respectively (Fig. 4A). Twelve weeks after inoculation, we observed a positive effect of the combination of three microorganisms (*O. maius*, *Hymenoscyphus* sp. and *P. fortinii*) on plant height (Fig. 4B). *Xylaria* sp. improved the growth of plants inoculated with a single strain of *O. maius*, with the combination of *O. maius* and *Hymenoscyphus* sp. and with the combination of *O. maius*, *Hymenoscyphus* sp. and *P. fortinii* 7 weeks after inoculation (Fig. 4C). However, 12 weeks after inoculation, *Xylaria* sp. improved the growth of plants inoculated only with a combination of three ericoid fungi (*O. maius*, *Hymenoscyphus* sp. and *P. fortinii*) (Fig. 4D).

### Semi-industrial scale of blueberry biotization in greenhouse

#### Growth parameters

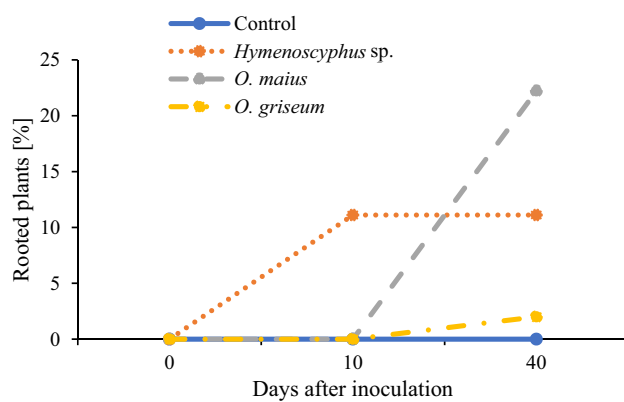
At the end of the season, the height of young plants reached 11.6 cm and was significantly better than that of control plants (35%; Fig. 5A). Additionally, inoculation of the young plants resulted in significantly increased leaf area (61%, from 3.8 to 6.1 cm<sup>2</sup>; Fig. 5B) and shoot fresh weight (33%, from 0.6 to 0.8 g; Fig. 5C). Plant dry weight was not affected by the inoculation of young plants (Fig. 5D). Root diameter and root volume were positively affected by inoculation (by 14% and 59%, respectively; Table 1).

In the case of older plants, we did not observe any differences in plant height upon inoculation (Fig. 5E). However, inoculated plants had larger leaf area (100%; Fig. 5F) and fresh (Fig. 5G) and dry (Fig. 5H) root weight; root fresh and dry mass were improved by 57% (from 2.1 to 3.3 g) and 72% (from 0.4 to 0.7 g), respectively. Plant shoots yielded 62% (from 2.7 to 4.2 g) more dry and 55% (from 7.3 to 11.8 g) more fresh biomass. In total, plant biomass production was improved by 60% in relation to dry mass production and 57%, for fresh mass.



**Fig. 2** Parameters of *V. corymbosum* inoculated with fungi isolated from blueberry hairy roots: plant height (A); plant fresh weight (B); plant dry weight (C); leaf number (D); leaf area (E); root length (F). For each treatment, 24 seedlings were inoculated and grown in peat

and perlite (5:1, v:v). Plants were harvested for analysis 90 days after inoculation. Significant statistical differences between inoculated and control plants are indicated by asterisks (Dunnett test,  $p \leq 0.05$ ,  $n = 3-80$ )



**Fig. 3** Effect of ericoid fungi on the rooting of *Vaccinium corymbosum* cuttings

### Plant vitality

The contribution of light reactions to primary photochemistry ( $\phi_{P0}/(1 - \phi_{P0})$ ) was significantly higher for young, inoculated plants than for control plants (Fig. 6A). In older plants, this parameter did not change upon inoculation. However, other tested parameters (absorbance performance index, fraction of reaction centre chlorophyll per

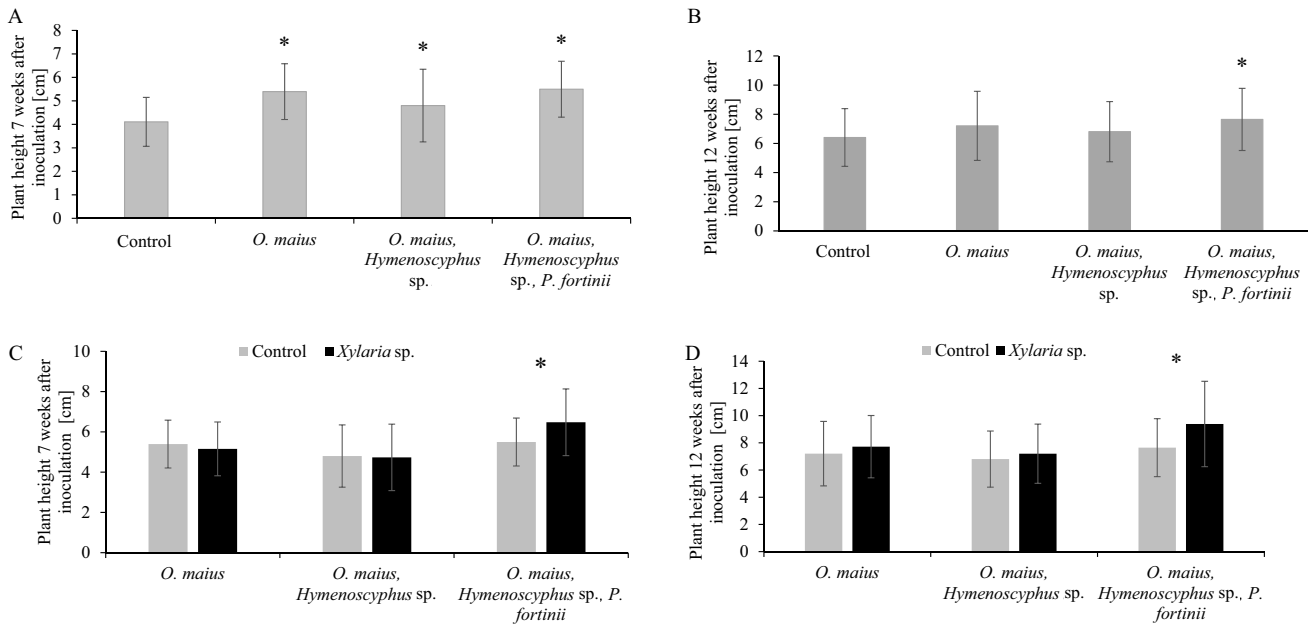
chlorophyll of the antennae, electron transport beyond primary quinone acceptor and contribution of the reduction of end equivalents) were significantly higher in inoculated plants than in control plants (Fig. 6B).

### Secondary metabolites in leaves

The concentrations of tartaric esters and flavonols in the leaves of young, inoculated plants reached 168 and 60 mg of standard equivalents and were significantly lower than those in control plants (Fig. 7A). The total phenolic concentration did not differ between the experimental treatments in young plants. In the case of older inoculated plants, the total phenolic, tartaric ester and flavonol concentrations reached 443, 214 and 65 mg of standard equivalents and did not differ significantly from the control, noninoculated plants (Fig. 7B).

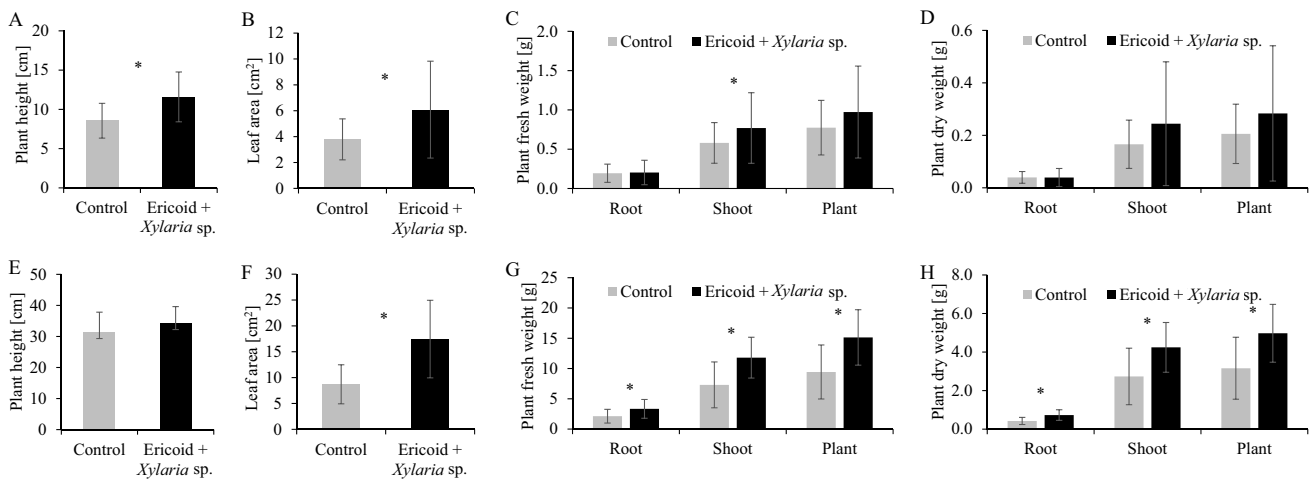
### Discussion

Thirteen endophytic fungal taxa were isolated from the hairy roots of *V. corymbosum*. Taxonomically, all the fungal strains belonged to the *Ascomycota*. Some of these taxa such as *O. griseum*, *O. maius*, *Hymenoscyphus*



**Fig. 4** The height of plants inoculated with single or multiple inocula composed of the ericoid fungi *Oidiodendron maius*, *Hymenoscyphus* sp. and *Phialocephala fortinii* seven (A) and 12 (B) weeks after inoculation and the ericoid fungi and the endophytic fungus *Xylaria* sp. seven (C) and 12 (D) weeks after inoculation. For each treatment, 36

unrooted *V. corymbosum* seedlings were inoculated and grown in peat and perlite (5:1, v:v). Bars indicate  $\pm$ SD; the statistical significance was tested using one-way analysis of variance (ANOVA) followed by the Dunnett (A, B) test and a *t*-test (C, D) at  $p \leq 0.05$  ( $N = 20$ )



**Fig. 5** Plant height (A, E); leaf area (B, F); fresh (C, G) and dry biomass (D, H) of *V. corymbosum* inoculated with inoculum composed of the ericoid fungi *Oidiodendron maius*, *Hymenoscyphus* sp. and *Phialocephala fortinii* and the endophytic fungus *Xylaria* sp. at the moment of transferring plants from in vitro peat (A–D) and at the

moment of transferring from small (0.4 L) to larger (1 L) pots (E–H). For each treatment, 250 seedlings were inoculated and grown in peat and perlite (5:1, v:v). Plants were harvested for analysis at the end of the growing season. Bars indicate  $\pm$ SD; statistical significance was tested using *t*-test at  $p \leq 0.05$  ( $N = 20$ )

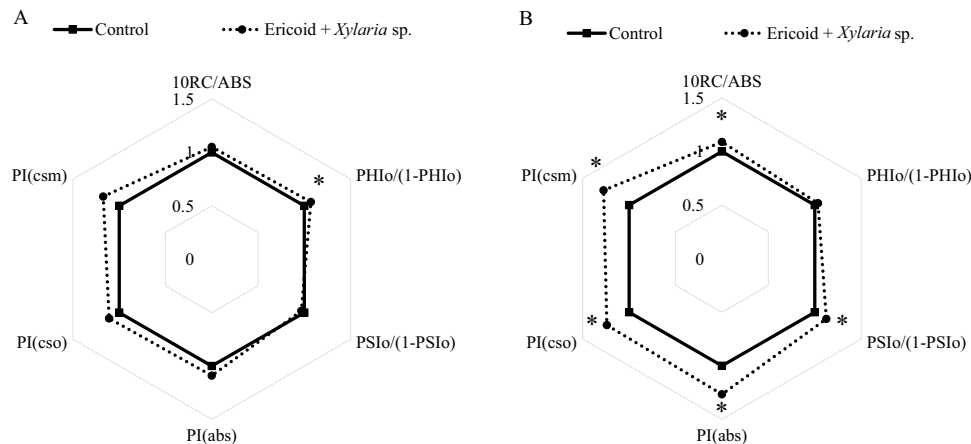
sp., *C. finlandica* and *Cadophora* sp. were previously shown to be mycorrhizal symbionts of ericoid plants (Dalpé 1986; Douglas et al. 1989; Cairney and Burke 1998; Vrålstad et al. 2002; Villarreal-Ruiz et al. 2004; Bizabani and Dames 2015). *M. variabilis* is known as an

anamorph of root-associated fungi belonging to *H. ericae* (Hambleton and Sigler 2005). Others, *L. orchidicola*, *P. fortinii*, *S. inflata*, *S. variegatus* and *T. harzianum*, are known as nonmycorrhizal, symbiotic fungi (Pearson and Read 1973; Hambleton and Currah 1997; Halmschlagler

**Table 1** Root parameters: length, diameter and volume of *V. corymbosum* inoculated (at the moment of transferring plant from in vitro to peat) with ericoid fungi—*Oidiodendron maius*, *Hymenoscyphus* sp. and *Phialocephala fortinii* and endophytic fungus *Xylaria* sp

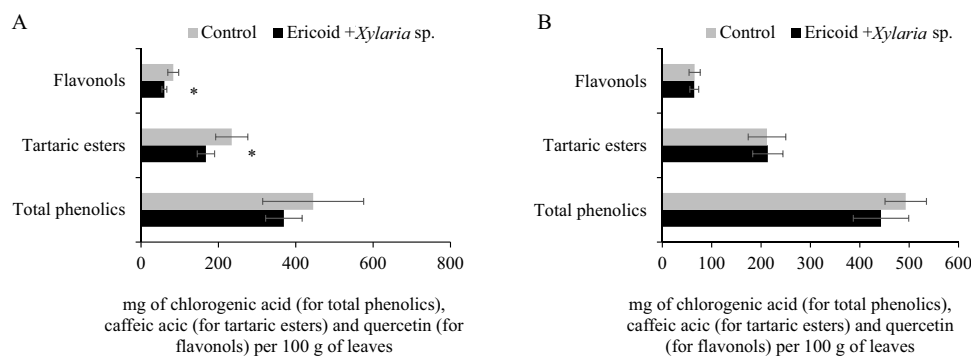
	Root length (cm)	Root diameter (cm)	Root volume (cm <sup>3</sup> )
Control	522.8 ± 86.1	0.20 ± 0.03	0.17 ± 0.06
Ericoid + <i>Xylaria</i> sp.	554.9 ± 173.1	0.24 ± 0.03*	0.27 ± 0.13*

For each treatment, 250 seedlings were inoculated and grown in peat and perlite (5:1, v:v). Plants were harvested for analysis at the end of growing season as 1-year-old. Statistical significance differences between inoculated and control plants are indicated by asterisk (*t*-test at  $p \leq 0.05$ ,  $N=8$ )



**Fig. 6** PSII efficiency of *V. corymbosum* inoculated with inoculum composed of the ericoid fungi *Oidiodendron maius*, *Hymenoscyphus* sp. and *Phialocephala fortinii* and the endophytic fungus *Xylaria* sp. at the moment of transferring plants from in vitro to peat (A) and at the moment of transferring from small (0.4 L) to larger (1 L) pots (B) compared to noninoculated plants (control). For each treatment, 250 seedlings were inoculated. Plants were grown in peat and perlite (5:1, v:v), irrigated with water. Plants were harvested for analysis at the end of the growing season. JIP-test parameters:  $PI_{abs}$ —absorbance

performance index,  $PI_{total}$ —total performance index,  $\phi_{P0}/(1-\phi_{P0})$ —contribution of light reactions for primary photochemistry,  $RC/ABS$ —fraction of reaction centre chlorophyll per chlorophyll of the antennae,  $\psi_0/(1-\psi_0)$ —electron transport beyond primary quinone acceptor and  $RE/ABS$ —contribution of the reduction of end equivalents are presented relative to entirely noninoculated plants; statistically significant differences between particular treatments and those entirely noninoculated plants using *t*-test,  $p \leq 0.05$  ( $N=10$ ) are indicated by asterisk



**Fig. 7** Total phenolic, tartaric ester and flavonol concentrations in leaves of *V. corymbosum* inoculated with inoculum composed of the ericoid fungi *Oidiodendron maius*, *Hymenoscyphus* sp. and *Phialocephala fortinii* and endophytic fungus *Xylaria* sp. at the moment of transferring plants from in vitro to peat (A) and at the moment of

transferring from small (0.4 L) to larger (1 L) pots (B). For each treatment, 250 seedlings were inoculated. Plants were grown in peat and perlite (5:1, v:v) irrigated with water. Plants were harvested for analysis at the end of the growing season. Bars indicate  $\pm$ SD; statistical significance was tested using *t*-test at  $p \leq 0.05$  ( $N=5$ )



and Kowalski 2003; Vohník et al. 2003; Fan et al. 2020). Recently, *Meliniomyces* spp. and *P. fortinii* have been shown to be colonizers not only of roots but also of aboveground compartments of *V. myrtillus* (Perotto et al. under review).

In the experiments, in which plants were inoculated with a single strain of ericoid fungi, the most beneficial for plant growth were *O. maius* and *P. fortinii*. Previously, the application of *O. griseum*, *Pezizella ericae* and *H. ericae* were shown to promote *V. corymbosum* growth (Scagel 2005; Scagel et al. 2005). Inoculation increased nutrient uptake, nutrient use efficiency and leaf biomass of Bluecrop, Earliblue, Georgia Gem and Patrion highbush blueberry cultivars (Scagel 2005). In another study, Bizabani and Dames (2015) showed that fungi from the *Cadophora* and *Lachnum* genera improved highbush blueberry growth. Thus, not only mycorrhizal fungi promoted highbush blueberry growth. Inoculation of blueberry with the endophytic fungus *Anteaglonium* improved root growth and shoot branching, which may be largely attributed to phytohormone signalling and biosynthesis in inoculated plants (Wu et al. 2021). Contrary to our results, Vohník et al. (2012) did not observe a biomass increase in *V. corymbosum* inoculated with the root symbiotic fungi: *O. maius*, *H. ericae* and *P. fortinii*. However, the addition of *Agrocybe praecox*, a saprotrophic basidiomycete, positively affected plant growth and biomass production. This effect may be associated with improved nutrient release from plant residues in the substrate by the lignin-degrading fungus (Vohník et al. 2012).

For the final composition of fungi used for the induction of blueberry growth, we added *Xylaria* sp. to the selected *O. maius*, *P. fortinii* and *Hymenoscyphus* sp.. *Xylaria* sp. is a representative of endophytic fungi shown to enhance mycorrhizal development in another plant species (Weźowicz et al. 2017). In our experiment, the addition of *Xylaria* sp. to inoculum composed of *O. maius*, *Hymenoscyphus* sp. and *P. fortinii* increased plant height. A previous study carried out on *Lactuca serriola* showed that the coinoculation with mycorrhizal and endophytic fungi was more beneficial for the plant than inoculation with a single fungus (Ważny et al. 2018). Blueberry biotization with ericoid and endophytic fungi on a semi-industrial scale improved plant growth parameters. The most pronounced increase was an increase in plant biomass production and a significant increase in plant leaf area. Significantly higher yields were obtained independently of the culture regime.

Accelerated growth increases plant demand for energy. Activation of photosynthesis and carbon assimilation in symbiotic plants has been shown previously (Sheng et al. 2008; Rozpądek et al. 2014, 2019; Ważny et al. 2018). This feature of plants treated with plant growth promoting

microorganisms has been described on numerous occasions, however, never completely. Here, biotization of plants improved many photosynthesis indices (absorbance performance index, fraction of reaction centre chlorophyll per chlorophyll of the antennae, electron transport beyond the primary quinone acceptor and contribution to the reduction of end equivalents). This aspect of plant-microorganism interactions may play a significantly more important role than only improving plant growth. We cannot exclude the possibility that it also plays an important role in plant stress protection and disease resistance. This, however, requires further investigation.

Antioxidant compounds in plant tissues include mostly phenolic compounds (phenolic acid, flavonoids, tannins, stilbenes), carotenoids and vitamins (Larson 1988; Cao et al. 1997; Pietta 2000). Genetic factors, ontogenesis, intrafruit variation, field conditions, postharvest management and fruit processing are the main factors that affect the antioxidant capacity of crops (Manganaris et al. 2014; Rozpądek et al. 2015). Field management systems can play a significant role in antioxidant levels. In previous years, this system has focused on intraspecies crosses (Diamanti et al. 2012) and chemical growth regulators (Percival and MacKenzie 2007). The utilization of biotization methods is much less popular in agronomic practice. Our study shows that plant biotization with mycorrhizal and endophytic fungi did not increase secondary metabolites (tartaric esters, flavonols and total phenolics) in plant leaves. Most importantly, it did not have a negative effect on metabolite content. The total phenolics content in the leaves of blackberry, red raspberry and strawberry investigated by Wang and Lin (2000) varied with cultivar and developmental stage; young leaves had a higher total phenolic content than old leaves. These authors showed a positive correlation between phenolic content and oxygen radical absorbance capacity (ORAC); however, the correlation of ORAC values in fruits and leaves from some cultivars appears to be unclear.

In conclusion, the results presented here indicate that biotization of *V. corymbosum* with ericoid mycorrhizal and endophytic fungi significantly improved the biomass and vitality of plants cultivated on a semi-industrial scale. The biotization technology of highbush blueberry shown in this paper has been patented and may be utilized in horticulture (Turnau et al. 2021). This technology can be a way to modify conventional field management systems in blueberry fruit production in the direction of environmentally friendly practices and for organic blueberry production. Large-scale production of blueberries is based on in vitro plant material, cultivated in pots for one or a few seasons before planting out. These plants are mainly colonized by fungi that are already present in the soil, mutualists and pathogens. Inoculation of the soil with selected microorganisms can

accelerate plant colonization by symbiotic microorganisms and, as a result, improve plant resistance to diseases. To improve plant growth and productivity (fruiting) with biotization, symbiotic microorganisms colonizing all plant tissues should also be taken into account.

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**Author contribution** RW, KT and PR conceived and designed the research. RW, RJJ and AD performed the research. RW analysed the data and wrote the original draft. RJJ, AD, PR and KT edited the manuscript. KT acquired funding. All authors read and approved the manuscript.

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**Data availability** Data generated during this study (nucleotide sequences) have been deposited in the GenBank database with the primary accession numbers OM729672–OM729684.

## Declarations

**Ethics approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare no competing interests.

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