

Article

# Buckwheat Milling Waste Effects on Root Morphology and Mycorrhization of Silver Fir Seedlings Inoculated with Black Summer Truffle (*Tuber aestivum* Vittad.)

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**Abstract:** Large amounts of buckwheat waste are generated annually by the industry and are used in several different ways. To date, there has been little research regarding its suitability as a medium for growing seedlings in nurseries. The aim of this study was therefore to analyze the suitability of common and Tartary buckwheat wastes (brans and husks) as media used for raising seedlings. A pot experiment with five different treatments was carried out, in which silver fir root parameters were analyzed and compared 6 and 12 months after summer truffle-spore inoculation. A significantly higher concentration of the antioxidant rutin was confirmed in Tartary buckwheat bran compared to other buckwheat waste used. We also confirmed a significantly positive effect of added Tartary buckwheat husks on specific root length, root tip density, and specific root tip density compared to added common buckwheat husks or Tartary buckwheat bran, for which a significantly negative effect on branching density was confirmed. A significantly negative effect of added buckwheat husks and Tartary buckwheat bran was confirmed for summer truffle mycorrhization level.

**Keywords:** buckwheat waste; root growth; silver fir; inoculation with ectomycorrhizal fungi; summer truffle; forest nursery

## 1. Introduction

The utilization of industrial by-products is a current major challenge. European and world food industries generate millions of tons of by-products annually [1]. Grain processing industries do not fully utilize potentially useful raw materials (husks, straw, bran) that contain significant amounts of valuable substances, including fiber and antioxidants. Buckwheat industrial by-products are characterized by a complex structure in which both lipophilic and hydrophilic compounds form the matrix and are complexed to various macromolecules, such as antioxidants and other bioactive substances. These interactions have a great influence on bioavailability and, hence, the antioxidant ability of bioactive compounds present in cereal by-products [2,3]. A major concern is to develop efficient environment-friendly technology to take advantage of unexploited cereal milling industry by-products.

Large amounts of waste are generated annually worldwide by the cereal processing industry. In Slovenia alone, over 3000 metric tons of buckwheat are produced or imported

and processed annually [4]. Based on an estimation that about 30% of the total processed material represents milling or husk waste remains, we surmise that about 700–1000 metric tons of by-products per year are still produced without a proper end use [5], which is especially true for buckwheat husks and bran. Worldwide, from the annual yield of 2,905,294 tons of buckwheat [1], the resulting waste amounts to about 1,000,000 tons per year, according to data reported by Bonaffacia et al. [5].

Traditionally, buckwheat husks have been used for insulation material in several-hundred-year-old houses (Denmark), as storage for dry sausages and salami (Valtellina, Italy), mattresses (China), and traditional pillows (China and Japan). Husks obtained after dehulling thermally pretreated buckwheat grains, which are disinfected by hydrothermal treatment and are free from flour particles, are most suitable for the latter usage. Husks that remain after milling may still contain some residues of endosperm starch particles.

However, these uses represent only a small fraction of the variety of available buckwheat by-products. Buckwheat by-products may also be burned either as a biofuel in heating plants or in an uncontrolled burn in the field, thus contributing to CO<sub>2</sub> release and smoke pollution.

The efficient management and valorization of cereal and buckwheat industrial wastes thus represent a priority research area. Meanwhile, there are numerous studies on how agro-industrial wastes from other cereals (e.g., rice, corn, wheat) can be used in industrial processes to make a variety of natural products [6–10]; under current practices, buckwheat by-products remain underexploited and have a low added value of downstream applications and products; hence, the development of new or improved approaches is a highly worthwhile research area.

Two buckwheat species, common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.), are used to produce flour for use in various foods and dishes. In China, Korea, and Japan, common and Tartary buckwheat are used mostly to make pasta products. Common buckwheat groats and dishes from groats are produced and used in Slavic countries, such as Slovenia, Croatia, Poland, Ukraine, Belarus, and Russia [11]. One recent development in Slovenia is the husking of Tartary buckwheat grain to make Tartary groats dishes.

Buckwheat seed contains high-quality starch, fiber, and various phenolic substances [3,12–14]. As reported by Ikeda et al. [15], phenolic substances have a significant inhibitory effect on the *in vitro* peptic and pancreatic digestion of globulin; hence, common and Tartary buckwheat secondary metabolites may have an impact on protein digestibility. Considerable interaction between polyphenols and proteins was observed after hydrothermal treatment [16].

Finding ways of reusing industrial waste material such as buckwheat bran and husks to support the economy in agricultural and forestry areas is urgently needed. One important challenge is the development of planting and gardening substrates for seedlings in forest nurseries, which could enhance not only the growth of root systems but also the general quality of seedlings.

To date, silver fir has received little attention as a forest tree in general, and specifically as a tree that can also be used in commercial truffle species mycorrhization processes. In recent years, Slovenian forests, as well as some others in Central Europe, have suffered severe damage due to gophers grazing on Norway spruce; silver fir became the first in a line of potential tree species that could replace the currently very vulnerable Norway spruce in forest systems. The aim of this study was, therefore, to analyze the potential of buckwheat bran and husks as additives to the substrate for preserving and further nurturing the mycorrhizal fungi that are present, which have a beneficial effect on seedling development. In addition, this study explores their use in the potentially more successful production of silver fir seedlings for planting, to enhance not only the growth of root systems but also the overall quality of the seedlings. Another specific challenge is

to find a suitable substrate by which to enhance the mycorrhization processes of the commercially important summer truffle.

## 2. Materials and Methods

### 2.1. Experimental Design

Four-month-old, certified silver fir seedlings with a cover root system and that were morphologically uniform were purchased from the LIECO nursery (Kalwang, Austria) and were transferred to pots. The criteria for seedling quality were described in a previous study by Unuk Nahberger et al. [17].

Before commencing the pot experiment, the root systems of 10 randomly selected silver fir seedlings were first checked for the possible presence of contaminant fungi. In a small proportion, not exceeding 5% of the analyzed root tips per seedling, the presence of the contaminant ectomycorrhizal fungi, *Thelephora* sp., was confirmed, whereas the ectomycorrhizal fungi, summer truffle, was not observed on the seedling root tips. We established 4 different treatments with added truffle spore inoculums: (i) treatment with common buckwheat bran, (ii) treatment with common buckwheat husks, (iii) treatment with Tartary buckwheat bran, (iv) treatment with Tartary buckwheat husks, and a control treatment using the basic substrate without spore inoculums. For each treatment, 25 silver fir seedlings were planted, a total of 100, in individual containers with a dimension of 52 cm × 38 cm × 50 cm. These containers were used instead of traditional pots (340 mL/650 mL) to ensure sufficient space for the growing root systems.

The basic control substrate used for the experiment was an artificial mixture composed of black peat (Roko, Hoče, Slovenia) (40%), common/Tartary buckwheat husks or bran (10%), vermiculite (33%), perlite (15%) and limestone dust (2%). Before adding the spore suspension, the substrate was irrigated and the pH was adjusted to range from 7 to 7.5. During the pot experiment, seedlings were not additionally fertilized.

The silver fir seedlings in the experiment were supplemented with an adequate amount of ectomycorrhizal fungus spores. For the spore material, the ripened fruit bodies of summer truffle were used. Before the preparation of the spore suspension, each sporocarp was surface-sterilized by irrigation in 70% ethanol (EtOH) for 10 min. After 10 min, ethanol was removed and sporocarps were washed under running tap water for 20 min. After the sporocarp was dried, the peridium and all macroscopically damaged parts were eliminated by peeling. To obtain the desired amount of 2 g of pure summer truffle spores per plant, 100 g of the sporocarps were crushed in a previously sterilized blender in sterilized water, in order to release the spores. The obtained dilution was mixed with ultrapure water (from Milli-Q Water System, Millipore SAS, Molsheim, France) to obtain a homogeneous distribution. The dilution was flushed into the substrate that had been prepared for inoculation.

Containers were kept in a growth chamber for one year under controlled conditions, with a daily regime of full daylight for 15 h and a temperature of 22 °C, and a night regime of darkness for 9 h and a temperature of 15 °C.

### 2.2. Root Morphology Assessment and Evaluation of Seedling Mycorrhization Level

Ten seedlings per treatment were analyzed and evaluated for root morphological parameters, as well as for seedling mycorrhization level, at 6 months and 12 months after spore inoculation.

For the root morphology assessment, the whole root system of each analyzed seedling was first gently washed and then scanned by an Epson Perfection V700 photo scanner (Seiko Epson Corp., Suwa, Nagano, Japan) in trays filled with distilled water. Scans were further analyzed using WhinRhizo software, to obtain the values for root length and the number of root tips and branches for each individual seedling.

A mycorrhization level assessment of plants was performed using an Olympus SZH (Olympus, Tokyo, Japan) dissecting microscope, following the procedure described by

Fischer and Colinas [18]. The root system of each plant was cut in half longitudinally, and one half was randomly chosen for examination. Furthermore, roots from the selected half were cut into segments of 2–3 cm and evenly spread over a 1 cm × 1 cm grid, in a shallow dish of distilled water. For counting purposes, squares were chosen randomly; in each selected square, the fine roots were counted and separated by category into nonmycorrhizal root tips (NM), summer truffle root tips (T), and other fungi (O). The roots were analyzed until 250 root tips per seedling were categorized. Mycorrhizal proportions were calculated, based on 250 counted root tips, specifically, the proportion of summer truffle ( $P_{\text{tuber}} = T/\text{total number of root tips}$ ), and the proportion of other (contaminant) fungi ( $P_{\text{other}} = O/\text{total number of root tips}$ ). For the calculation of relative abundance, individual proportions were amplified by 100.

After an evaluation of the seedling mycorrhization level, the roots were air-dried, then oven-dried at 70 °C for five days, and were kept desiccated over silica gel until weighing. Each root system was weighed separately using a SCALTEC SBC-31 (Denver Instruments, Bohemia, NY, USA) analytical scale. Morphological parameters, obtained with WhinRhizo in combination with root biomass, were used to calculate the specific root length (SRL, the ratio between root length and root biomass), root tip density (the ratio between the number of root tips and root biomass), specific root tip density (the ratio between the number of root tips and root length) and branching density (the ratio between the number of branches and root biomass).

### 2.3. Molecular Analysis

After the mycorrhization evaluation, ectomycorrhizal root tips from single ectomycorrhiza at various stages of development, also coming from different seedlings and different treatments, were included in the molecular identification. DNA extractions were performed with the DNeasy Plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Due to observed difficulties with the summer truffle ITS region amplification [17,19], the following fungus-specific primer pairs were used: ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCGCTTARTGATARGC-3') [20,21]; and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS7 (5'-ACTCGCCGTTACTGAGGCAAT-3') [22]. The PCR reactions involving the first pair, ITS1f/ITS4, were performed, as described by Grebenc and Kraigher [23], while the PCR reactions involving the second pair, ITS5/ITS7, were performed as described in [17].

From agarose gels, cut amplified DNA fragments were purified using the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions, while DNA fragment sequencing was performed elsewhere at a commercial sequencing laboratory (Macrogen Inc., Seoul, South Korea). The obtained sequences were processed using Geneious version 11.1.4 (<https://www.geneious.com>, (12.7.2021) [24]. The reads were assembled into contigs at 90% base pair similarity, and the BLASTN algorithm from the NCBI website (National Center for Biotechnology Information—<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (12.7.2021) was used to assess the similarity of obtained ITS sequences to sequences in GenBank. Representative sequences from summer truffle root tips have been submitted to GenBank with the following accession numbers: MN270406–MN270412.

### 2.4. Basic Chemical Analyses of Substrate

Basic chemical analyses for organic carbon, mineral carbon, and nitrogen content in substrates from the control treatment and those treatments with added Tartary and common buckwheat husks or bran were performed at the zero point, after 6 months, and 12 months after spore inoculation.

The organic carbon level was analyzed via elementary analysis according to ISO standard 10694 (Soil quality—determination of organic and total carbon after dry combustion). The temperature used for combustion was 1140 °C. The carbon gained from the analysis was the total carbon. Additionally, the mineral carbon was determined via

the volumetric method, according to ISO 10693 (Soil quality—determination of carbonate content). The organic carbon was calculated by subtracting the mineral carbon from the total carbon.

The total nitrogen was ascertained via elemental analysis, according to ISO 13878 (Soil quality—determination of total nitrogen content by dry combustion). The combustion temperature was 1140 °C.

The instrument used for the determination of total carbon and nitrogen was an elemental analyzer, Elementar VarioMAX Cube CNS (Elementar Analysensysteme, Langensfeld, Germany). Usually, the weights of the samples varied from 180 to 400 mg. The mineral carbon level was determined using a calcimeter (Royal Eijkelpoort Soil & Water, Giesbeek, The Netherlands).

### 2.5. Rutin and Quercetin Concentration in Buckwheat Husks/Bran

Samples of common and Tartary buckwheat husks and bran were first milled using a Retsch mixer mill (Retsch, Haan, Germany), and weighed out to 100 mg for each sample. The samples were further extracted with 25 mL of 80% methanol by shaking at room temperature for 1 h. To establish the rutin and quercetin concentrations of the samples, HPLC analyses were performed on a Luna Omega Polar 5 µm C18 250 × 4.6 mm (Phenomex) column, using a Waters 2695 Separation Module and 2996 PDA detector. The mobile phase consisted of acetonitrile (gradient) (A) and 0.1% phosphoric acid in ddH<sub>2</sub>O (B). The gradient elution was as follows: 0–1 min, isocratic elution (20% A and 80% B); 1–5 min, linear gradient elution (25% A and 75% B); 5–15 min (30% A and 70% B); and 20–25 min (40% A and 60% B). The injection volume was 10 mL and the flow rate was 1 mL min<sup>-1</sup> at a column temperature of 30 °C. The detection of rutin and quercetin was conducted at 265 nm (rutin) and 372 nm (quercetin). The limits of detection for rutin and quercetin were at 1 mg mL<sup>-1</sup>, respectively. The limits of quantification for rutin and quercetin were 3.6 and 3.3 mg mL<sup>-1</sup>, respectively.

### 2.6. Statistical Analyses

Statistical analyses were performed with R 3.5.1 [25]. Normal distribution and the homogeneity of variance were tested using the Shapiro–Wilk normality test and the Bartlett test and were improved using the approach of Tukey’s ladder of powers via the package “rcompanion”, where needed ( $p \leq 0.05$ ). For the statistical evaluation of differences between treatments, a one-way analysis of variance (ANOVA) was applied to test the effects of added buckwheat husks/bran on the root morphological parameters. A one-way ANOVA was conducted separately according to the time after spore inoculation, where the Tukey HSD test was used as a post hoc test of ANOVA. A Kruskal–Wallis ANOVA and the Dunn post hoc test were used to assess the effect of added Tartary buckwheat husks/bran on the summer truffle mycorrhization level, compared to treatment with no added buckwheat products. The  $p$ -values were adjusted using the Bonferroni method.

## 3. Results and Discussion

### 3.1. Chemical Composition of the Substrate

Chemical analyses of the substrate were performed for organic carbon (C), mineral C, and nitrogen (N) content at the zero point, 6 months, and 12 months after spore inoculation. The significantly highest content of organic C was recorded in the substrate with added common bran ( $23.6 \pm 0.4\%$ ) compared to other treatments at the zero point. The treatment with added common husks resulted in a significantly lower content of organic C ( $19.4 \pm 0.1\%$ ) compared to the control treatment, but it was not significantly different from treatments with added Tartary buckwheat bran or husks at the zero point (Table 1). At six and 12 months after spore inoculation, the significantly highest content of organic C was recorded in the treatment with added common buckwheat bran, as

compared to treatments with added Tartary buckwheat bran and husks, for which the significantly lowest content of organic C was recorded (Table 1).

In contrast to organic C, the significantly highest content of mineral C was recorded in the substrates with added Tartary buckwheat bran, while the lowest content of mineral C was recorded in the substrate from the control treatment at the zero point. Six months after spore inoculation, two homogeneous groups were formed in the substrate: the group with significantly higher mineral C content (those treatments with added Tartary bran or Tartary husks) and the group with significantly lower mineral C content, i.e., treatments with added common bran or husk, and the control treatment. However, 12 months after spore inoculation, there were no statistically significant differences in the mineral C content between the analyzed treatments (Table 1).

Interestingly, the highest content of N at the zero point was recorded in the substrates from those treatments with added Tartary or common bran (0.98% and 0.817%, respectively) versus N content from the control treatment or treatments with added common or Tartary husks, for which a significantly lower N content was recorded (Table 1). Six months after spore inoculation, the N content divided treatments into the same homogeneous groups, as at the beginning of the experiment (i.e., the significantly highest N contents were in substrates from treatments with added buckwheat bran), whereas at 12 months after spore inoculation, there were no statistically significant differences between treatments (Table 1).

**Table 1.** Organic carbon, mineral carbon, and nitrogen content (average  $\pm$  SD), and C/N ratio in substrates from different treatments, at zero point, 6 months, and 12 months after spore inoculation (CB = common buckwheat, TB = Tartary buckwheat). Different letters mark statistically significantly different results, according to the Tukey HSD test for all treatments and time points ( $p \leq 0.05$ ).

| Time after Spore Inoculation (Months) | Treatment | Organic Carbon Content (%) | Mineral Carbon Content (%) | Nitrogen Content (%) | C/N Ratio |
|---------------------------------------|-----------|----------------------------|----------------------------|----------------------|-----------|
| zero point                            | Control   | 22.5 $\pm$ 0.5a            | 1.4 $\pm$ 0.01c            | 0.505 $\pm$ 0.006b   | 44:1      |
| zero point                            | CB bran   | 23.6 $\pm$ 0.4a            | 1.8 $\pm$ 0.02b            | 0.817 $\pm$ 0.008a   | 29:1      |
| zero point                            | TB bran   | 17.2 $\pm$ 0.5bc           | 2.6 $\pm$ 0.05a            | 0.980 $\pm$ 0.03a    | 18:1      |
| zero point                            | CB husks  | 19.4 $\pm$ 0.07b           | 1.9 $\pm$ 0.03b            | 0.426 $\pm$ 0.001bc  | 45:1      |
| zero point                            | TB husks  | 15.6 $\pm$ 0.3c            | 2 $\pm$ 0.01b              | 0.354 $\pm$ 0.008c   | 44:1      |
| 6                                     | Control   | 19.5 $\pm$ 0.1b            | 1.2 $\pm$ 0.03b            | 0.455 $\pm$ 0.002b   | 43:1      |
| 6                                     | CB bran   | 21.5 $\pm$ 0.2a            | 1.6 $\pm$ 0.02b            | 1.027 $\pm$ 0a       | 21:1      |
| 6                                     | TB bran   | 14.7 $\pm$ 0.1c            | 2.2 $\pm$ 0.1a             | 0.888 $\pm$ 0.01a    | 17:1      |
| 6                                     | CB husks  | 19.7 $\pm$ 0.2b            | 1.5 $\pm$ 0.02b            | 0.496 $\pm$ 0.009b   | 40:1      |
| 6                                     | TB husks  | 14.8 $\pm$ 0.1c            | 2.1 $\pm$ 0.09a            | 0.400 $\pm$ 0.08b    | 37:1      |
| 12                                    | Control   | 17.7 $\pm$ 0.3b            | 1.4 $\pm$ 0.02b            | 0.418 $\pm$ 0.009b   | 42:1      |
| 12                                    | CB bran   | 21.6 $\pm$ 0.03a           | 1.5 $\pm$ 0.2b             | 0.770 $\pm$ 0.01a    | 28:1      |
| 12                                    | TB bran   | 12.6 $\pm$ 0.02c           | 2.0 $\pm$ 0.07ab           | 0.679 $\pm$ 0.008a   | 19:1      |
| 12                                    | CB husks  | 18.4 $\pm$ 0.2b            | 1.6 $\pm$ 0.01b            | 0.501 $\pm$ 0.004ab  | 37:1      |
| 12                                    | TB husks  | 13.3 $\pm$ 0.2c            | 2.2 $\pm$ 0.1a             | 0.382 $\pm$ 0.01b    | 35:1      |

### 3.2. Rutin and Quercetin Concentrations in Buckwheat By-Products

Common and Tartary buckwheat husks and bran were also analyzed for their rutin and quercetin concentrations. The significantly highest rutin concentration was recorded in the substrate from the treatment with added Tartary buckwheat bran, versus the treatment with added common buckwheat husks, for which the significantly lowest rutin concentration was recorded (Table 2). Tartary buckwheat bran was also utilized as a source of rutin by the authors of [26], while the study by Vojtiškova et al. [27] reported higher values of rutin concentrations in the wholemeal flour compared to peels (husks) of common buckwheat, which is consistent with our results, as higher concentrations of rutin

were detected in bran compared to husks in the case of common as well as Tartary buckwheat. Tartary buckwheat seeds contain higher concentrations of rutin compared to common buckwheat, as identified in the studies by [13,14,28].

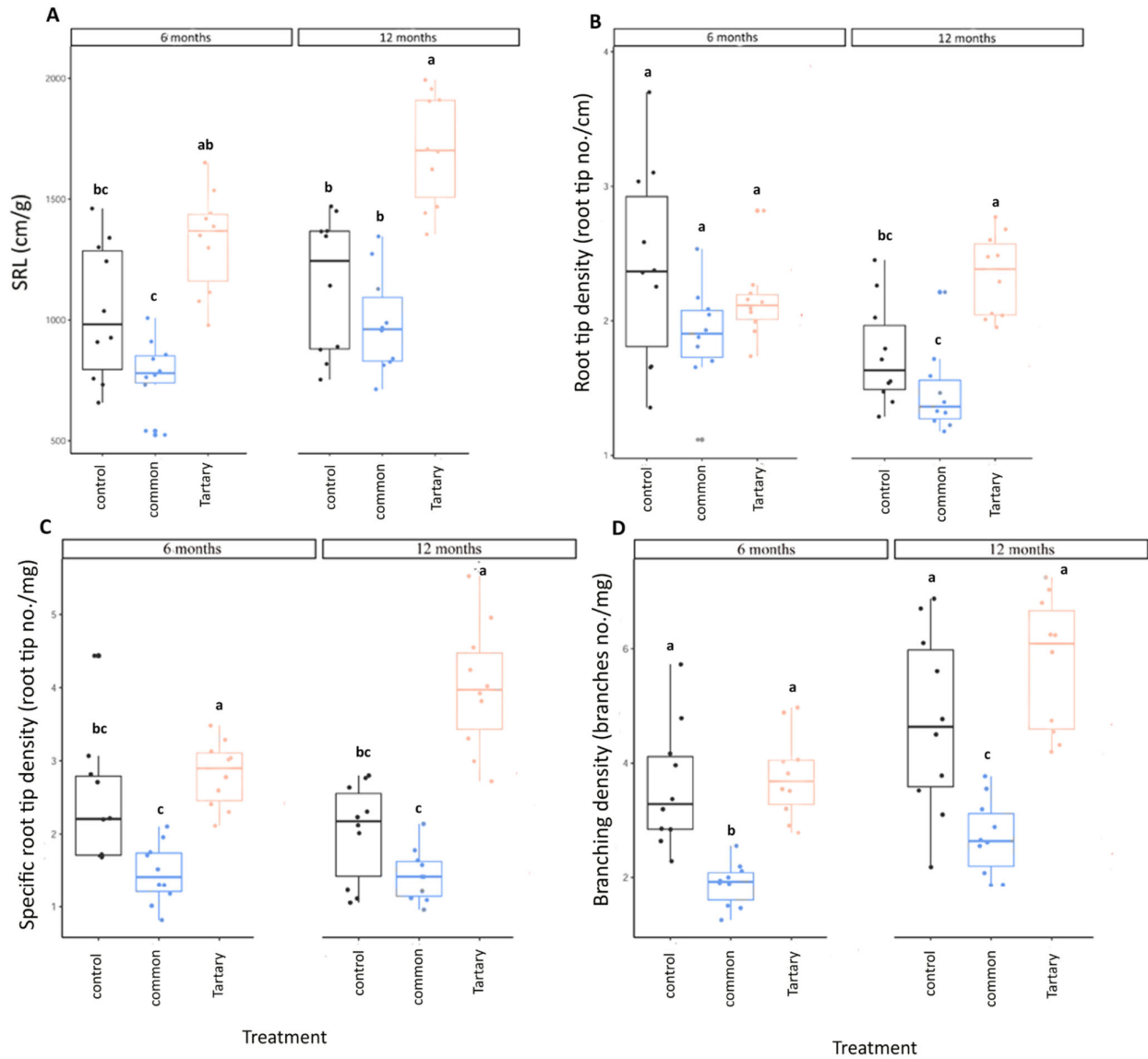
The statistically significant highest quercetin concentration was also recorded in the substrate from treatment with added Tartary buckwheat bran versus treatment with added common buckwheat husks, for which the lowest quercetin concentration was recorded (Table 2). In contrast to our results, the study by Fabjan et al. [29] measured very high concentrations of rutin and only traceable amounts of quercetin in buckwheat. However, in our study, higher concentrations of quercetin compared to rutin were measured in both common and Tartary buckwheat seeds. These substances were measured because of their potential biological effects on fungi and their known presence in buckwheat plants.

**Table 2.** Rutin and quercetin concentrations (average  $\pm$  SD) in common buckwheat (CB) and Tartary buckwheat (TB) bran and husks. Different letters mark statistically significantly different results, according to the Tukey HSD test ( $p \leq 0.05$ ).

| Buckwheat Product | Rutin<br>(mg/g Dry Weight) | Quercetin<br>(mg/g Dry Weight) |
|-------------------|----------------------------|--------------------------------|
| CB bran           | 0.08 $\pm$ 0.04b           | 0.012 $\pm$ 0.002b             |
| TB bran           | 0.51 $\pm$ 0.02a           | 0.701 $\pm$ 0.021a             |
| CB husks          | 0.02 $\pm$ 0.03b           | 0.010 $\pm$ 0.006b             |
| TB husks          | 0.07 $\pm$ 0.02b           | 0.031 $\pm$ 0.001b             |

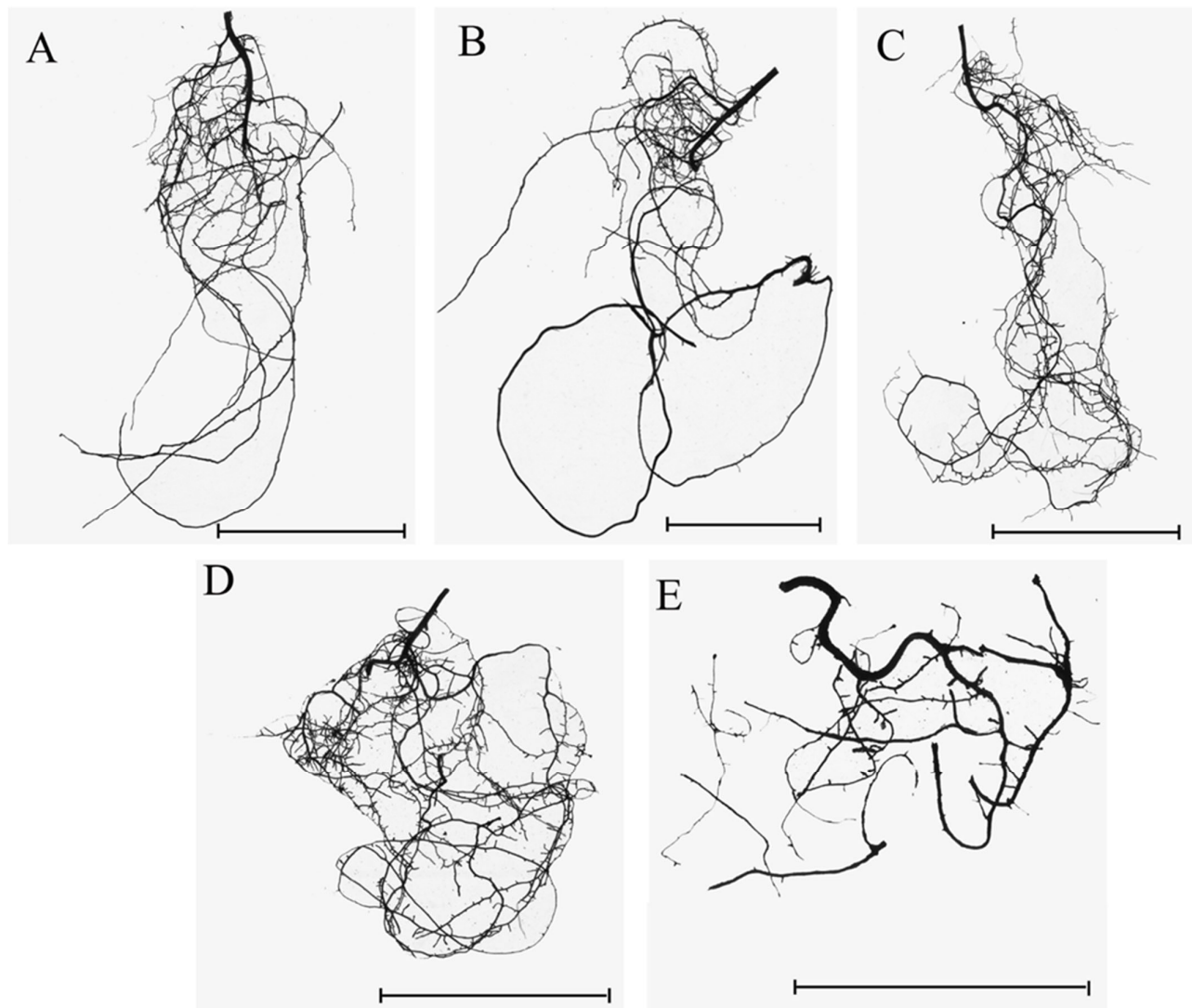
### 3.3. Impact of Husks on Root Growth

Significant effects from adding buckwheat husks were recorded on the silver fir seedlings' root morphological parameters at 6 months and 12 months after spore inoculation. The SRL (cm/g) was significantly positively affected by added Tartary husks as a statistically significant effect was confirmed 12 months after spore inoculation (Figures 1A and 2A–C). Common buckwheat husks had no effect on SRL 6 months or 12 months after spore inoculation (Figure 2B). Adding Tartary buckwheat husks also had a significantly positive effect on root tip density 12 months after spore inoculation, whereas no significant effect from adding Tartary husks 6 months after spore inoculation was recorded (Figure 1B). As with SRL, we did not record any significant effect from adding common buckwheat husks on root tip density at any time point (Figure 1B). Similarly to the previous root parameters, a significant effect from adding Tartary buckwheat husks on specific root tip density was confirmed at 6 months as well as 12 months after spore inoculation, while the addition of common buckwheat husks had no significant effect on specific root tip density at any time point (Figure 1C). In contrast, common buckwheat husks had a statistically significant negative effect on branching density at 6 months as well as 12 months after spore inoculation, while adding Tartary buckwheat husks had no statistically significant effect on branching density (Figure 1D).



**Figure 1.** The impact of buckwheat husks on root parameters at 6 months and 12 months after spore inoculation: (A) specific root length (SRL) (cm/g); (B) root tip density (no. of root tips/cm); (C) specific root tip density (no. of root tips/mg) and (D) branching density (no. of branches/mg). Different letters mark statistically different results (Tukey HSD,  $p \leq 0.05$ ).





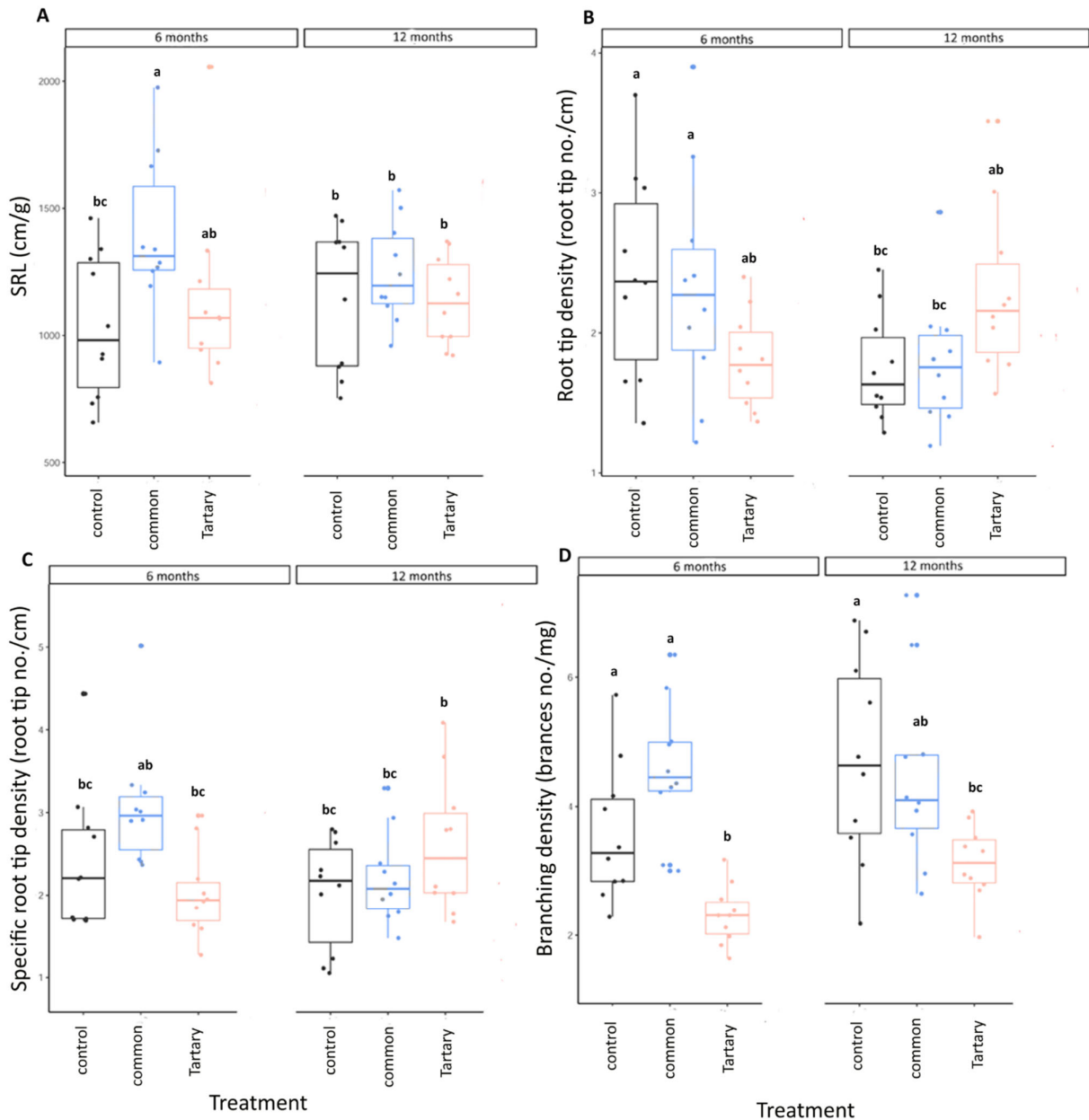
**Figure 2.** Examples of root system scans of silver fir seedlings with different treatments, 12 months after spore inoculation: (A) control treatment; (B) treatment with added common husks; (C) treatment with added Tartary buckwheat husks; (D) treatment with added common bran; (E) treatment with added Tartary buckwheat bran. The bar denotes a length of 5 cm.

In general, Tartary husks had significantly positive effects on several root parameters and root growth (Figure 2C); however, these cannot be linked to either the chemical composition of the substrate or to the rutin and/or quercetin concentrations in husks/bran. One possible explanation for the findings observed could be linked to the concentrations of mineral nutrients in husks/bran since, in accordance with the study by Vojtiškova et al. [27], husks are characterized by higher concentrations of several mineral elements, e.g., lead (Pb), sodium (Na), calcium (Ca) and potassium (K), which could enhance the mineral nutrition of seedlings. Ca and K are classified as macronutrients that enhance plant growth. Potassium is one of the most valuable elements for the growth and development of plants [30], as is calcium, which also contributes to normal root system development [31,32]. In their study, Viegas et al. [33] observed a drastically reduced growth of roots and the plant in the absence of Ca. Higher concentrations of these elements in buckwheat husks could therefore enhance overall root growth, which results in the positive effect of added Tartary buckwheat husks on the root parameters. Vojtiškova et al. [27] measured

the content of mineral elements in common buckwheat products, but further analyses would be needed to be able to confirm this possibility.

#### *3.4. Impact of Bran on Root Growth*

The significant effects of added buckwheat bran were confirmed only for SRL and branching density (Figure 3), where SRL was shown to be affected by adding common bran 6 months after spore inoculation, with a significantly positive effect, as compared to added Tartary buckwheat bran, which had no significant effect (Figure 3A). In contrast, branching density was affected by adding Tartary buckwheat bran but not by adding common buckwheat bran (Figure 2B,C). Six and 12 months after spore inoculation, statistically significant negative effects from adding Tartary bran were confirmed (Figure 3D). The observed findings could be linked to the higher measured concentrations of quercetin in Tartary buckwheat husks, as several studies have suggested and also confirmed that flavonoids, specifically quercetin, can act as auxin transport inhibitors [34–38], which results in lateral root defects and is negatively correlated with the degree of branching on root systems [39] (Figure 2D,E).



**Figure 3.** Impact of buckwheat bran on root parameters at 6 and 12 months after spore inoculation: (A) Specific root length (SRL) (cm/mg); (B) root tip density (no. of root tips/cm); (C) specific root tip density (no. of root tips/mg); and (D) branching density (no. of branches/mg). Different letters mark statistically significant results (Tukey HSD,  $p \leq 0.05$ ).

### 3.5. Buckwheat Husks Had a Negative Impact on Summer Truffle Mycorrhization Level

Through the morphological and molecular analyses of root tips, the presence of the ectomycorrhizal fungi summer truffle was confirmed as early as 6 months after spore inoculation. Statistically significant negative effects from adding common and Tartary buckwheat husks on the summer truffle mycorrhization level were confirmed at 6 months and 12 months after spore inoculation (Figure 4A). In contrast to husks, statistically significantly negative effects on summer truffle mycorrhization level were confirmed only for Tartary buckwheat bran at 6 months and 12 months after spore inoculation, while we

did not confirm any effect from adding common buckwheat bran on summer truffle mycorrhization level at any time point (Figure 4B).

Based on all the analyzed parameters in our study, the substrate with added common bran stood out as it was characterized by a significantly highest content of organic C and N contents at all three sampling times after spore inoculation. The highest C and N contents in the substrate with added common bran resulted in a lower C/N ratio, which is an important soil characteristic not only for mycelia formation but also for the fruiting of *Tuber* species [40]. The C/N ratio relationship reflects the amount of mineralization in the soil and gives an indication of biological activity [41]. In our study, soil from treatments with added Tartary and common buckwheat husks was characterized by a C/N ratio that was well above the upper limit (maximum values of 26) of summer truffle. Therefore, a very high C/N ratio of the substrate with added common and Tartary husks could have a decisive impact on summer truffle mycorrhiza formation. Authors in different truffle cultivation manuals recommended a C/N ratio for the cultivation of truffles of between 18 and 15, with an optimum of around 10 [40,41]. The C/N ratio of soil from treatment with added Tartary buckwheat bran was the most optimal for the mycelia production of summer truffle, but because of the high concentration of quercetin and its negative impact on branching density, mycorrhizal mycelia formation could be inhibited due to those characteristics. Although the control treatment was also characterized by a relatively high C/N ratio, we suggest that the combination of the C/N ratio, concentrations of rutin and quercetin (which were high in Tartary buckwheat bran and husks), and possible differences in the concentrations of several mineral nutrients (for example, calcium, which is an important factor determining the production of mycelia and carpophore of several *Tuber* species [42]) resulted in the non-mycorrhiza formation of summer truffles in treatments with added Tartary and common buckwheat husks, as well as in treatment with added Tartary bran.

Another possible explanation for the non-formation of mycorrhiza by the summer truffle in our study could also be due to the antimicrobial activity of buckwheat secondary metabolites, as has already been reported by several authors [43–46]. Polyphenols are the major bioactive compounds that have previously shown antibacterial, antiviral, and antifungal actions [46–48]. Several authors have reported the antifungal activities of buckwheat phenolic acids against different pathogenic fungi, causing foodborne diseases [49–52]. Based on the study by Li et al., common husks have a higher phenolic content compared to Tartary husks, whereas Tartary bran has a higher phenolic content compared to common bran. Therefore, the high phenolic content of common husks and Tartary bran could show an antifungal activity, which may result in the non-mycorrhiza formation of summer truffle in those specific treatments.

However, we did record and observed abundant ectomycorrhizal root tips in the treatment with added common buckwheat. Based on those findings, and the polyphenol content of common/Tartary bran and husks reported in the study by Li et al., we would expect to detect some mycorrhiza formation in the treatment with added Tartary buckwheat husks as well.

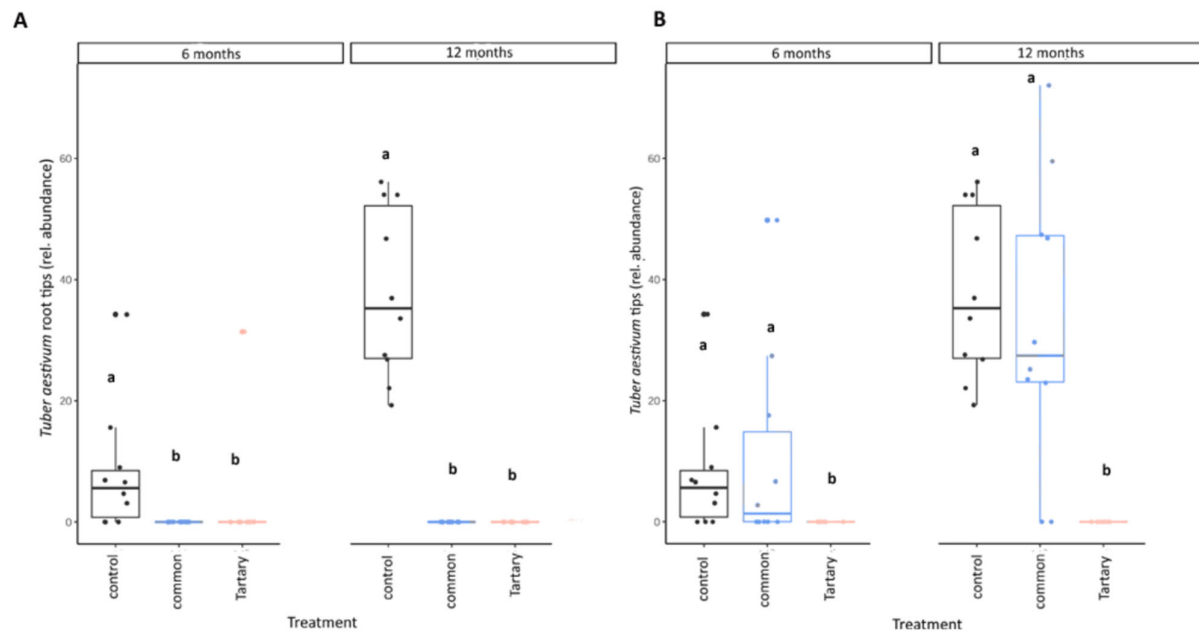
Besides polyphenols, several authors have also reported the antifungal activity of different flavonoids, e.g., rutin, quercetin, and isoquercetin [45,46,53]. Based on those studies, a combination of the higher quercetin content in Tartary buckwheat husks compared to common bran, together with polyphenols, could result in antifungal activities and non-mycorrhizal formation in terms of treatment with added common bran.

Although the antifungal activity of phenolic substances and flavonoids has been widely reported, all conducted studies analyzed the antifungal activities of buckwheat secondary metabolites against pathogenic fungi; however, there are no records regarding possible antifungal actions against symbiotic fungi, for example.

To conclude, although we expected to observe positive effects from adding Tartary and common buckwheat husks and bran on silver fir root parameters, as well as on summer truffle mycorrhization level, we have confirmed only the positive effects of added

Tartary buckwheat husks on silver fir root parameters. This, however, has confirmed our suggestions regarding the high potential of buckwheat by-products (in our case, Tartary husks) for use in forestry nursery applications, as seedlings grown in a substrate with added Tartary buckwheat husks showed significantly better-developed root systems, which is important particularly when growing seedlings for the reforestation of natural forest ecosystems. Unfortunately, we have confirmed the negative effects of adding common buckwheat husks, and bran in general, on several silver fir root parameters and on the summer truffle mycorrhization level; therefore, we conclude that common buckwheat husks and bran are unsuitable, in general, for forest nursery applications.

Common and Tartary buckwheat are crops cultivated in the mountain areas of Central Europe, close to the forests, including forest tree nurseries. The extraction of rutin or other polyphenols from buckwheat waste offers potential possibilities, but at the present time, this is not yet performed. Large quantities of buckwheat husks and bran are presently seen as a waste product without any commercial value, available without any purchase cost, and with negligible transportation expenses.



**Figure 4.** Summer truffle mycorrhization level at 6 months and 12 months after spore inoculation: (A) impact of added buckwheat husks; (B) impact of added buckwheat bran. Different letters mark statistically significant results (Kruskal–Wallis,  $p \leq 0.05$ ).

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