Screening of Azotobacter, Bacillus and Pseudomonas Species as Plant Growth-Promoting Bacteria

Authors:

Mariana Minu?, Mariana Diaconu, Mihaela Ro?ca, Petronela Cozma, Laura Bulgariu, Maria Gavrilescu

Date Submitted: 2023-02-17

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In this study, bacteria from the genus of Azotobacter, Bacillus and Pseudomonas were isolated from the roots of Phaseolus vulgaris and used as plant growth-promoting bacteria for Sinapis alba L., Brassica napus L., Amaranthus retroflexus L., Linum usitatissimum L., Panicum miliaceum L. and Rumex patientia L. plants. The results showed that all three bacteria had different effects on plants growth considering both sterile and non-sterile soil. Bacillus sp. induced the greatest influence in terms of the root length of Sinapis alba L. grown in sterile soil (with 28%), while considering non-sterile soil, Pseudomonas sp. increased the root and shoot length by 11.43% and 25.15%, respectively, compared to the blank sample. Azotobacter sp. exerted the highest beneficial influence on Brassica napus L. growth in non-sterile soil, since the root and shoot lengths were stimulated with 27.64% and 52.60%, respectively, compared to uninoculated plants. Bacillus sp. had a positive effect on the growth of the shoot length of Amaranthus retroflexus L. (with 30.30% in sterile soil and 3.69% in non-sterile soil compared to the control). Azotobacter sp. stimulated the growth of the root length of Rumex patientia L. with 35.29% in sterile soil and also the shoot length of Panicum miliaceum L. in non-sterile soil by 20.51% compared to the control. Further, the roots and shoots of Linum usitatissimum L. grown in non-sterile soil and in the presence of Pseudomonas sp. increased by 178.38% and 15.08%, respectively, compared to the flax grown in sterile soil. Statistically, according to Tukey's Honestly Significant Difference (HSD) test results, not all observed differences in plants grown with the selected bacteria are significantly different compared to the control.

Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):	LAPSE:2023.0115
Citation (this specific file, latest version):	LAPSE:2023.0115-1
Citation (this specific file, this version):	LAPSE:2023.0115-1v1

DOI of Published Version: https://doi.org/10.3390/pr11010080

processes

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Screening of *Azotobacter*, *Bacillus* and *Pseudomonas* Species as Plant Growth-Promoting Bacteria

Mariana Minuț [1], Mariana Diaconu [1], Mihaela Roșca [2], Petronela Cozma [1,*], Laura Bulgariu [1] and Maria Gavrilescu [1,3,*]

[1] Department of Environmental Engineering and Management, "Cristofor Simionescu" Faculty of Chemical Engineering and Environmental Protection, "Gheorghe Asachi" Technical University of Iasi, 73 Professor Dimitrie Mangeron Blvd., 700050 Iasi, Romania
[2] Department of Horticultural Technologies, Faculty of Horticulture, "Ion Ionescu de la Brad" Iasi University of Life Sciences, 3 Mihail Sadoveanu Alley, 700490 Iasi, Romania
[3] Academy of Romanian Scientists, 3 Ilfov Street, 050044 Bucharest, Romania
* Correspondence: petronela_cozma@tuiasi.ro (P.C.); mgav@tuiasi.ro (M.G.)

Abstract: In this study, bacteria from the genus of *Azotobacter*, *Bacillus* and *Pseudomonas* were isolated from the roots of *Phaseolus vulgaris* and used as plant growth-promoting bacteria for *Sinapis alba* L., *Brassica napus* L., *Amaranthus retroflexus* L., *Linum usitatissimum* L., *Panicum miliaceum* L. and *Rumex patientia* L. plants. The results showed that all three bacteria had different effects on plants growth considering both sterile and non-sterile soil. *Bacillus* sp. induced the greatest influence in terms of the root length of *Sinapis alba* L. grown in sterile soil (with 28%), while considering non-sterile soil, *Pseudomonas* sp. increased the root and shoot length by 11.43% and 25.15%, respectively, compared to the blank sample. *Azotobacter* sp. exerted the highest beneficial influence on *Brassica napus* L. growth in non-sterile soil, since the root and shoot lengths were stimulated with 27.64% and 52.60%, respectively, compared to uninoculated plants. *Bacillus* sp. had a positive effect on the growth of the shoot length of *Amaranthus retroflexus* L. (with 30.30% in sterile soil and 3.69% in non-sterile soil compared to the control). *Azotobacter* sp. stimulated the growth of the root length of *Rumex patientia* L. with 35.29% in sterile soil and also the shoot length of *Panicum miliaceum* L. in non-sterile soil by 20.51% compared to the control. Further, the roots and shoots of *Linum usitatissimum* L. grown in non-sterile soil and in the presence of *Pseudomonas* sp. increased by 178.38% and 15.08%, respectively, compared to the flax grown in sterile soil. Statistically, according to Tukey's Honestly Significant Difference (HSD) test results, not all observed differences in plants grown with the selected bacteria are significantly different compared to the control.

Keywords: beneficial effects; non-sterile soil; plant growth-promoting bacteria (PGPB); sterile soil

Citation: Minuț, M.; Diaconu, M.; Roșca, M.; Cozma, P.; Bulgariu, L.; Gavrilescu, M. Screening of *Azotobacter*, *Bacillus* and *Pseudomonas* Species as Plant Growth-Promoting Bacteria. *Processes* **2023**, *11*, 80. https://doi.org/10.3390/pr11010080

Academic Editor: Guining Lu

Received: 20 November 2022
Revised: 21 December 2022
Accepted: 22 December 2022
Published: 28 December 2022

1. Introduction

Soil is a complex system that supports plant growth and animal and human life, being negatively affected by various abiotic and biotic factors [1–4]. For example, high concentrations of heavy metals, an excess or deficiency of nutrients, high temperature or the presence of pathogens may reduce soil fertility, affect the structure and constrain the formation of sustainable agricultural soil systems, and also may cause nutritional and hormonal imbalance, physiological disorders and other changes in plants. Finally, the growth and development of the plants, the yield and biomass production are highly affected [5–8].

One of the most promising strategies to achieve a sustainable agriculture system is the use of rhizosphere bacteria [9]. Bacteria were among the first life forms to appear on the planet, with nearly 6.5 million species identified on the Earth and 2.2 million species in the oceans [10]. Various types of bacteria are found on plants parts, which can have beneficial, harmful or neutral effects on plant growth and development. Bacteria

with beneficial effects are known as plant growth-promoting bacteria (PGPB) [8]. In general, the PGPB belong to *Azotobacter*, *Agrobacterium*, *Arthrobacter*, *Azospirillium*, *Bacillus*, *Burkholderia*, *Bradyrhizobium*, *Clostridium*, *Klebsiella*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Mesorhizobium*, *Pseudomonas*, *Phyllobactum*, *Streptomyces*, *Serratia*, *Rhizobium*, *Thiobacillus* and *Xanthomonas* families [8,11,12], with the predominant species being members of the *Bacillus* and *Pseudomonas* families [13].

The rhizosphere can comprise up to 1011 microbial cells per gram of root and 30,000 prokaryotic species that may improve plant productivity [14]. Due to their characteristics, diversity and relationships to plants, PGPB could be used to alleviate abiotic and biotic stresses on plants [13,15,16]. In addition, the growth-promoting substances that are produced in high quantities by the action of rhizosphere microorganisms influence the overall morphology and physiology of different crops [17]. In this regard, in the last decade, the use of plant growth-promoting bacteria increased the crop yield by almost 10% [18]. Bacteria promote plant growth under both normal and stress conditions using direct mechanisms such as nitrogen fixation, phosphate solubilization, potassium solubilization, phytohormone production, iron sequestration or indirect mechanisms for the protection of plants against various pathogens by antibiotic release, the induction of systemic resistance and competition [19,20].

The use of PGPB is also important for the intensification of phytoremediation processes when various species of plants are used to clean up soil contaminated with different toxic pollutants (for example, heavy metals). PGPB have the ability to colonize the roots of plants, to stimulate plant growth by increasing the nutrient uptake (nitrogen, phosphorus) and phytohormone content (auxin, cytokinin), to survive, develop and compete with pathogenic species by the secretion of substances that inhibit their activity [8,21,22]. Plants stimulate microbial activity by increasing the secretion of elements such as carbohydrates, amino acids and other growth factors, thus ensuring the essential nutrients for microbial growth [23]. PGPB can also possess the ability to survive in various environmental situations and can even develop different defense mechanisms, such as antioxidant defense systems. The antioxidant enzymes produced are able to prevent the oxidative stress caused by stress factors through the reduction of excess reactive oxygen species (ROS) [24,25]. For instance, PGPB have been applied in combination with metal-tolerant plants to improve the efficiency of the phytoremediation of metal-polluted soils by various mechanisms (e.g., nitrogen fixation, phosphorus solubilization, production of siderophores, acetic acid, ammonia or synthesis of phytohormones) [26,27]. It is also important to note that PGPB are able to alleviate metal toxicity and alter metal bioavailability in soils through metal biosorption, bioaccumulation, mobilization, redox reaction, transformation and precipitation. They can also provide tolerance to a variety of climatic stresses (salinity, extreme temperature). Thus, understanding the interaction between plants and microbe association would enhance the process of shifting from the laboratory to the field and would accelerate phytoremediation under various environmental stressors [27].

The soil type may influence the effects of PGPB on plant growth and development. For example, in the studies elaborated by Rajkumar et al. [28] and Rajkumar and Freitas [29], the results showed that *Pseudomonas* sp. and *Bacillus* sp. appeared to consistently promote the growth of *Brassica juncea* in sterilized soil. However, the effects are more variable in non-sterilized soil according to Grandlic et al. [30,31], mainly due to the inference with the microorganisms that are already present in the soil. Moreiro et al. [32] applied *Ralstonia eutropha* 1C2 and *Chryseobacterium humi* ECP37 bacteria on maize plants to promote their growth in sterile and non-sterile soil. Inoculation in sterile soil led to a significant increase of dry biomass compared to non-sterile soil. Selecting suitable bacterial strains as inoculants for large-scale phytoremediation is also important, as stated by Moreiro et al. [32]. Soil sterilization has been shown to alter the soil microbial structure and microbiological properties of the rhizosphere, but at the same time, it may promote plant growth, probably by reducing the inoculum of soil-borne plant diseases [33]. Moreover, soil sterilization could be used as a methodology to select and isolate beneficial bacteria specific to certain

crops [33]. Furthermore, in sterile soil, only the effects of inoculated bacteria on the plants can be observed, whereas in non-sterile soil, the effects are variable due to competition and the complementary activities of the indigenous bacteria from soil that can have a major influence on the PGPB activities [22].

Therefore, it is very important to study and clarify the correlation between the presence of PGPB in soil, the quality of the soil and the ability of plants to grow in sterile and non-sterile conditions. Based on our knowledge, at the Romanian level, few studies have investigated the growth of PGB in both sterile and non-sterile soil, and a comparative study in this regard has not yet been developed. For example, in the study conducted by Ştefănescu [34], *Bacillus megaterium* increased the germination of soybean plant growth in sterile soil. The dry biomass and the growth of the plant were improved considering different type of soils amended with phosphogypsum (which is a residue of phosphoric acid). Based on his research, the selected bacteria will be further used for the bioremediation of phosphogypsum-contaminated soil. Diaconu et al. [1] used *Azotobacter chroococcum* and *Pichia spas* on cress plants (*Lepidium sativum* L.) to test the phytotoxicity of Cd and Cr, this being a preliminary study for the phytoremediation of soil polluted with heavy metals.

In this context, the main objective of the present paper was to establish the potential of *Azotobacter*, *Bacillus* and *Pseudomonas* species to promote the growth of *Sinapis alba* L. (white mustard), *Brassica napus* L. (rapeseed), *Amaranthus retroflexus* L. (redroot pigweed), *Linum usitatissimum* L. (flax), *Panicum miliaceum* L. (proso millet) and *Rumex patientia* L. (patience dock) plants in both sterile and non-sterile soil. The plants were basically selected considering the characteristics that must be fulfilled in order to be successfully applied in soil phytoremediation such as fast growth, a branched root system, the production of a high biomass amount, as well as the ability to tolerate and bioaccumulate heavy metals. The selected plants are widely known to be metal-tolerant and accumulator plants as stated by different authors: white mustard and oilseed rape [35–37], flax [38,39], dock [40,41] redroots pigweed [42,43] and proso millet [44,45]. Through this dual approach (sterile and non-sterile soil), is expected that the transferability of the process to more complex environmental conditions can be assessed and whether soil conditions influence the performance of the bacteria used can be ascertained. Thus, the present study may contribute toward developing an understanding of the efficiency of the bacteria under different soil conditions. Finally, the results of this work will represent the foundations for further studies that will additionally exploit the plant–microorganism relationship for increasing the efficiency of the phytoremediation of soils polluted with heavy metals.

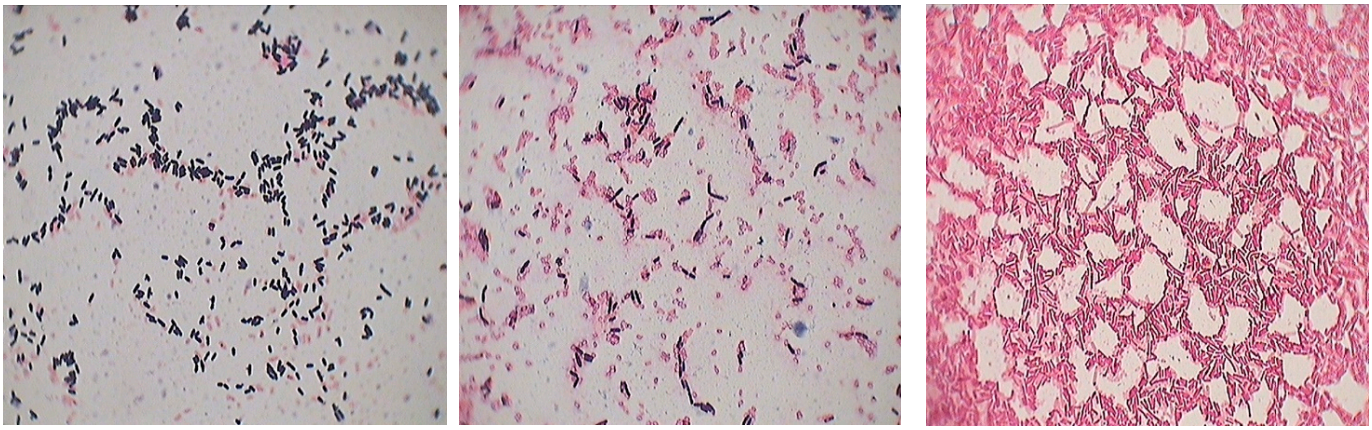
2. Materials and Methods

2.1. Isolation and Identification of Bacteria Genus

Generally, leguminous plants are able to establish symbiotic relationships with both rhizobial and non-rhizobial bacteria. These bacteria contribute to the formation of plant root nodules, colonizing them and fixing atmospheric nitrogen [46]. Several species of bacteria from various genera (*Acinetobacter*, *Paracoccus*, *Bacillus*, *Phyllobacterium*, *Azotobacter*, *Pseudomonas*) have been isolated from different parts of bean plants (roots, stems and seeds) [46–49]. Taking into account that the soil area near the vicinity of the plant roots presents the highest density and diversity of microorganisms, it was considered appropriate to isolate them from the rhizosphere of bean, maize and tomato plants. According to our tests, bacteria from the genera *Azotobacter*, *Bacillus* and *Pseudomonas* were only identified in bean plants. Therefore, *Azotobacter*, *Bacillus*, and *Pseudomonas* species were isolated from the rhizosphere area of bean plants (*Phaseolus vulgaris*) grown in a soil where no phytosanitary treatments were applied. More specifically, soil samples were collected from a local garden where only manure or compost were applied to increase soil fertility.

The roots of the bean plants were placed in 10 mL phosphate buffer (0.1 M and pH 7), shacked and centrifuged at 5000 rpm for 10 min in order to extract the three bacteria from the roots' surface. The resulting concentrate was subsequently mixed with 10 mL phosphate buffer, diluted to 10^8 with 9 g/L sterile saline solution and used for the isolation of

dedicated microorganisms. The *Azotobacter* strain was isolated on Jensen medium (20 g/L sucrose; 1 g/L K_2HPO_4; 0.5 g/L $MgSO_4$; 0.5 g/L NaCl; 0.1 g/L $FeSO_4$; 0.005 g/L Na_2MoO_4; 2 g/L $CaCO_3$; 15 g/L agar), the *Bacillus* strain on Luria–Bertani medium (10 g/L tryptone; 5 g/L yeast extract; 5 g/L NaCl; 15 g/L agar; pH—6.8–7) and the *Pseudomonas* strain on Acetamide Nutrient Broth medium (10 g/L acetamide; 1.39 g/L K_2HPO_4; 0.73 g/L KH_2PO_4; 0.5 g/L $MgSO_4$ x $7H_2O$; 5 g/L NaCl; 0.012 g/L phenol red; 20 g/L agar; pH—6.8–7.0) [50]. All culture media were sterilized at 121 °C for 15 min. The growth of microorganisms on specific media was achieved at 30 °C for 4–8 days. Moreover, the reaction between the crystal violet and the surface of the microbial cells (Gram-positive or Gram-negative character), the shape and arrangement of cells, the presence or absence of the spores, as well as their arrangement in the cells [51] were visualized using a microscope (Microscope—Motic Digital Microscope—DMB Series). In Figure 1, the microscopic aspects of the *Azotobacter*, *Bacillus* and *Pseudomonas* species used in this study are presented.



(**a**) (**b**) (**c**)

Figure 1. Microscopic aspects of: (**a**) *Azotobacter* sp.—Gram-positive; (**b**) *Bacillus* sp.—Gram-positive; (**c**) *Pseudomonas* sp.—Gram-negative.

2.2. Soil Sterilization

The soil utilized in the experiments was a universal substrate composed of peat soil and humus for garden plants (S.C. FLORISOL PRODUCT S.R.L supplier). According to the supplier, the soil was taken from the Dersca–Dorohoi peat field (site located in the Moldova region, Romania) and contains 192 mg/L P, 1350 mg/L K, 410 mg/L N, has a pH of 6.5–7 and a humidity of 60–70%. The non-sterile soil was dried at laboratory temperature (22–24 °C) for 7 days. The sterile soil was also dried at room temperature for 7 days and after that it was sterilized for 3 h at 105 °C (using a forced convection oven, Labtech LDO-080F). The purpose of sterilization was to eliminate all of the potential microorganisms from the soil in order to evaluate the effects of the inoculated bacteria on plant growth. Later, the presence of microorganisms in the soil was verified by performing growth tests on solid medium in Petri dishes [51].

2.3. Experimental Protocol Used for Synergism Studies

In order to establish the effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on the plant growth, the experiments were carried out in 30 mL polypropylene pots filled with 15 g of sterile or non-sterile peat soil.

The microorganisms were previously tested to detect their ability to be applied as plant growth-promoting bacteria. The methods used for selecting the plant growth-promoting bacteria from the rhizobiome of the plant species have been mainly based on traditional techniques considering cultivation of the microorganisms on specific nutrient media and under specific growth conditions. Thus, the isolated microorganisms were initially tested for siderophore production by applying the universal chemical test (chromium azurosul-

fonate test) as described by Schwyn and Neilands [52] and by determining indoleacetic acid (IAA) synthesis using the method proposed by Gordon and Weber [53] (data not shown).

The proposed experiments were performed following the protocol presented in Figure 2. The plants were raised from 9 September to 3 October 2019 under laboratory conditions. The inoculum of each bacterium was prepared with YPG sterile medium (yeast peptone glucose), whose composition was as follows: 40 g/L glucose, 10 g/L peptone, 5 g/L NaCl and 5 g/L yeast extract [51].

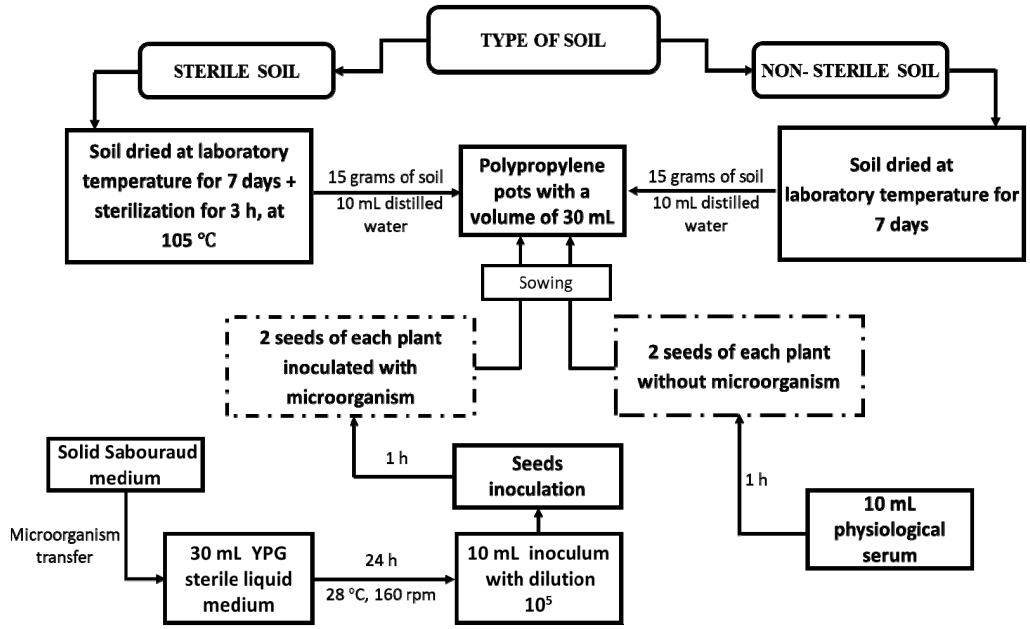


Figure 2. Experimental protocol for screening of *Azotobacter*, *Bacillus* and *Pseudomonas* species as PGPB.

The plant seeds used in this study were purchased from various organic product manufacturers or were harvested from the spontaneous flora of Romania, from areas where no phytosanitary treatments and no chemical fertilizers were applied. *Sinapis alba* L. seeds were purchased from Solaris, *Linum usitatissimum* L. and *Panicum miliaceum* L. from Germline, *Brassica napus* L. from Monsanto (DEKALB Expansion hybrids), which to our knowledge are certified as organic seeds. *Amaranthus retroflexus* L. and *Rumex patientia* L. seeds were harvested from spontaneous flora. *Amaranthus retroflexus* L. (weed) is a common plant from Romania that may be found on cultivated land, gardens, waste disposal sites, roadsides, riverbanks and other open habitats where annual weeds predominate. *Rumex patientia* L. (patience dock) is rarely cultivated, being an unpretentious plant adapted to a temperate climate, and may be picked directly from nature. The seeds of weed and patience dock were not available on the bio market; thus, these were taken from a garden where no phytosanitary treatments were performed. The *Amaranthus retroflexus* L. and *Rumex patientia* L. seeds were collected when the plants were at the maturity stage (inflorescence). Previously, all six plants' seeds were sterilized with 95% ethanol (*v*/*v*) for 20 s, followed by 20% sodium hypochlorite (NaClO) (*v*/*v*) for 10 min. Further, the seeds were rinsed seven times with sterile distilled water and dried for 5 days at laboratory temperature (22–24 °C) [54]).

2.4. Plant Length and Dry Biomass Measurement

The effects of the *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Sinapis alba* L. (white mustard), *Brassica napus* L. (rapeseed), *Amaranthus retroflexus* L. (redroot pigweed), *Linum usitatissimum* L. (flax), *Panicum miliaceum* L. (proso millet) and *Rumex patientia* L. (patience dock) plants were established by visualizing the appearance of the roots and shoots by measuring their length and by weighing the root and shoot dry biomass. Depending on the

growth rate, the plants were harvested after 25 days (*Sinapis alba* L., *Brassica napus* L., *Linum usitatissimum* L., *Panicum miliaceum* L. and *Rumex patientia* L.) and 30 days (*Amaranthus retroflexus* L.), respectively.

The roots of the plants were gently removed from the soil, washed with distilled water and gently tapped to eliminate any excess water. All plants were measured in terms of root and shoot biomass content by drying the fresh biomass for 15 h at 105 °C in a forced convection oven (Labtech LDO-080F), followed by weighing the dry biomass content of each sample [55].

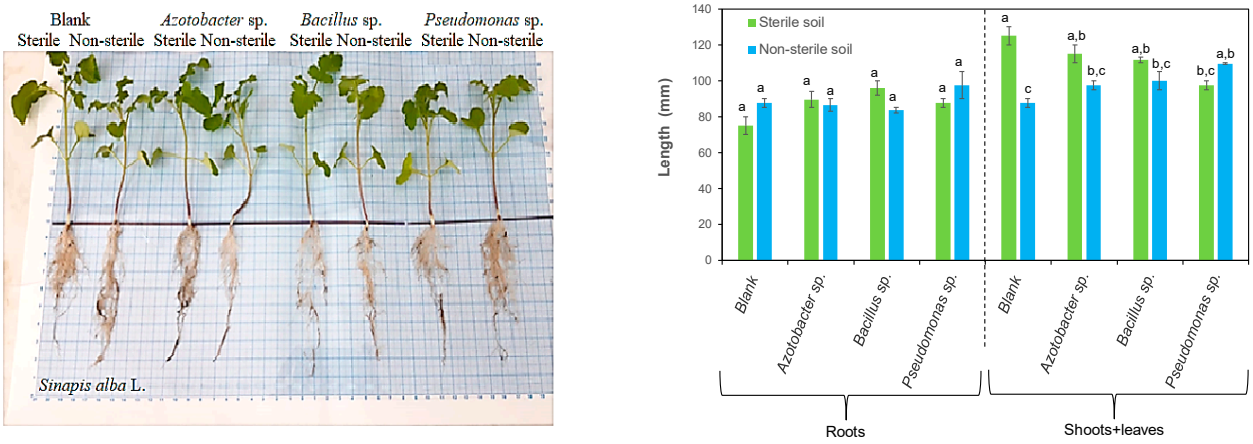
2.5. Statistical Analysis

The datasets collected were screened by analysis of variance (ANOVA) in Minitab 17 software. The Tukey's Honestly Significant Difference (HSD) test at a significance level of $p \leq 0.05$ was used to compare the mean difference between each group. The graphical representations of the data were created using an Excel 2013 spreadsheet.

3. Results

3.1. Effects of Bacteria on Plant Length

The *Sinapis alba* L. roots and shoots had different aspects in the presence of the selected bacteria that colonized the seeds, also depending on the soil in which the plants were grown (sterile vs. non-sterile soil). In terms of visual aspects, it was observed that the shoots and roots had the same vigor; however, their length, as well as the branching of the root system, depended on the microorganism and soil type (Figure 3a). From Figure 3a, it can be clearly seen that the root system of *Sinapis alba* L. grown in non-sterile soil and in the presence of *Pseudomonas* sp. was much more branched compared to the other plants' root system. Moreover, from the measurements of the length of the plant roots and shoots, it was observed that in sterile soil, all three microorganisms stimulated the growth of the root length, with *Bacillus* sp. having the greatest effect (28% higher than the control sample). However, the selected microorganisms caused a shortening in the shoot length. In the non-sterile soils, only *Pseudomonas* sp. induced an increase in the root length (by 11.43% compared to the control sample), while the other microorganisms had no effect on the length. In the presence of the selected bacteria, the shoots of the plants grown in the non-sterile soil were longer compared to the control: *Azotobacter* sp. stimulated the shoot length of the plants by approximately 11.43%, *Bacillus* sp. by 14.29% and *Pseudomonas* sp. by 25.15% (Figure 3b).



(**a**) (**b**)

Figure 3. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Sinapis alba* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).

Similar effects were observed in rapeseed plants grown in the presence of the selected bacteria. In the sterile soil, the *Azotobacter, Bacillus* and *Pseudomonas* species had slightly positive effects on the increase in the length of the roots and, on the contrary, inhibitory effects on the shoots, which were visibly shorter compared to the control. In the non-sterile soil, *Azotobacter* sp. had the greatest influence on plant growth, with longer root and shoot elongations of approximately 17 mm and 40.5 mm, respectively, compared to the control sample (Figure 4b).

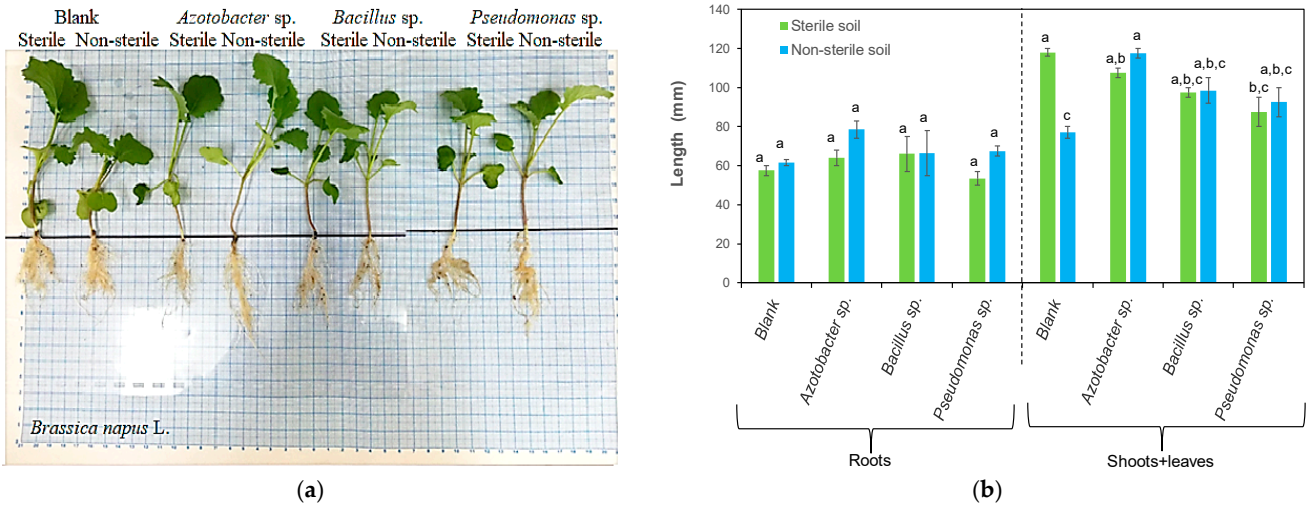


Figure 4. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Brassica napus* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).

The studies performed to establish the effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on the growth of *Amaranthus retroflexus* L. plants showed that their development in the sterile soil was more efficient than in the non-sterile soil (Figure 5a). The shoots of the plants from the sterile soil were shorter by 23.97%, 28.91%, 4.44% and 13.64%, respectively, compared to the shoots of the plants grown in non-sterile soil. It was also found that *Azotobacter* sp. had an inhibitory effect on the plants grown in the sterile soil, assuming that this bacteria species did not promote the growth of *Amaranthus retroflexus* L. In the non-sterile soil, no significant difference was observed between the root and shoot length of *Amaranthus retroflexus* L. inoculated with *Azotobacter* of uninoculated plants (Figure 5b). Therefore, among the selected species, *Bacillus* sp. showed the highest performance in stimulating growth in terms of the length of the plant parts: the shoots were longer by 30.30% in the sterile soil and by 3.69% in the non-sterile soil compared to the control sample (Figure 5b).

Azotobacter sp., attached to the plant seeds' surface in the sterile soil, diminished the root length of *Panicum miliaceum* L. and *Linum usitatissimum* L. by 33.17% and 16.39%, respectively, but at the same time, increased the root length of *Rumex patientia* L. by 35.29% (Figures 6–8). *Bacillus* and *Pseudomonas* species, attached to the surface of the plant seeds, induced a decrease in the flax plants' root length by 24.75% and 38.13%, respectively, while insignificant changes in the root length of proso millet and patience dock were observed. The selected bacteria caused insignificant visible changes compared to the control sample on flax, proso millet and patience dock shoot growth in the sterile soil, and an increase of only 12% in the millet shoots with *Bacillus* sp. was observed.

In the non-sterile soil, the roots and shoots of the flax plant were longer than those grown in the sterile soil, thus denoting the importance of the microorganisms present in soil in the development of this plant. The roots of the plants were longer by 48.83% (control), 88.80% (*Azotobacter* sp.), 100% (*Bacillus* sp.) and 178.38% (*Pseudomonas* sp.) and

the shoots by 31.43% (control), 23.40% (*Azotobacter* sp.), 4.93% (*Bacillus* sp.) and 15.08% (*Pseudomonas* sp.) compared to those of the plants grown in the sterile soil.

According to the data presented in Figures 6 and 7, the indigenous microorganisms that are naturally available in the selected soil together with *Azotobacter*, *Bacillus* and *Pseudomonas* species did not seem to have significant beneficial effects on the proso millet and patience dock plants compared to the flax plant. The proso millet and patience dock roots inoculated with *Azotobacter* and *Pseudomonas* were highly developed; however, in the presence of *Bacillus* sp., they had the same length or were even shorter. The *Azotobacter* sp. attached to the surface of proso millet seeds in the non-sterile soil stimulated the shoot length by 20.51% and diminished the root length by 23.68%. The *Bacillus* and *Pseudomonas* strains induced a decrease in the plant root and shoot length compared with the control sample. The *Linum usitatissimum* L. roots and shoots were not significantly longer in the presence of the selected bacteria compared to the control sample, except for the shoots of the plants grown in the presence of *Bacillus* sp., which were lower by 23.19%.

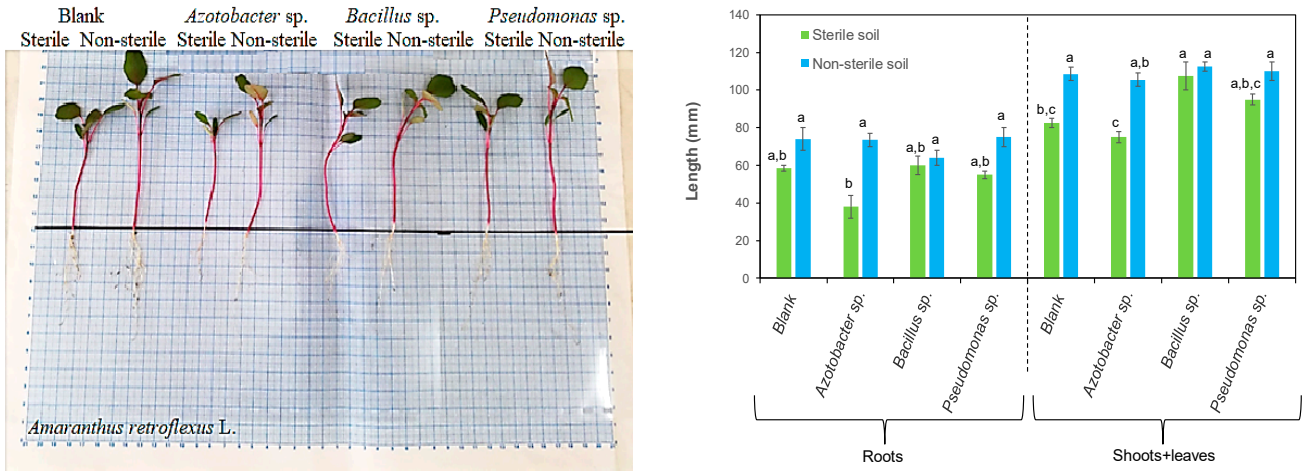


Figure 5. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Amaranthus retroflexus* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).

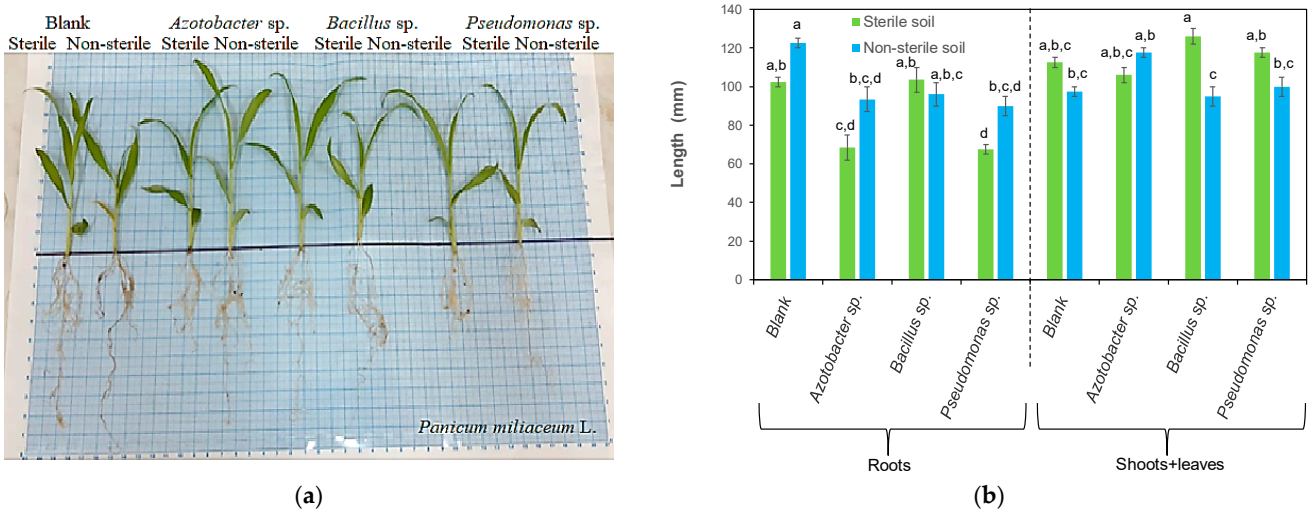
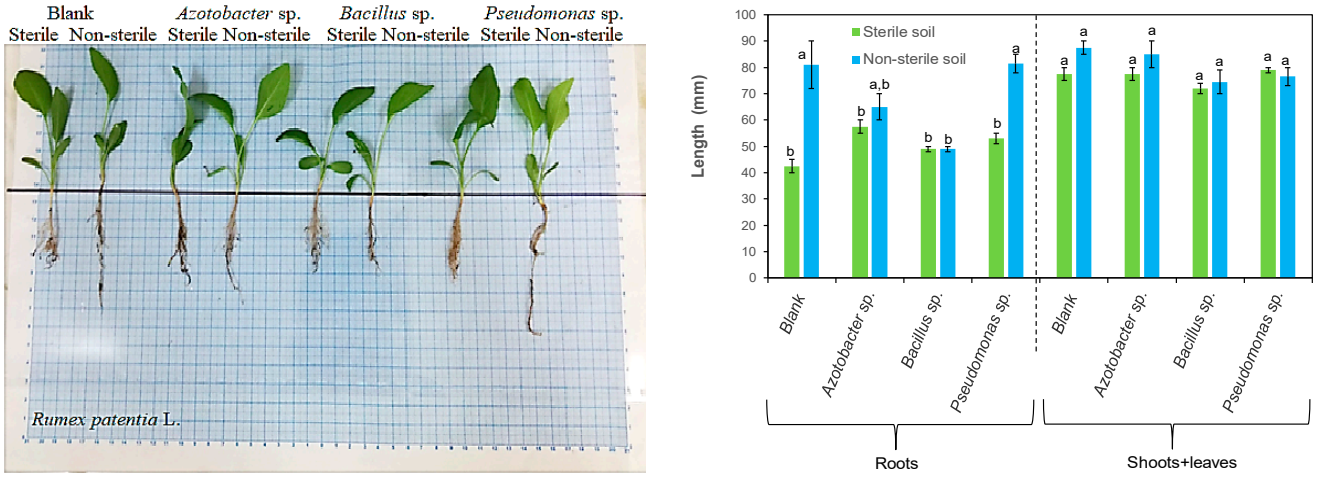
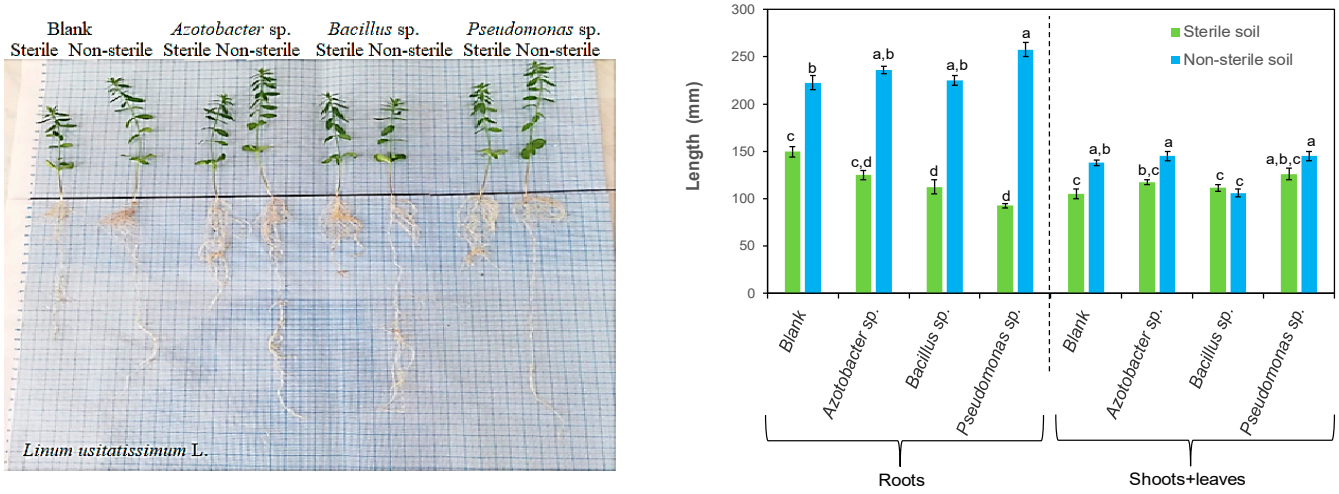


Figure 6. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Panicum miliaceum* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).



(**a**) (**b**)

Figure 7. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Rumex patientia* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).



(**a**) (**b**)

Figure 8. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Linum usitatissimum* L.: (**a**) visual aspect of plants and (**b**) the root and shoot/leaf length (error bars represent the experimental error; means that do not share a letter are significantly different according to the Tukey's HSD test).

3.2. Effects of Bacteria on Plant Dry Biomass

Information about the effects that the microorganisms had on plant growth and development was also provided by weighing the root and shoot dry biomass of the plants (Figure 9). Thus, the *Amaranthus retroflexus* L. and *Linum usitatissimum* L. dry biomass amounts were lower compared to the control sample regardless of the bacteria or soil used.

In the sterile soil, the root and shoot dry biomass of *Amaranthus retroflexus* L. grown in the presence of the *Azotobacter*, *Bacillus* and *Pseudomonas* strains was lower by 38.64%, 5.77%, 38.34%, and 16.59%, 2.12% and 16.31%, respectively, compared to the control samples.

Under the same conditions, the root dry biomass weight of *Linum usitatissimum* L. was smaller by 30.68%, 15.38% and 8.57%, and the shoot dry biomass weight by 22.62%, 11.74% and 4.87%, respectively, compared to the control samples. In the non-sterile soil, the selected bacteria caused a reduction in the *Amaranthus retroflexus* L. root and shoot dry biomass by 8.85–46.86% and 0.09–30.33%, respectively, with the most pronounced

effects being found for the plants grown in the presence of *Bacillus* sp. Similar effects were observed in the case of flax plants, with the greatest negative effects being given by *Pseudomonas* (−17.66% for roots and −11.03% for shoots).

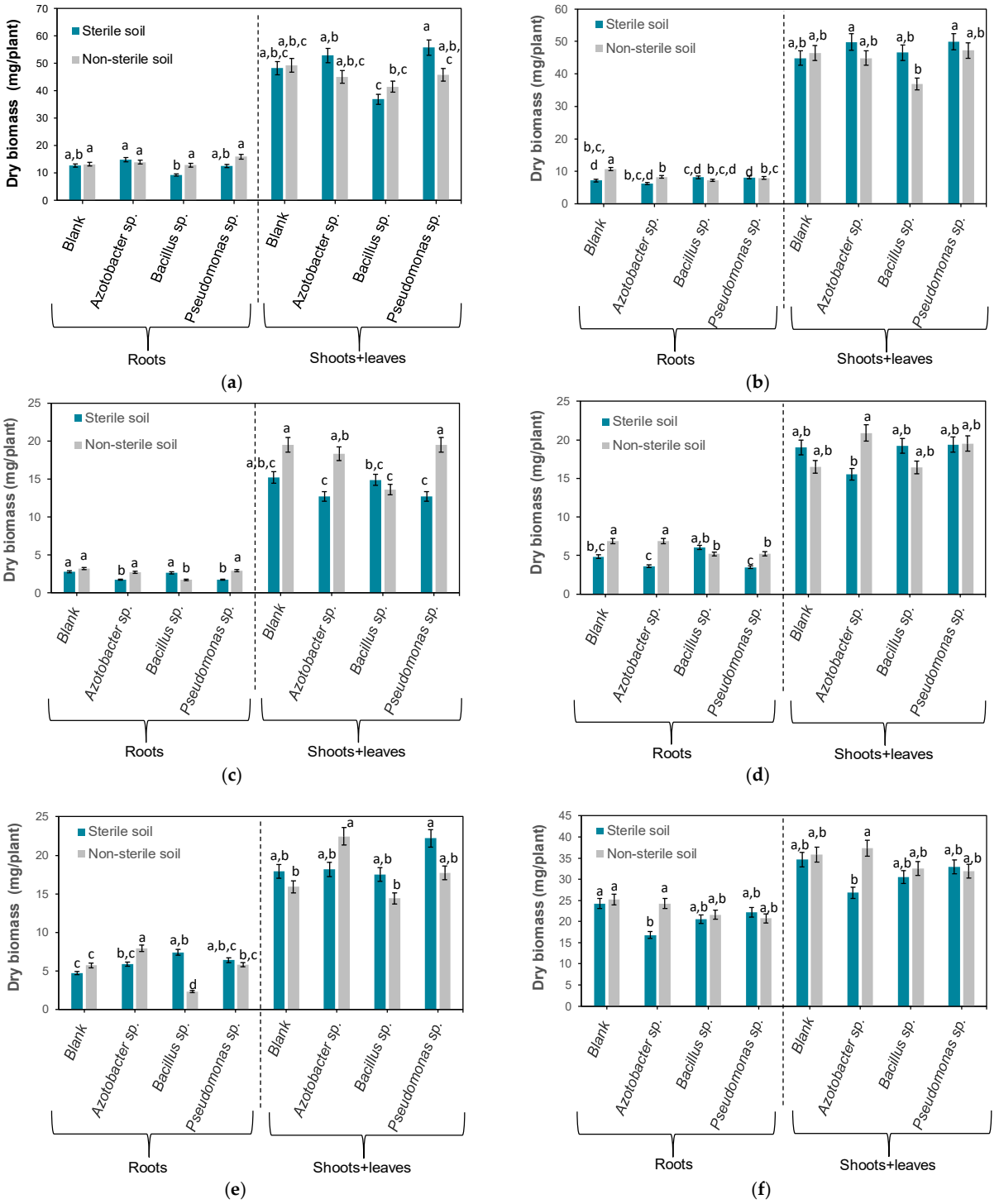


Figure 9. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on: (**a**) *Sinapis alba* L., (**b**) *Brassica napus* L., (**c**) *Amaranthus retroflexus* L., (**d**) *Panicum miliaceum* L., (**e**) *Rumex patientia* L. and (**f**) *Linum usitatissimum* L. on dry biomass weight after 25 days (*Sinapis alba* L., *Brassica napus* L., *Linum usitatissimum* L., *Panicum miliaceum* L., *Rumex patientia* L.) and 30 days (*Amaranthus retroflexus* L.) of growth (error bars represent the percentage error; means that do not share a letter are significantly different according to the Tukey's HSD test).

In the sterile soil, an increase in the dry biomass was observed for the roots and shoots of: *Sinapis alba* L. and *Rumex patientia* L. grown with *Azotobacter*; *Brassica napus* L., *Rumex patientia* L. and *Panicum miliaceum* L. grown with *Bacillus* sp.; and *Brassica napus* L. and *Rumex patientia* L. grown with *Pseudomonas*. In the sterile soil, the dry biomass of *Rumex patientia* L. roots was 24.80%, 57.15% and 35.53% higher in the presence of *Azotobacter*, *Bacillus* and *Pseudomonas* strains, respectively (Figure 9e), due to the fact that these bacteria stimulated the root elongation and branching system (Figure 6). In the non-sterile soil, in the presence of *Bacillus* sp., the dry biomass of *Sinapis alba* L., *Brassica napus* L., *Panicum miliaceum* L. and *Rumex patientia* L. was lower by 2.2%, 32.23%, 24.41% and 59.22%, respectively, for the roots and by 15.81%, 20.65%, 0.3% and 9.46%, respectively, for the shoots compared to the control sample. The rapeseed roots' length inoculated with selected bacteria in the non-sterile soil was longer than the roots of the uninoculated plants. However, the root system was less developed (weakly branched), being thus justified the lower dry biomass amount of the roots compared to the control sample (Figure 9b).

The *Azotobacter* sp. development in the non-sterile soil caused an increase in the root dry biomass amount of *Sinapis alba* L. (6.19%), *Panicum miliaceum* L. (0.07%) and *Rumex patientia* L. (39.09%) and a decrease in the *Brassica napus* L., *Amaranthus retroflexus* L. and of *Linum usitatissimum* L. shoot biomass amount. Thus, the results presented in Figure 9 show that each bacterium induced different effects on the plant development, effects that are dependent on the plant species as well as on the presence of other microorganisms in soil.

3.3. Statistical Analysis of Experimental Data

In order to evaluate the effects induced by the *Azotobacter*, *Bacillus* and *Pseudomonas* species on *Sinapis alba* L., *Brassica napus* L., *Amaranthus retroflexus* L., *Panicum miliaceum* L., *Rumex patientia* L. and *Linum usitatissimum* L., the means of their root and shoot lengths as well as their dry biomass were compared using Tukey's Honestly Significant Difference (HSD) test at a significance level of $p \leq 0.05$. According to the Tukey test results, no significant differences were observed between the means of the root length of the *Sinapis alba* L., *Brassica napus* L. and *Amaranthus retroflexus* L. controls and the means of the plants grown in the presence of the selected bacteria. In the sterile soil, only the shoot lengths of *Sinapis alba* L. and *Brassica napus* L. grown under the influence of *Pseudomonas* were significantly shorter than those of the control. The mean length of *Amaranthus retroflexus* L. shoots under the effect of *Bacillus* sp. in the sterile soil was statistically significantly longer than the control. The studies in the non-sterile soil showed that only the shoot lengths of *Brassica napus* L. under the influence of *Azotobacter* sp. were statistically significantly longer compared to the control sample. The Tukey's test showed that in the case of *Panicum miliaceum* L., *Azotobacter* and *Pseudomona*s sp. had a significant negative effect on the root length in both soil types. In *Rumex patientia* L. plants, according to the statistical analysis, significant differences compared to the control were only observed in the root length of the plants grown in the presence of *Bacillus* sp. in the non-sterile soil. The *Pseudomonas* and *Bacillus* strains in the sterile soil significantly affected the *Linum usitatissimum* L. root length, while the shoot length of this plant was not affected.

In terms of the dry biomass, significant differences were induced by the selected bacteria on *Brassica napus* L. roots in the non-sterile soil compared to the control. The dry biomass of *Amaranthus retroflexus* L. roots grown with *Azotobacter* sp. in the sterile soil was significantly lower than the control and also compared to the roots and shoots of *Bacillus* sp. in the non-sterile soil. *Bacillus* sp. in the sterile soil significantly reduced the root biomass of *Panicum miliaceum* L. and *Rumex patientia* L. compared to the control. More statistical information about the differences between the means of each group have been highlighted in Figures 3–9 by the letters above the bars. The means that do not share a letter are significantly different according to the Tukey's HSD test.

4. Discussion

The selected plants are very widespread in Romania, have a fast growth rate and can adapt to different environmental conditions. *Sinapis alba* L. and *Brassica napus* L. are annual plants from the *Brassicaceae* family, especially cultivated for their seeds [56]. *Amaranthus retroflexus* L. is part of the spontaneous flora, being mainly widespread in agricultural crops as an annual plant belonging to the *Amaranthaceae* family [57]. *Linum usitatissimum* L. is an annual, herbaceous and fibrous plant from the *Linaceae* family cultivated in temperate climates. *Panicum miliaceum* L. is an annual and herbaceous plant from the *Poaceae* family, while *Rumex patientia* L. is a perennial plant that belongs to the *Polygonaceae* family, being rarely cultivated and may be picked directly from nature [39,40,58].

According to numerous studies, the *Azotobacter*, *Bacillus* and *Pseudomonas* species belong to the class of microorganisms denoted as plant growth-promoting rhizobacteria [13,59–61]. For example, the *Bacillus* species are the most abundant bacteria from the plant rhizosphere [61], and according to Hashem et al. [13], the species are capable of protecting the plants against stress factors and pathogens, are able to increase the lifetime of plants and to secrete metabolites and a variety of hydrolytic enzymes (cellulases, β-glucanases, proteases) that are involved in plant growth promotion. It is known that *Bacillus* are able to synthesize many secondary metabolites, hormones, cell-wall-degrading enzymes and antioxidants, thus assisting the plant in its defense against pathogen attacks. This bacterium can also solubilize the P from soil, enhance nitrogen fixation, and produce siderophores that promote its growth and suppress the activity of pathogens [13]. According to Muis [62], the inoculation of seeds with *Bacillus subtilis* offers protection to plants for the whole growth cycle as a result of the colonization of the entire root system. Adam et al. [63] reported that *Bacillus subtilis* Sb4-23 reduced nematode activity in tomato by activating induced systemic resistance. Ndeddy and Babalola [64] showed that *Bacillus subtilis* KP717559 inhibited the growth of the *Fusarium solani* fungus on *Brassica junc*ea. Sarwar et al. [65] identified different species of *Bacillus* able to produce siderophores that increased the bioavailability of the iron in soil by at least 69%.

The *Azotobacter* species (through the synthesis of biologically active substances, the production of phytopathogenic inhibitors, nitrogen fixation in soil and by balancing the nutrient uptake) may produce positive effects on crop growth and yield [17]. *Azotobacter* species are reported to synthesize auxins, cytokinins and gibberellins, substances that have been found to be associated with enhancement of plant growth by the positive influencing of seed germination and the root and shoot lengths of different crops [66]. Romero-Perdomo et al. [60], in their paper, showed that the application of a mixed culture of *Azotobacter* species (*Azotobacter chroococcum* AC1 and *Azotobacter chroococcum* AC10) reduced the need for nitrogen fertilizers by up to 50%. Kumar et al. [67] reported that *Azotobacter chroococcum* strains solubilized the phosphate in soil and improved the growth of *Triticum aestivum*. *Pseudomonas* species are considered the most promising group of microorganisms involved in the control of plant diseases and plant growth promotion [61]. According to Príncipe et al. [68], *Pseudomonas fluorescen*s was able to produce tailocins and to control the disease produced by *Xanthomona*s in tomato. It was also reported that *Pseudomonas fluorescens Pf-5* may produce different antibiotic compounds such as cyclic lipopeptides, amphisin, pyrrolnitrin, pyoluteorin, phenazine, tensin or tropolone [69,70], indole-3-acetic acid (IAA) [71] and ACC deaminase [72].

As discussed above, this present investigation confirms the positive effects of PGPB strains on plant growth in different crops and the results of this work are in line with other outcomes from the literature. For example, Ashnaei's [73] study showed that the inoculation of *Oryza sativa* with *Bacillus subtilis* UTSP40 produced the elongation of roots compared to the control, but decreased shoot growth, while the dry weight of the roots and shoots was lower compared to the dry biomass of the control sample. Moreover, in the study conducted by Prajapati et al. [22], the inoculation of *Oryza sativa* L. with *A. chroococcum* showed a significant decrease in the dry root weight compared to the control sample. The author suggested that the decrease in the dry weight of the plants

grown in sterile soil in the presence of inoculants may be explained by the variable water content, which depends on the plant's water status [15]. Liu et al. [26] revealed that the use of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens* strains significantly increased the biomass content of *Medicago sativa*, *Pennisetum purpureum* and *Oenothera erythrosepala* plants. In the study by Bhatia et al. [74], *Pseudomonas fluorescens* I and *Pseudomonas fluorescens* II enhanced the root biomass content of sunflowers. According to Rajkumar and Freitas [29], *Pseudomonas* sp. Ps29C increased the dry weight biomass of *Brassica juncea* by 17% in sterile soil. *P. fluorescens* NUU2 significantly stimulated the shoot and root length and dry weight of wheat [75]. Kuramshima et al. [37] showed that *B. subtilis* 11VM and *B. subtilis* 26D stimulated white mustard shoot and root growth by 7–12% and 9.7–11%, respectively. The use of *Azotobacter* caused a significant increase in the root and shoot length and dry biomass of maize and bamboo [59]. Chauchan et al. [76] reported that *A. chroococcum* increased the root and shoot length of *Gossypium hirsutum*, *Cyamopsis tetragonoloba* and *Lycopersicum esculentum* plants. Lally et al. [55] observed that *P. fluorescens* L.321 increased the *Brassica napus* length by 5.99%. Biari et al. [77] found that treatment with *Azotobacter* significantly increased the height and dry weight of the *Zea mays* L. shoot. Gholami et al. [78] showed that soil condition influenced the growth of *Zea may* L. treated with *P. fluorescens* R-93 and *P. fluorescens* DSM 50090, with the highest stimulating effects on growth and development being observed in the case of plants grown in non-sterile soil compared to sterile soil. According to Khalid et al. [24], the growth and development of *Triticum aestivum* L. depended on the plant genotype, the bacteria used for plant growth promotion and also on the environmental conditions. In addition, Burd et al. [79] and Nezarat and Gholami [80] suggested that the promotion of plant growth in terms of roots, shoots and leaves by PGPB might be associated with the cumulative mechanisms used by the bacteria, such as the production of siderophores, ACC deaminase and IAA, as well as nutrient (phosphate, potassium) solubilization. The production of auxins can lead to a better development of the plant parts through nutrient uptake, and this may suggest that beneficial bacteria presented more competitive ability compared to the indigenous bacteria available in the non-sterile soil [81].

More results about the effects that the *Azotobacter*, *Bacillus* and *Pseudomonas* species induced on different plants are shown in Table 1, along with those obtained in this study.

Table 1. Effects of *Azotobacter*, *Bacillus* and *Pseudomonas* species on plant growth and development.

Bacteria	Plants	Beneficial Effects	Ref.
Bacillus polymyxa, *Bacillus pantothenticus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus circulans*, *Pseudomonas cichorii*, *Pseudomonas putida*, *Pseudomonas syringae*	*Zea mays* L.	Improved seed germination and plant growth	[82]
Pseudomonas poae, *Pseudomonas trivali*	*Brassica campestris* L. spp. Pekinensis	Stimulated plant roots	[83]
Azotobacter chroococcum	*Brassica juncea* L.	Increased seed germination	[84]
Bacillus subtilis strain SJ-101	*Brassica juncea* L.	Increased plant growth	[85]
Azotobacter chroococcum, *Azotobacter virelandii* and *Azotobacter beijerinckii*	*Rhizophora mangle*	Increased the roots, shoots, leaves of plants and chlorophyll content	[86]
Azotobacter chroococcum	*Triticum aestivum* L.	Increased the plant biomass, the length of wheat and the wheat yield	[67]
Bacillus subtilis PCL1608 *Bacillus subtilis* PCL1612	*Persea americana* mill, *Solanum lycopersicum*	Produced antifungal lipopeptides and good colonization abilities	[87]

Table 1. *Cont.*

Bacteria	Plants	Beneficial Effects	Ref.
Bacillus RC01, *Bacillus* RC02, *Bacillus* RC03, *Bacillus* M-13	*Hordeum vulgare* L.	Increased the root and shoot biomass	[88]
Pseudomonas fluorescens	*Arachis hypogea* L.	Induced systemic resistance, antifungal activity	[89]
Pseudomonas fluorescens ACC5 *Pseudomonas fluorescens* biotype F (ACC73)	*Triticum aestivum* L.	Increased root weight, grain yield, number of tillers per plant and straw yield	[90]
Bacillus subtilis KP717559	*Brassica juncea*	Increased root and shoot length and the content of fresh and dry biomass	[64]
Pseudomonas brassicacearum Am3, *Pseudomonas putida* Bm3, *Pseudomonas marginalis* D	*Brassica napus*	More vigorous plant	[72]
Pseudomonas fluorescens YsS6	*Brassica rapa*	Promoted root elongation Plants much healthier and higher	[91]
Azotobacter sp.	*Sinapis alba* L.	Increased the root, shoot and leaf length and the root, shoot and leaf dry weight	This study
Azotobacter sp.	*Brassica napus* L.	Increased the root, shoot and leaf length and the root, shoot and leaf dry weight	This study
Azotobacter sp.	*Amaranthus retroflexus* L.	Did not improve the growth	This study
Azotobacter sp.	*Panicum miliaceum* L.	Increased the shoot and leaf length and dry weight Vigorous shoots	This study
Azotobacter sp.	*Rumex patientia* L.	Increased the root length and the root, shoot and leaf dry weight Stimulated root branching	This study
Azotobacter sp.	*Linum usitatissimum* L.	Increased the root, shoots and leaf length and the shoot and leaf dry weight Stimulated root branching	This study
Bacillus sp.	*Sinapis alba* L.	Increased the root, shoot and leaf length	This study
Bacillus sp.	*Amaranthus retroflexus* L.	Increased the root, shoot and leaf length	This study
Bacillus sp.	*Brassica napus* L.	Increased the root, shoot and leaf length and their dry weight biomass	This study
Bacillus sp.	*Panicum miliaceum* L.	Increased the shoot and leaf length and the root dry weight Vigorous shoots	This study
Bacillus sp.	*Rumex patientia* L.	Increased the root length and dry weight Stimulated root branching	This study
Bacillus sp.	*Linum usitatissimum* L.	Increased the root shoot and leaf length Branched roots	This study
Pseudomonas sp.	*Sinapis alba* L.	Increased the root, shoot and leaf length Increased the root, shoot and leaf dry weight	This study
Pseudomonas sp.	*Brassica napus* L.	Increased the root, shoot and leaf length Increased the root, shoot and leaf dry weight Stimulated root branching	This study
Pseudomonas sp.	*Amaranthus retroflexus* L.	Increased the shoots + leaves length	This study
Pseudomonas sp.	*Panicum miliaceum* L.	Increased the shoots + leaves length and dry weight	This study

Table 1. *Cont.*

Bacteria	Plants	Beneficial Effects	Ref.
Pseudomonas sp.	*Rumex patientia* L.	Increased the root, shoot and leaf length and dry weight Stimulated root branching Vigorous shoots	This study
Pseudomonas sp.	*Linum usitatissimum* L.	Increased the root, shoot and leaf length Stimulated root branching Vigorous shoots	This study

5. Conclusions

The use of plant growth-promoting bacteria represents an advantageous strategy than can be applied to improve the growth and development of plants, as well as to ensure the protection of the plants against different abiotic and biotic factors for improving phytoremediation technology. Several studies reported the importance and the leading role of beneficial bacteria for plant growth and the necessity of using PGPB as an essential process to ensure a sustainable agricultural soil system.

In the present study, the *Azotobacter*, *Bacillus* and *Pseudomonas* species had different effects on the growth of *Sinapis alba* L., *Brassica napus* L., *Amaranthus retroflexus* L., *Panicum miliaceum* L., *Rumex patientia* L. and *Linum usitatissimum* L. plants grown in both sterile and non-sterile soil. In the sterile soil, only the effects of the inoculated bacteria were observed without the interference of the indigenous bacteria naturally available in the soil. Regarding the non-sterile soil, the existence of bacteria in the soil may have an influence on plant growth due to the incompatibility of the inoculated bacteria with the indigenous bacteria. In addition, it is important to highlight that the results were not constant in both types of soil, and the positive effects of the inoculated *Azotobacter*, *Bacillus* and *Pseudomonas* species on plant growth and development were less pronounced in the non-sterile soil. These findings demonstrate that sterile soil can enhance the performance of bacteria. Moreover, the indigenous bacteria from the soil may affect the results of bacteria inoculation, and this fact must be taken into account in future experiments, especially in the case of soil phytoremediation, in order to select the most suitable/resistant bacteria.

The results showed that the growth of the plants in the presence of the selected bacteria induced different effects on the roots and shoots, suggesting that the plant species showed various sensitivity to the bacteria strains. *Bacillus* and *Pseudomonas* induced the greatest influence on the roots of mustard grown in the sterile and non-sterile soil. *Azotobacter* sp. exerted the highest beneficial influence on rapeseed grown in the non-sterile soil. In both the sterile and non-sterile soil, *Bacillus* sp. had a positive effect on the growth of redroot pigweed shoots. *Azotobacter* sp. stimulated the growth of the roots of patience dock in the sterile soil and also the proso millet and flax plants grown in the non-sterile soil. *Pseudomonas* sp. contributed to a greater increase in the flax roots and shoots in the non-sterile soil compared to those in the sterile soil.

The statistical analysis of the results by Tukey's Honestly Significant Difference (HSD) test revealed that the differences observed in the plants grown with or without the selected bacteria, in sterile or non-sterile soil, are sometimes insignificant compared to the control. For example, in the sterile soil, only the mean length of *Amaranthus retroflexus* L. shoots under the effect of *Bacillus* sp. was statistically significantly longer than the control, while in the non-sterile soil, *Azotobacter* and *Pseudomonas* sp. had a significant negative effect on the root length of *Panicum miliaceum* L. in both soil types.

In conclusion, the results highlight the importance of identifying suitable PGPB that can enhance plant growth, especially for their application to improve the efficiency of the phytoremediation process. This study may also contribute toward an understanding of the efficiency of the bacteria under different soil conditions and can represent a basis

for further evaluation in order to exploit the potential of plant–bacteria interactions for environmental remediation.

Author Contributions: Conceptualization, M.M., M.R., M.D. and M.G.; methodology, M.D., M.R. and M.M.; validation, M.G., L.B. and M.D.; formal analysis, M.R., M.M. and P.C.; investigation, M.M., M.D. and M.R.; resources, M.G. and M.D.; data curation, L.B. and M.G.; writing—original draft preparation, M.M., M.R. and P.C.; writing—review and editing, M.M., M.R., P.C. and M.G.; visualization, M.D., M.G. and L.B.; supervision, M.R., M.D. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant from the Romanian National Authority for Scientific Research, CNCS—UEFISCDI, project number PN-III-P4-ID-PCE2016-0683, Contract no. 65/2017, and a grant of the Romanian Ministry of Education and Research, CCCDI—UEFISCDI, project number PN-III-P2-2.1-PED-2019-5239, Contract no. 269PED/2020, within PNCDI III.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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