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Article

Synthesis of indoleacetic acid, gibberellic acid and ACC-deaminase by *Mortierella* strains promote winter wheat seedlings growth under different conditions

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Abstract: The endogenous pool of phytoregulators in plant tissues supplied with microbial secondary metabolites may be crucial for the development of winter wheat seedlings during cool springs. Phytohormones may be synthesized by psychrotrophic microorganisms in lower temperatures occurring in temperate climate. Two fungal isolates from the Spitzbergen soils after the microscopic observations and ITS region molecular characterization were identified as *Mortierella antarctica* (MA DEM 7) and *Mortierella verticillata* (MV DEM32). To study the synthesis of indoleacetic acid (IAA) and gibberellic acid (GA) *Mortierella* strains were grown on media supplemented with precursors of phytohormones (tryptophan or methionine) at 9, 15 and 20°C for 9 days. The highest amount of IAA synthesis was observed in MV DEM 32 9-day culture at 15°C with 1.5 mM of tryptophan. At the same temperature the significant promoting effect (about 40% root and shoot fresh weight) of this strain on seedlings was observed. However, only MA DEM 7 had the ACC-deaminase activity with the highest efficiency in 9°C and at this temperature synthesized IAA without tryptophan also at the same conditions the strain confirmed the strong promoting effect (about 40% root and 24% shoot fresh weight) on seedlings. Both strains synthesized GA in all tested terms and temperatures. Tested *Mortierella* strains had some important traits to consider them as microbial biofertilizers component improving plant growth in difficult temperate climate.

Keywords: *Mortierella*, phytohormones, winter wheat seedlings, psychrotrophs

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1. Introduction

Next to the *Penicillium* and *Aspergillus* genera, *Mortierella* are very common filamentous fungi isolated from environment [1, 2]. Soil ecological studies describing fungal diversity reported an occurrence of *Mortierella* strains in soil, rhizosphere, rivers and lakes on different continents [3, 4, ,5, 6, 7, 8, 9]. Nagy et. al. [1] described *Mortierella* as widespread in the temperate zone. Most of the deeply investigated strains were isolated from low-temperature environments. Psychrophilic microorganisms grow well at low temperatures (about 5°C or below), but their optimum is about 15°C or lower, while the psychrotrophs grow at low temperature with their optimum above 15°C and their maximal growth temperatures is above 20°C [10, 11].

Winter wheat is one of the most important cereal crops in Europe, which is always sown in fall. In Poland the optimal seedling window for this plant is between second half of September and first half of October when the average of daily temperatures correspond with optimum growth temperature of psychrotrophs [12,13].

The indoleacetic acid (IAA), gibberellic acid (GA) and ACC-deaminase are plant growth regulators synthesized by plants as well as for many soil microorganisms [14] Tryptophan is considered to be the precursor of auxins in higher plants and microbial synthesis of IAA which is essential for all important processes including seedlings growth. Among many functions gibberellic acid may stimulate the synthesis of hydrolytic enzymes activity during the germination of cereal grain. The phytohormone - ethylene regulates plant development and stress resistance. Microbial ACC-deaminase convert the precursor of ethylene (ACC) to the ammonia and α-ketobutyrate and protect plants from too high concentration of phytohormone [15, 16, 17].

Microbial production of phytohormones and phytoregulators like ACC-deaminase is one of the most important direct mechanism contributing to rapid and durable colonization of soil and as a result enhancing the plant growth [18, 19, 20]. More attention should be focused on microorganisms promoting plant growth at lower temperatures and in broad range of temperature during the selection of microbial components of biofertilizers intended to applicate in climate conditions of Eastern Europe. There are many very detailed studies on the synthesis of polyunsaturated fatty acids by fungi of the genus *Mortierella,* but very little is known about production of phytoregulators by the *Mortierella* strains [21, 22, 23, 24]. Therefore, in this study we identified two *Mortierella* strains growing in the wide temperature range. We also have investigated synthesis of indoleacetic acid, gibberellic acid, ACC-deaminase and evaluated the effect of these microorganisms on the growth of winter wheat seedlings in the temperatures significant in the temperate zone.

2. Results

2.1. Identification and Optimal Growth Temperature of Fungal Isolates

Mycelium of tested MA DEM 7 and MV DEM 32 fungi growing on PDA formed a rosette, cottony, white aerial colonies (Figure 1A, B). Additionally, the MV DEM 32 mycelium was yellowish with age. As the result of microscopic observations of both tested microcultures incubated at 20°C for 7 days we did not obtain fungal sporulation. After 7-days incubation at 4°C the MV DEM 32 isolate formed characteristic sporangiophores with few-spored sporangia (Figure 1C). This strain was characterized by the presence needle-shaped sporangiophores with a 2-3 branches and few-spored sporangia.

To obtain the sporulation by starvation of *Mortierella* strains we have also tried to culture them on water agar, but they did not grow on this medium. We did not notice good sporulation of MA DEM 7 isolate.

To identifficate of fungal isolates the ITS sequences were investigated and sent to NCBI Gen Bank. 100% similarity to *Mortierella antarctica* (strain MA DEM 7) was detected. The MV DEM 32 strain exhibited 100% homology with *Mortierella verticillata* Linnem. – the synonym for this species is *Mortierella marburgensis* Linnem [25, 26, 27] was detected. According to the best of our knowledge this is the first work describing *Mortierella verticillata* isolated from the Spitzbergen soil. The consensus of the ITS fragment (query) was submitted and deposited in the GenBank under accession numbers: MH 289781 (strain MA DEM 7); MH 304896 (strain MV DEM 32)

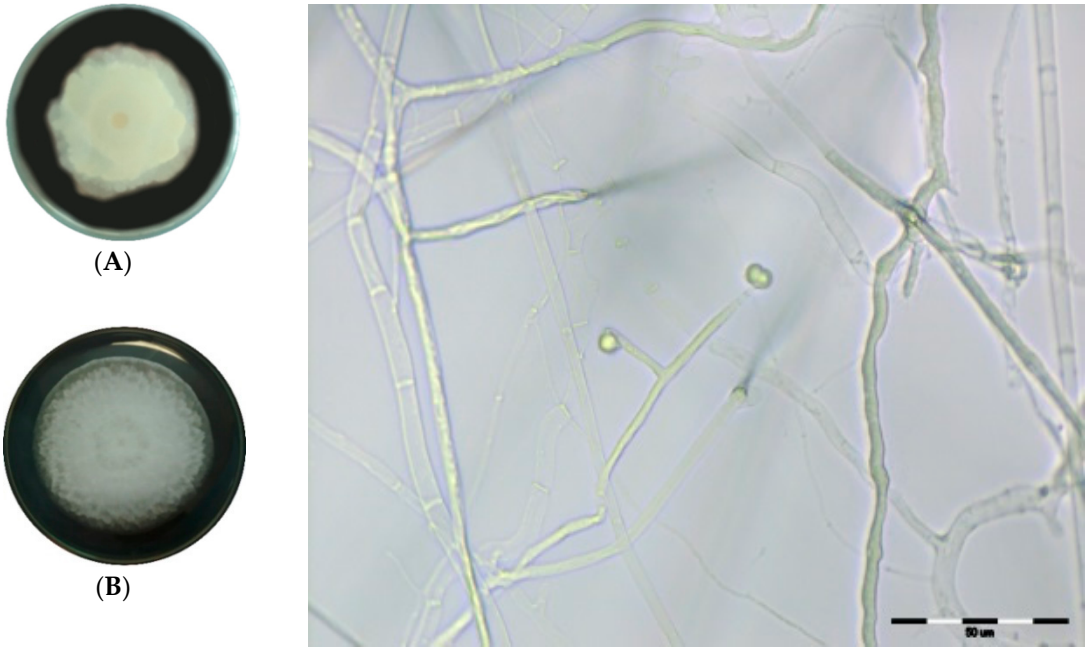

Figure 1. Mycelial growth of (**A**) *M. antarctica* DEM 7; (**B**) *M. verticillata* DEM 32 on PDA medium (**C**) Sporangiophores with few-spored sporangia *M. verticillata* DEM 32, x 400 on PDA medium

2.2. The Effect of Temperature on the Radial Growth of Mortierella Strains

The ability of two *Mortierella* strains to growth at five different temperatures was tested to determinate the optimal temperature conditions (Figure 2A, B). The experiment was just shut down after 6 days when the fungal colony started to reaches the diameter of 9 cm wide on Petri dish. The results showed that *M. antarctica* DEM 7 and *M. verticillata* DEM 32 strains were able to grow at temperature range between 4˚C and 28˚C however the MA DEM 32 demonstrated minimal growth at 28˚C (the diameter of colony on the 6th day of experiment was only 1 cm). The best temperature for mycelial growth of MA DEM 7 and MV DEM 32 was 15˚C in addition with the highest growth rate, respectively (1.19 ±0.08; 1.35±0.09) (Table 1).

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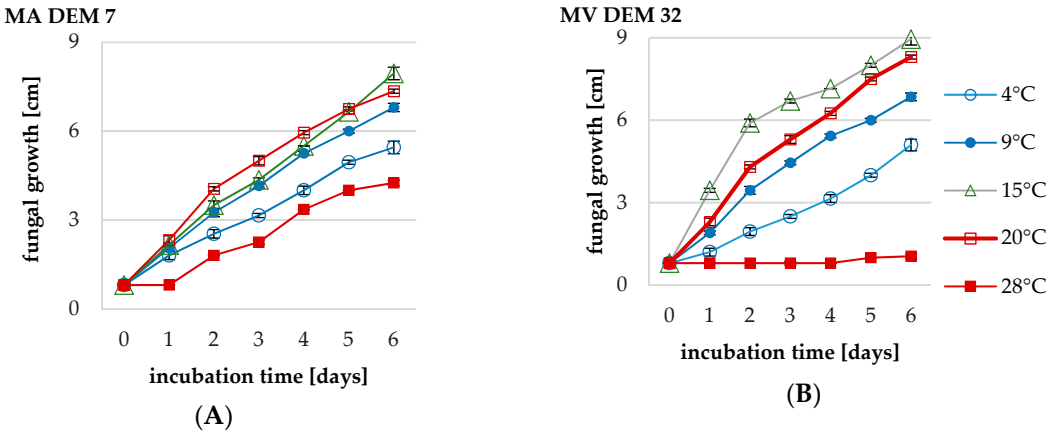

Figure 2. Mycelial growth [cm] at PDA medium measured daily for 6 days (**A**) the diameter of the *M. antarctica* DEM 7; (**B**) the diameter of the *M. verticillata* DEM 32 at five temperature conditions. Values are means of three replicates. Bars represented standard deviations (SD)

We found that *Mortierella* growth rate at 20˚C between 5th and 6th day of incubation was slow. At 4˚C the colony of MA DEM 7 and MV DEM 32 started to grow from the very beginning of the experiment. The optimal growth temperature for *Mortierella* strains (MA DEM 7 and MV DEM 32) on PDA medium oscillated between 15˚C and 20˚C (Figure 3A, B).

Table 1. The mycelial growth rate [cm/day] of *M. antarctica* DEM 7 and *M verticillata* DEM 32 at five temperature conditions

Temperature	MA DEM 7	MV DEM 32
4˚C	0.77^{b} ±0.06	0.71^{b} ±0.02
9˚C	1.0^{c} ±0.05	1.0^{c} ±0.04
15˚C	1.19^{d} ±0.08	1.35^{d} ±0.09
20˚C	1.09^{cd} ±0.05	1.25^{cd} ±0.06
28˚C	0.7^{a} ±0.02	0.041^{a} ±0.00

Means followed by the same letters at the same columns are not significantly different

2.3. *Phytoregulators Activity Assayed at Different Temperature Conditions*

Two fungal strains *M. antarctica* DEM 7 and *M. verticillata* DEM 32 were tested for their ability to synthesize IAA. The CDM (Chapek-Dox modified) medium supplemented with initial dose of Trp (1.5 mM or 3.0 mM) was more suitable for IAA production for MV DEM 32 strain compared with CDM without Trp at 15°C and 20 °C (Figure 3B, D, F). The highest amount of IAA was measured after 7. and /or 9. day of incubation *Mortierella* strains at all temperature conditions (Figure 3B, C, D, E, F) except with MA DEM 7 strain incubated at 9°C (Figure 3A). It was found that the concentration of IAA in *M. verticillata* cultures was correlated with the initial concentration of Trp at 9°C and 20°C (Figure 3B, F). The optimal growth conditions for both tested fungi were obtained in CDM supplemented with 1.5 mM Trp at all tested temperatures except with MA DEM 7 strain incubated at 9°C (Figure 3A). Only in the absence of Trp *M. antarctica* strain synthetized (about 0.75 µg IAA/mL), but the dry weight of fungal biomass obtained at such conditions was the highest (about 38 mg d.w./mL). After incubation at 15°C and 20 °C the MA DEM 7 strain, higher

130 concentration of IAA was obtained, but only in cultures with the initial dose of Trp 3.0 mM (Figure
131 3B, F).
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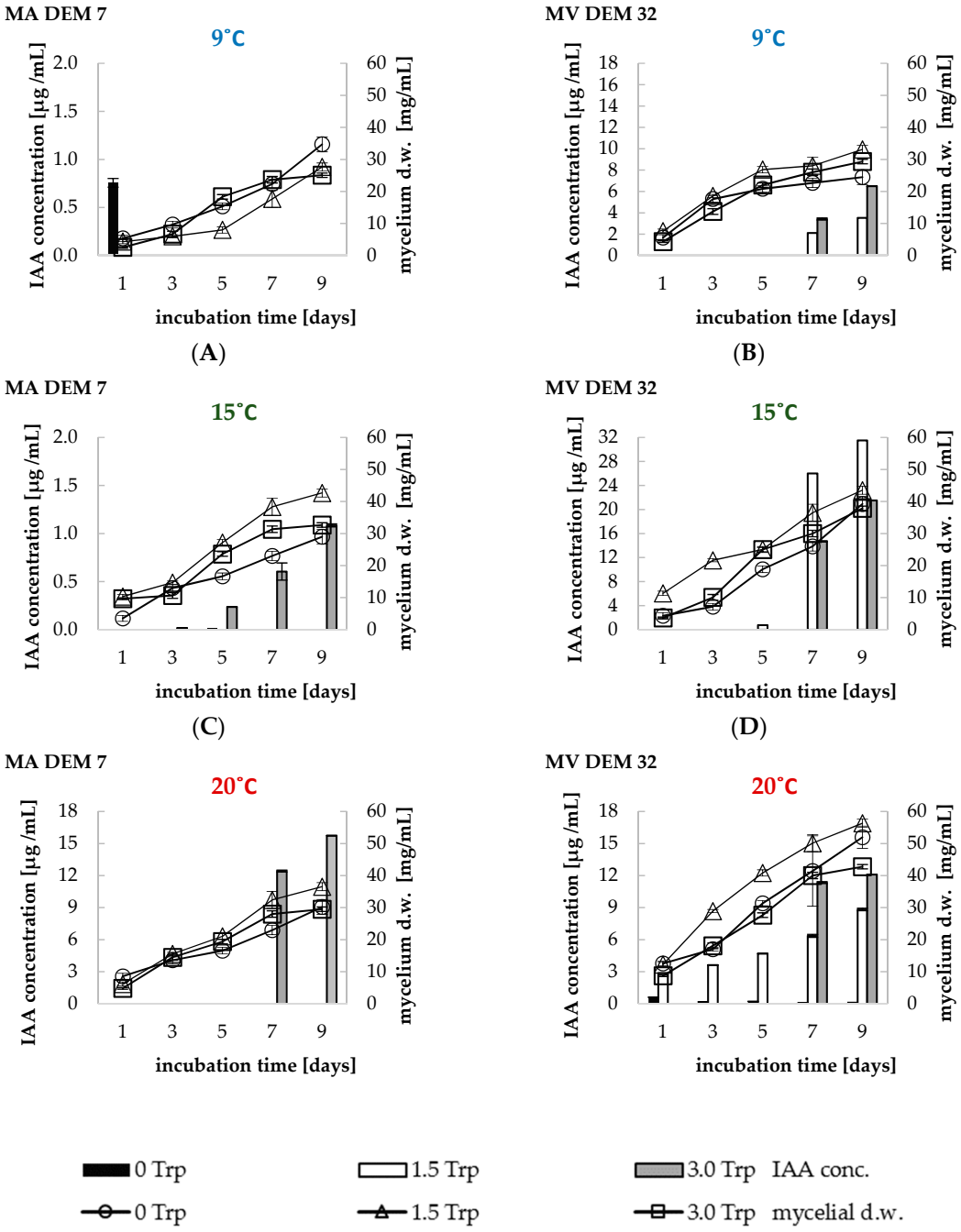

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134 **Figure 3.** The indoleacetic acid (IAA) concentration and fungal biomas in *M. antarctica* DEM 7 (**A**, **C**, **E**) and
135 *M.verticillata* DEM 32 (**B**, **D**, **F**) cultures grown in CDM medium supplemented with 1.5 mM, 3.0 mM or
136 without Trp in different temperature. Bars represented standard deviations (SD)

137 Both *Mortierella* strains started GA synthesis after 24 hours of incubation on media supplemented
138 with Trp and without amino acid in all free temperatures (Figure 4A, B). The optimal conditions of
139 phytohormone synthesis depended on the culture conditions. Under all tested conditions the highest
140 concentration of GA was found in the 9. days MV DEM 32 isolate culture with 3.0 of Trp after

incubation at 20 °C (about 3 µg GA/mL^{-1}) (Figure 4B). The 3 times lower, maximum concentration of phytohormone, in MA DEM 7 isolate was measured at the end of experiment but in the culture without prior addition of tryptophan (Figure 4A). The optimal growth conditions for both tested *Mortierella* strains were similar (Figure 4A, B).

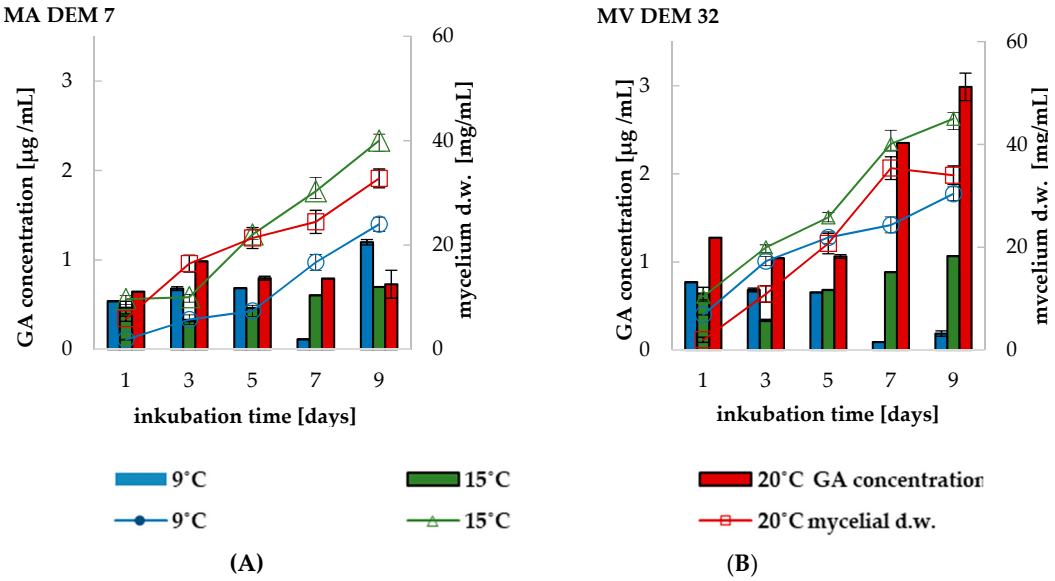

Figure 4. The gibberellic acid (GA) concentration and mycelial growth in *M. antarctica* DEM 7 (**A**) and *M. verticillata* DEM 32 (**B**) cultures grown in CDM medium supplemented with 3.0 mM Trp in different temperature conditions. Bars represented standard deviations (SD).

Two *Mortierella* strains were able to grow on CDM (data not shown). Only *M. antarctica* DEM 7 expressed ACC-deaminase activity from 0.9 to 6 µM of α-ketobutyrate/mg protein/h with the highest amount at 9°C (Figure 5).

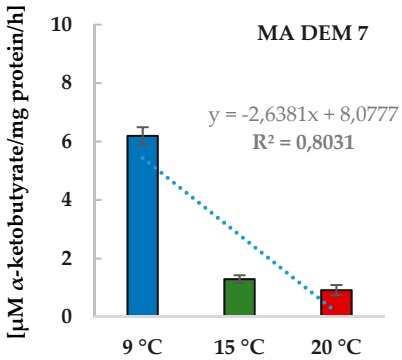

Figure 5. The ACC-deaminase activity of *M. antarctica* DEM 7 culture in different temperature conditions (9-day culture). Results are represented as µM of α-ketobutyrate mg/protein/h. Bars represented standard deviations (SD).

2.4. Growth of Winter Wheat Seedlings with Mortierella Strains Under Various Conditions

To find out if the tested *Mortierella* strains promoting plant growth we germinated winter wheat seeds in the presence of fungal inoculum with or without the initial dose of tryptophan (Figure 6A, B). Cultivation of winter wheat grain at 15 °C with MA DEM 32 strain in the presence of 3.0 mM Trp led

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to increase in the total root fresh biomass over 40% as compared to the control with Trp (Figure 6A). The same stimulating effect of MA DEM 7 strain on root fresh weight was observed after 10 days of inoculation at 9 ^{0}C but without precursor of IAA – tryptophan additionally the shoot fresh weight of these plants was also higher as compared to the non-inoculated control and other plants growing without Trp (Figure 6B). The highest shoot fresh weight was measured in seedlings grown with Trp added and with MV DEM 32 inoculum. After 5 days of development the seedling shoot fresh weight was almost 40 % higher than the fresh weight those grown aseptically with Trp.

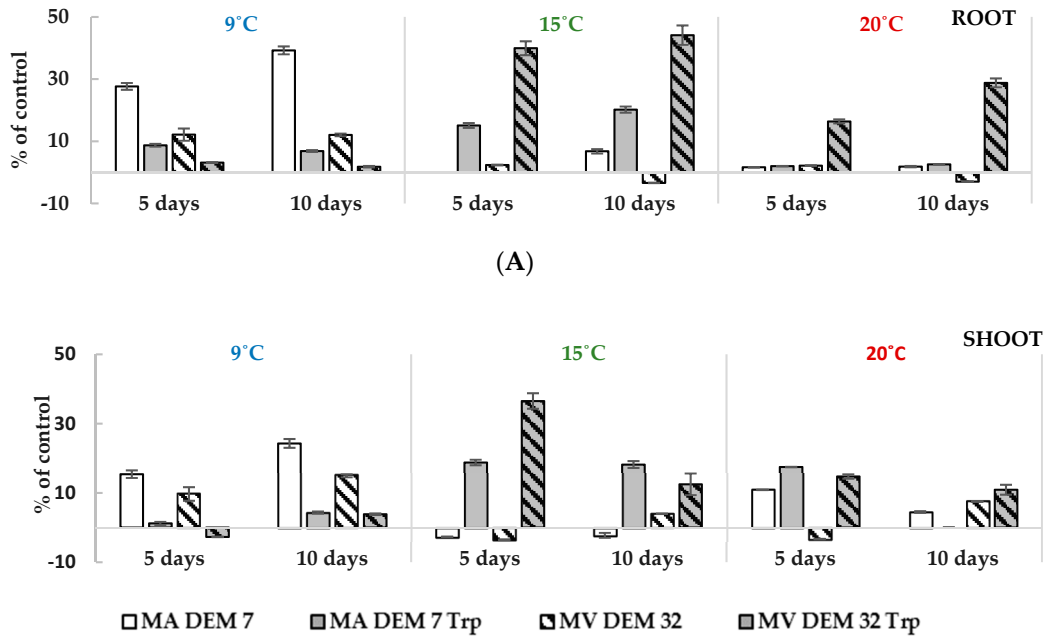
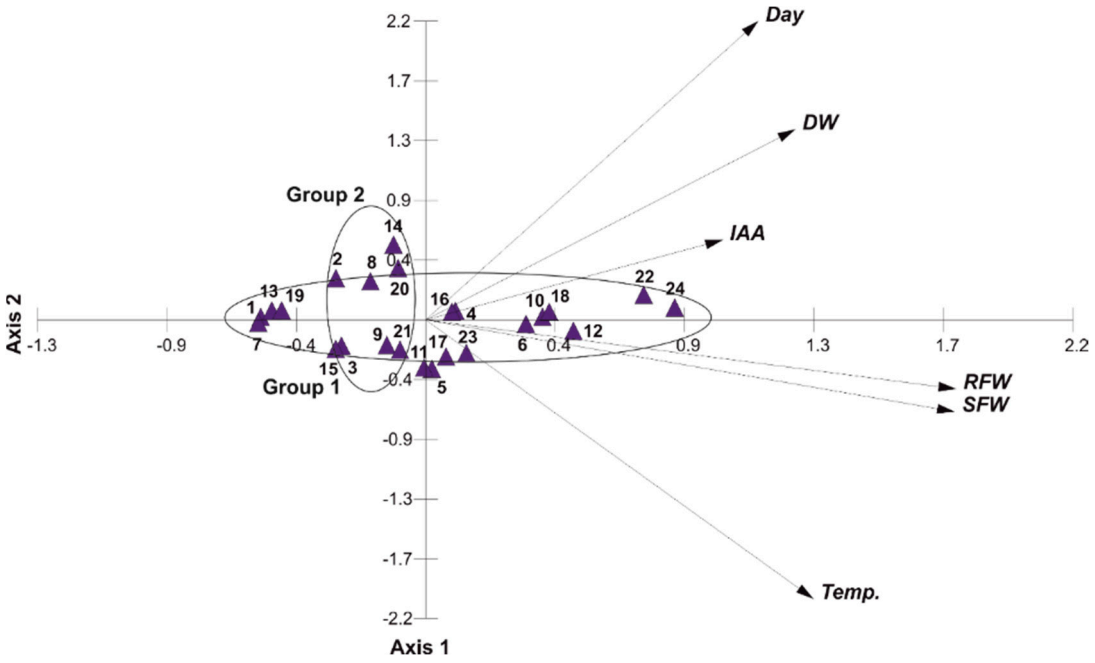


Figure 6. Effect of *Mortierella* strains on winter wheat seedlings root (**A**) and shoot (**B**) fresh weight in different temperature conditions with 3.0 mM Trp or without Trp. Results are presented as percent of controls treated or non-treated with Trp. Values are means of six replicates of each treatment (with 20 seedlings). Bars represented standard deviations (SD).

The PCA ordination analysis facilitated determination of the relationships among IAA concentration, strain dry weight, and the roots and shoots fresh weight in two *Mortierella* strains, treated and not treated tryptophan, cultivated at 9, 15 and 20 °C and analyzed after 5 and 10 days (Figure 7). All the variables analyzed were statistically significant at the level of $p<0.05$. Four axes of the diagram explained 98.1% of the data variability, i.e. the first axis – 56.1%, and the second one – 20%.

The two main trends of the variation could be seen. The first trend was related to the first axis and was positively correlated with all variables tested. The strongest correlation with this axis was shown by RFW and SFW. Axis 1 determined the gradient of all analyzed parameters. Group 1 represents an increasing dependence of FW starting from 7 and 1 to 22 and 24. Group 2 was negatively correlated with the first axis and correlated with the temperature. Axis 2 was strongly positively correlated with the day of the analysis. IAA concentration increased from 7 to 22 and 24.



1 - MA DEM 7/9/5	13 - MV DEM 32_9_5
2 - MA DEM 7_9_10	14 - MV DEM 32_9_10
3 - MA DEM 7_15_5	15 - MV DEM 32_15_5
4 - MA DEM 7_15_10	16 - MV DEM 32_15_10
5 - MA DEM 7_20_5	17 - MV DEM 32_20_5
6 - MA DEM 7_20_10	18 - MV DEM 32_20_10
7 - MA DEM 7 Trp_9_5	19 - MV DEM 32 Trp_9_5
8 - MA DEM 7 Trp_9_10	20 - MV DEM 32 Trp_9_10
9 - MA DEM 7 Trp_15_5	21 - MV DEM 32 Trp_15_5
10 - MA DEM 7 Trp_15_10	22 - MV DEM 32 Trp_15_10
11 - MA DEM 7 Trp_20_5	23 - MV DEM 32 Trp_20_5
12 - MA DEM 7 Trp_20_10	24 - MV DEM 32 Trp_20_10

Figure 7. Ordination diagram showing the results of the PCA for the two strains: MA DEM 7 and MV DEM 23, both with out and with tryptophan, treated three temperatures (9, 15 and 20 °C; Temp.) and analyzed in two days (5 and 10 day; Day). RFW and SFW - average root and shoot fresh weight, respectively; IAA - average concentration; SW - strain dry weight.

3. Discussion

We have obtained good sporulation after 7 days by incubating the microculture of *M. verticillata* DEM 32 at 4°C to trigger reproduction. According to Domsch et al. [25] sporangiophores of *M. verticillata* are smoof, more or less verticillately branched. Watanabe [27] noticeded 1-3 sporangiospores per *M. verticillata* sporangium. Original isolate of *Mortierella verticillata* has a few 2-spored sporangia [25]. Our observation of MV DEM 32 strain confirmed the presence of needle-shaped sporangiophores, at the base with a few (1-5) branches was described by Skirgiełło et. al. [26] (Figure 1C). According to phylogenetic analysis confirmed by DNA amplification and nucleotide sequence, Wagner et. al. [28] classified *Mortierella antarctica* in the group 6: alpina and polycephala. Domsch et al. [25] noticed that *Mortierella alpina* forms many-spored sporangia. Del Frate and Caretta [29] described the same features, but for *M. antarctica. M. alpina* forms unbranched sporangiophores with a widened and often irregularly swollen base [25]. According to Del Frate and Caretta [29] *M. antarctica* has additionally a basal collarette. In this study, *Mortierella antarctica* DEM 7

failed to sporulate and we did not observe some of the morphological characters of this species and genetic analysis was required. Based on analysis of ITS1 and ITS4 fragments two strains were identified as *Mortierella antarctica* (strain MA DEM 7) and *Mortierella verticillata* (strain MV DEM 32).

Five temperatures were used to find the optimum for mycelial growth of *Mortierella antarctica* DEM 7 and *Mortierella verticillata* DEM 32 (Figure 2A, B). Strains exhibited an optimal growth temperature between 15˚C and 20˚C. The results also confirm that two *Mortierella* fungi are able to grow at lower temperatures (4˚C and 9˚C) and might be classified as psychrotrophs similarly to other isolates of *Mortierella* investigated before [9, 29]. Shmidt et al. [7] announced that 317-1 strain from subalpine forest, belonging to *Mortierellales* has its optimal growth temperature between 15˚C and 20˚C. The strain *Mortierella alpina* ITA-CCMA-952 isolated from Antarctic moss and tested by Melo et. al. [4], grew at a very wide range of temperatures with the optimum between 15˚C and 25˚C. The strain incubated at 4˚C at the 6th day of incubation reached about 2.8 cm of diameter, but the colony of this Antarctic fungus did not grow through the first two days of the experiment. After 6 days at 15˚C the *M. alpina* ITA-CCMA-952 colonies reached only 4.0 cm, which was only about 50 % of final MA DEM 7 and MV DEM 32 diameter. These data show that the optimal growth temperature of strains isolated from similar habitat can differ significantly.

There are many reports announced that plant growth promoting fungi (PGPF) improving crop productivity [4, 20, 30, 31, 32, 33]. Indoleacetic acid, the main auxin is crucial for majority of plant growth and development processes. The production of microbial IAA may depend on the presence of its precursor – tryptophan, however some microorganisms and plants can also synthesize IAA by Trp-independent mechanism [15, 34]. According to Quiroz-Villareal et. al. [35] this amino acid was identified as one of the component of root exudates. The IAA synthesis is widespread among soil microorganisms involving them with a promoting of plant growth. It was found that the concentration of IAA in *Mortierella* cultures was correlated with the initial dose of Trp at 15^{0}C and 20^{0}C. The optimal dose of Trp recommended for fungal cultures is from 2 to 4 mM of Trp and the maximal concentration of IAA was usually measured in 60 and 72 hour of incubation [36, 37]. Our study confirmed that in the absence of Trp (1.5 or 3.0 mM) *M. antarctica* and *M verticillata* strains synthesized respectively about 16 μg IAA/mL and 32 μg IAA/mL (Figure 3C, D). The newest publication describing the significantly increased levels of IAA in tissue of maize seedlings growing in soil inoculated with *Mortierella elongata* [38]. The concentration of IAA in plant tissue inoculated with this strain, after 29 days was about 27 % higher than this indicated in non-inoculated seedlings. Furthermore, the whole genome sequencing of *Mortierella elongata* showed the genetic capacity to synthesize of IAA. Wani et. al. [32] investigated the IAA synthesis of fungal isolates from *Croccus sativus* in India. At 7 th day of incubation the *Mortierella alpina* CS10E endophytic strain synthesized 21.6 mg IAA/L while our *M. verticillata* DEM 32 strains at the same time produced about 26 mg/L [Figure 3D].

Gibberellic acid (diterpenoid acid) is one of the major phytoregulator among others controlling seed germination, root and shoot growth of plants [39]. GA effects on plant growth at very low concentration and it is known to be synthesizing by plants and some microorganisms. There are many reports indicating the ability to GA synthesis by filamentous fungi belonging to genera: *Aspergillus, Penicillium, Fusarium and Rhizopus* [15, 39, 40]. According to the best of our knowledge this is one of the first work investigating the synthesis of gibberellic acid by *Mortierella* strains. The concentration of GA indicated in MV DEM 32 cultures was correlated with temperature of incubation (Figure 4B)

and the highest value of phytohormone measured at 20°C was about 3 times higher in comparison with the amounts of GA in the cultures incubated at 9°C or/and 15 °C. The MV DEM synthesis of GA at 20°C was greater every day in contrast the highest amount of GA was measured at 1st day of incubation. It is important to take individual time of phytoregulators accumulation in liquid cultures when selecting PGPF strains. Additionally, the dry weight of MV DEM 32 mycelium measured at 9th day cultures incubated at 9°C and 20°C (about 37 mg/mL). These results shows that synthesis of GA depends on the temperature. Fluctuations in the results of phytohormone synthesis observed in culture of MA DEM 7 strain in all temperatures (Figure 4A) suggest that some part of gibberellic acid could be utilized [32]. Our earlier studies with filamentous fungi suggest that tryptophan (the precursor of IAA and GA) may be consumed [15].

ACC-deaminase is one of the efficient markers for microorganisms, which promotes plant growth by decreasing the level of ethylene in plant tissues [42]. One of the products of ACC hydrolysis is ammonia, source of nitrogen available for microorganisms and plants. The positive effect of inoculation with rhizobacteria containing ACC-deaminase was observed [14]. In the presented study MA DEM 7 strain increased fresh biomass of seedlings root at 9°C (Figure 5A). The linear correlation between temperature of incubation and ACC-deaminase activity (R^2=0.803) implies that activity of phytoregulator increased with decrease of incubation temperature.

The production of phytoregulators may enhance the germination, root and shoot plant growth [43]. Our results of IAA activity by MA DEM 7 and MV DEM 32 suggested that both strains could effectively promote plant growth at lower temperature.

In order to study the real *Mortierella* impact on the growth of winter wheat seedlings we inoculated seeds with strains in the presence of tryptophan. Plant roots are very sensitive to IAA concentration this is the first plant organ responses for increasing amount of exogenous auxin [44]. In this study inoculation with tested *Mortierella* fungi at 15°C and 20°C with 3.0 mM Trp, after 5 days showed the increasement in fresh weight of root and this effect was visible also after 10 days (Figure 6A, B) compared to non-inoculated seedlings.

In Ul Hassan and Bano experiment [32] the seeds of *Triticum aestivum* were treated with aqueous solution of Trp. After 57 days of pot experiments the weight of fresh aerial parts of plants treated with *Pseudomonas moraviensis* or *Bacillus cereus* increased even about 20 % as compared to the control. In this study, inoculation with *Mortierella* strains improves shoot growth of winter wheat seedlings (*Triticum aestivum* L. cv Arkadia) under a various temperature conditions (Figure 6A). Additionally, we noticed the higher root and shoot biomass of plants mainly cultivated at 15°C and 20°C with initial dose of Trp but at 9°C we measured the highest fresh mass of plants cultivated without Trp. This data suggests that at lower temperatures the presence of the precursor of phytohormones is not the main factor influencing at phytoregulators synthesis.

Winter grains germinate before winter comes and plant root is crucial for good plant adaptations to critical factors indicates in temperate zone. Some of psychrotolerant *Mortierella* strains may enhancing plant growth at the beginning of vegetative period in temperate zone. Further studies using these strains should be continued for the exact contribution of the other PGP traits of *Mortierella* strains at lower temperatures. The potential of these strains in practical use (in field experiment) should be undertaken. According to our research, the *M. verticillata* DEM 32 strain indicated phytohormones synthesis and stimulated growth of plant biomass especially at 15°C and 20°C in the presence of tryptophan (Figure 4B, 7). What is interesting *M. antarctica* DEM 7at 9°C showed its

highest ACC-deaminase activity and GA synthesis comparing with all temperatures examined. The selection of microbial components of biofertilizers should be based on the wide spectrum of biological diversity [45] On this account, the PGPF activity in lower temperatures should be also taken into consideration. There is still a need of research on efficient strains potential component of agricultural product.

4. Materials and Methods

4.1. Microscopic and Molecular Identification of Fungal Isolates

The experiment was carried out on two *Mortierella* fungi MA DEM 7 and MV DEM 32 isolated from soil humus horizon collected from micro−relief forms in tundra in the Bellsund region, west coast of Spitzbergen, in the Svalbard Archipelago (77°33′ N, 14°31′ E). Soil properties and microbial activity were presented and discussed in the study by Kurek et al. [10].

The morphological identification of pure cultures of isolates to the species level was accomplished using established procedures including macroscopic characteristics on the plates and microscopic observation in the microcultures. In the macroscopic observations of cultures on plates and slants on PDA medium the macromorphological traits were considered colonies size and structure: velvety, woolly or the presence of concentric petals or stipes, pigment (revers, awers). The microscopic observations were conducted in microcultures. The micromorphological features included observation of shape and size of sporangiophores, sporangia and amount of sporangiospores in sporangium and the presence and morphology of chlamydospores. Fungal microcultures were cultivated in sterile Petri dishes with sterile triangle-shaped glass rod and microscope slide. Agar discs (10 mm in diameter) were cut with sterile cork-borer from PDA medium and placed on the center of the slide. Small blocks of medium were inoculated with mycelial hyphe of the *Mortierella* fungi. After that sterile cover slide was put onto inoculum. Sterile distilled water (3 mL) was transferred gently on the bottom of Petri dish in order to maintain humidity. Microcultures were incubated at 20°C for 7 days and at 4°C for 7 days. To obtain a better sporulation of tested fungi we tried to culture microorganisms on water agar (agar media containing no nutrients) and incubated at 4°C and 20°C according to Su et.al. [46].

The morphological characteristics and classification the species level was identified according to the keys and descriptions given by Domsch et al. [25], Watanabe [27] and Skirgiełło et al. [27]. The microscopic observations were conducted in microcultures on agar discs using an Olympus BX-41 laboratory microscope [47].

4.1.1. Genomic DNA Extraction, Primers and Sequencing

Fungal species were identified using ITS (internal transcribed spacer) region sequencing method. The total genomic DNA was extracted from fungal mycelium using CHELEX resin (Biorad) and enzymes for digesting the cell wall, i.e. lyticase (1 mg/1 ml) and Proteinase K (20 mg/ml). The ITS region was amplified using two primers described by White et al. [48]. The ITS1 (59-TCC GTA GGT GAA CCT GCG G-39) and ITS 4 (59-TCC TCC GCT TAT TGA TAT G-39) make use of conserved regions of the 18S (ITS 1) and the 28S (ITS 4) rRNA genes to amplify the intervening 5.8S gene and

the ITS 1 and ITS 2 noncoding regions. The primers were synthesized using the UNMC, Eppley Molecular Biology Core Laboratory. The amplicons were resolved by capillary electrophoresis using the Applied Biosystems Inc. ABI3730xl DNA genetic analyser in the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics oligo.pl in Polish Academy of Sciences, Warsaw, Poland. Contiguous sequences (contigs) were assembled from chromatogram sequence reads using Seqman (DNAStar) and a consensus sequence was generated. The sequences were aligned with a software aligner and analyzed to identify the fungi according to BLAST search of the sequences and performed against NCBI-GenBank database for comparison. The nucleotide sequences are available under the GenBank accession numbers: MH 289781 (strain MA DEM 7); MH 304896 (strain MV DEM 32).

4.2. Determination of Optimal Fungal Growth Temperature

The starting cultures of *M. antarctica* DEM 7 and *M. verticillata* DEM 32 were incubated at 4, 9, 15, 20,28°C on PDA medium for six-day. To determinate the optimal growth temperature the *Mortierella* isolates were grown on PDA medium (Potato Dextrose Agar - Sigma Aldrich 70139) at five temperatures (4, 9, 15, 20 and 28 °C). The mycelia discs (0.8 cm) from starting cultures were transferred to PDA medium in Petri dishes (total diameter 9 cm). The diameters of the colonies of *Mortierella* strains were measured daily for six days. Data obtained for mycelial growth were collected from six replicates.

4.3. Maintenance of Strains

The *Mortierella* strains were stored on PDA (Potato Dextrose Agar, Sigma-Aldrich, 70139) slants at 4°C, transferred every 3 months, deposited in the Fungal Collection of the Department of Environmental Microbiology (DEM) at Maria Curie-Sklodowska University, Lublin, Poland.

4.4. In Vitro Screening of Mortierella Strains for Phytoregulators Synthesis

4.4.1. Preparation of Fungal Inoculum

To prepare liquid inoculum two *Mortierella* strains (MA DEM 7 and MV DEM 32) were grown on CDM (Czapek-Dox Modified) medium composed of: glucose, 10.0 g; $NaNO_3$, 3.00 g; K_2HPO_4, 1.00 g; $MgSO_4$, 0.50 g; KCl, 0.50 g; and $FeSO_4$, 0.01 g in 1000 mL, pH 7.0 [49]. The strains were cultivated in darkness at 20 °C and 60% relative humidity in an Innova 4900 growth chamber (New Brunswick Scientific, USA) at 120 rpm for 3 days. Mycelial pellets from shaken cultures were filtered from the growth medium and then washed and resuspended in 9 mL sterile, deionized water. The mycelial suspensions were fragmented under sterile conditions, five times for 20 s each time at 1-min intervals at 10.000 rpm in a laboratory blender (IKA® T 18 basic ULTRA - TURRAX®) and adjusted by dilution to the desired concentration (about 1×10^5 cfu/mL). To confirm the number of fungal cfu/mL, 1 mL of the homogenate the biomass was suspended in 9 mL of sterile distilled water and mixed vigorously with a vortex and the serial dilutions were prepared. The number of fungal cfu/mL was determined by the plate dilution technique on CDM medium and counted after 7 days of incubation at 20°C.

386
387 *4.4.2. The Phytohormones and ACC-deaminase Formation*
388
389 To study of the formation, the IAA (indoleacetic acid) and GA (gibberellic acid) *Mortierella,*
390 strains were cultivated in 250 mL Erlenmeyer flasks with 50 mL of a CDM liquid media (pH 7.0). The
391 CDM medium was supplemented with the solutions of IAA precursor - tryptophan (Trp) sterilized
392 using syringe filters (0.22 μm) in dose 0 mM, 1.5 mM and 3.0 mM. Cultures were incubated at 9, 15
393 and 20°C. To study the GA synthesis strains were cultivated on CDM medium with initial dose of
394 3.0 mM Trp. The ACC-deaminase activity was studied on CDM medium with 1.5 g $NaNO_3$. The
395 phytohormones concentration and ACC-deaminase activity were measured in 1., 3., 5., 7. and 9. days
396 filtrates of fungal cultures. The dry weight of mycelial fragments in the liquid culture was determined
397 after collecting on Whatman no. 1 filters and weighting them after drying at 60°C for 24 h. To
398 determine the ability of the *Mortierella* strains to synthesize IAA, GA and ACC-deaminase the
399 supernatants of the liquid culture were centrifuged (10,000×g for 10 min). The IAA concentration was
400 estimated according to the method of Glickmann & Dessaux [50], using Salkowski's modified reagent
401 [15, 51]. One mL of the culture supernatant was mixed with 1 mL of Salkowski's reagent. The
402 absorbance of the pink color was developed after 30 min of incubation in darkness at 20°C and read
403 at λ = 530 nm. The concentration of IAA was calculated from the regression equation of standard
404 curve of pure indole-3-acetic acid (Sigma 57330). To quantify the GA using the modified method of
405 Brückner et al. [30, 52], Hasan [41] the pH of filtrates was adjusted to 2.8 with 1 M HCl and extracted
406 3 times with equal volume of ethyl acetate. The ethyl acetate fractions were pooled and vacuum-
407 evaporated (using Eppendorf Concentrator plus) at 20°C. The dry pellet was resuspended in
408 ethanol:H_2SO_4 (9:1) mixture. The absorbance was read at λ=254 nm and the GA concentration was
409 calculated from the regression equation of standard curve of pure gibberellic acid (Sigma 63492) and
410 expressed as μg/mL over control [15]. All the spectrophotometric analyses were conducted on Varian
411 Cary 1E UV-Visible Spectrophotometer.
412 The ACC deaminase activity was assayed according to a modified method of Belimov et al.
413 [42] and Shaharoona et al. [14] measuring the amount of α-ketobutyrate, product relelased after
414 hydrolysis of ACC. The number of μmol of α-ketobutyrate produced by this reaction was determined
415 by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate (Sigma-
416 Aldrich, USA) ranging between 0.1 to 1.0 μM. The ACC deaminase activity was expressed in mM of
417 a-ketobutyrate mg/protein/h. The protein concentration was quantified by the Bradford method [53].
418 For the assessment of ACC deaminase activity *Mortierella* strains were grown on CDM medium at 9,
419 15 and 20 °C. At the end of the induction period, the cultures were centrifugated at 15 000 rpm for 5
420 min, and washed with 0.1M Tris-HCl (pH 7.5). After centrifugation the mycelia were fragmented
421 under sterile conditions, three times for 10 s each time at 1-min intervals at 10.000 rpm in a laboratory
422 blender (IKA® T 18 basic ULTRA - TURRAX®). To 200 μL of homogenized material 25 μL of toluene
423 was added and vortexed vigorously for 30 s. ACC (20 μL of 0.5 M solution) was added to the
424 suspension, vortexed and after 15 min incubation at 20°C, 1 mL of 0.56 N HCl was mixed. The lysates
425 were centrifuged (10 000 g, 10 min) and 1 mL of the supernatant was mixed with 800 mL of 0.56 N
426 HCl and with 300 mL of 2,4-dinitrophenylhydrazine (0.2 g in 100 mL of 2 N HCl). The mixtures were
427 incubated for 30 min at 20°C, following the addition and mixing of 2 mL of 2 N NaOH, the absorbance
428 of the mixture was measured using spectrophotometer at 540 nm.

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4.5. Winter Wheat Seedlings Inoculation with Mortierella Strains – Experimental Design

Seeds of winter wheat (*Triticum aestivum* L. cv Arkadia) purchased from DANKO Plant breeding Choryń Poland) were used in the experiments. Grains were surface-sterilized according to the procedure described by Jaroszuk-Ściseł and Kurek [54]. For 30 min the seeds were washed in tap water, then soaked for 10 min in a solution of $HgCl_2$ (0.1%, w/v), submerged for 10 min in a solution of H_2O_2 (30%, v/v) and washed three times with sterile deionized water. Surface-sterilized seeds were rinsed ten times in homogenized mycelial inoculum of MA DEM 7 and MV DEM 32 (about 1×10^5 cfu/mL) prepared as described before (chapter 4.4.1.) and transferred to sterile Petri dishes taking 20 seeds per dish. Six experiment treatments were used: without Trp, with water addition, inoculated with MA DEM 7 or MV DEM 32; with Trp solution (3.0 mM Trp/mL), inoculated with MA DEM 7 or MV DEM 32 and non-inoculant treatments (with or without Trp) as controls. The effect of fungal strains on winter wheat seedlings growth was observed under different temperature conditions (incubation at 9, 15 and 20 °C). The root and shoot fresh total weight of winter wheat seedlings was measured in g after 5 and 10 days of incubation.

4.6. Statistical Analysis

The experiments were carried out in three independent replicates and the results were expressed as mean ± SD. Standard deviations (presented as deviation bars) and determined using Microsoft Excel 2016 (Microsoft Corp., Redmond, Washington, USA). Where appropriate, the data were subjected to one-way ANOVA analysis of variance, significance was evaluated at $p < 0.05$. The responses of *Mortierella* strains to the selected temperatures and days of cultivation were evaluated by the principal component analysis (PCA) in the MVSP3.21 package. The analyses were based on the average values.

5. Conclusions

Based on morphological properties and sequence homology of the MA DEM 7 and MV DEM 32 filamentous fungi, two isolates from Spitzbergen soil were identified as *Mortierella antarctica* and *Mortierella verticillata* respectively. Our results confirmed that tested *Mortierella* strains are able to grow and synthetize the phytoregulators in conditions encountered in a temperate climate**.** After inoculation of winter wheat with *Mortierella* strains we have observed a positive effect on seedlings root and shoot fresh mass.

Author Contributions:

Ewa Ozimek and Jolanta Jaroszuk Ściseł conceived experiments and the experimental design. Ewa Ozimek and Anna Słomka administrated the project. Renata Tyśkiewicz, Artur Nowak and Agnieszka Hanaka participated in the plants cultivation, collecting the plant tissue and data analysis. Justyna Bohacz and Teresa Korniłłowicz-Kowalska conducted the morphological identification of fungi. Ewa Ozimek, Jolanta Jaroszuk Ściseł, Agnieszka Hanaka, Justyna Bohacz and Teresa

Korniłłowicz-Kowalska prepared the original draft manuscript. All authors approved the final version of the manuscript for submission.

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Abbreviations

IAA	indoleacetic acid
GA	gibberellic acid
PGPF	plant growth promoting fungi
PDA	potato dextrose agar
CDM	Chapek-Dox modified medium

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