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Evaluation of the Plant Growth Promoting Effect of Root Contact, Diffusible and Volatile Compounds Produced by Rhizobacteria and Microalgae on *Arabidopsis Thaliana*

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Abstract

Bacteria and microalgae have beneficial impact on plant growth and survival through host functional and adaptive traits via complex mechanisms. Volatile and non-volatile metabolites produced by microorganisms have a continuous effect on plants by providing nutrients and regulating various plant metabolic and signaling pathways. The aim of this study was to assess the plant promoting effect of two *Chlorella* spp. microalgae under mixotrophic conditions, as well as the effects of plant growth promoting rhizobacteria (PGPR) *Bacillus* sp. WCC-B36, *Azospirillum* sp. WCC-ASP12 and *Azotobacter* sp. WCC-IZA56 on the model plant *Arabidopsis thaliana*. Growth and quality parameters were followed in three different co-cultivation systems as (i) direct root contact supplemented with density effect, (ii) contact with diffusible compounds and (iii) effects of volatile compounds. Direct effect mediated by rhizobacteria promoted significant shoot and root length growth with well-developed root architecture at low bacterial densities ($<10^5$ CFU – colony forming unit mL^{-1}), which became more pronounced over time. At a higher microbial density ($>10^7$ CFU mL^{-1}), plant growth was retarded regardless of the bacteria present. This suggests that the microenvironment surrounding the colonies was altered and there was competition for nutrients. Our results indicate that the metabolites, diffusible and volatile organic substances produced by the microalgae enhanced lateral root growth and root hair formation, while inhibited primary root elongation. Volatile and diffusible substances of *Chlorella* sp. CHL13 and *Bacillus* sp. WCC-B36 have the most significant effect on seedlings and primary root growth.

Keywords

Arabidopsis, *Chlorella*, mixotrophic microalgae, plant promoting rhizobacteria, volatile compounds

1 Introduction

The microbiome of the soil, particularly the microorganisms of the rhizoplane and the rhizosphere have a beneficial impact on plant growth and survival. The microorganisms that are capable of colonizing roots, promoting plant growth and affecting plant health, are referred to as plant growth promoting rhizobacteria (PGPR) [1–3]. Different types of interactions can develop between PGPRs, plants and the microbiome of the rhizoplane, which can have beneficial, negative or neutral effects [4]. These can profoundly influence plant traits and the community structure of the rhizoplane microbiome [5]. Root exudates, which contain sugars, amino acids, organic acids, flavonoids, proteins, and fatty acids, act as attractive or repellent signals for the rhizosphere microbiota and influence these interactions [6]. Rhizobacteria can play an essential role in enhancing plant nutrient acquisition through diverse processes, e.g., nitrogen fixation, phosphate solubilization, and organic matter mineralization [7, 8]. In addition to nutrient mobilization, the phytohormone and siderophore production by the rhizobacteria are direct mechanisms against biotic and abiotic stress [9]. Rhizobacteria can induce systematic resistance in plants and suppress phytopathogens by the production of antibiotics, antagonistic substrates (e.g., hydrogen cyanide, siderophores etc.) and lytic enzymes (e.g., chitinase, proteases) [10]. In addition, bacteriocin production, microbe-to-plant signaling and sulphur deficiency alleviation were recently considered as additional plant growth promotion (PGP) mechanisms [7]. The molecules responsible for direct

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and indirect mechanisms can be non-volatile (siderophores, the majority of phytohormones) or volatile gases diffusing through the soil pores. Rhizobacteria can be characterised by their specific volatile compound (VOCs) profile which depends on their metabolic capacity, growth stage and available nutrients [11]. The emission of VOCs can be constitutive or induced by stress factors [12]. Many microorganisms (such as *Streptomyces*, *Stenotrophomonas*, *Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia* etc.) are able to emit low-molecular weight VOCs (e.g., terpenes, jasmonate etc.) that regulate various metabolic and signaling pathways as potential signal molecules [13] that interact with plants in soil environment without physical contact [14–16].

The beneficial effect of rhizobacteria and their application as soil inoculant are well-known, besides rhizobacteria, fungi, protozoa and algae co-existing in the rhizosphere and the rhizoplane also play a crucial role in plant health and productivity [7, 10]. In the past few years, algae have gained attention as a source of nutrition for humans and animals, as well as for their use in human cosmetics, as an excellent photosynthetic biofuel and as a biostimulant due to their ability to produce high levels of proteins, lipids and biomass [17]. The macroalgae-produced enzymes, amino acids, secondary bioactive metabolites, vitamin precursors, vitamins, essential nutrients, and plant hormones like auxins and cytokinins [17] were used as biofertilizers with promising effects in crop production. The microalgae Chlorophyceae, including members of the genus *Chlorella* producing large amount of carbohydrates, proteins, lipids and growth hormones are potential biofertilizers that can increase growth, yield and induce systemic acquired resistance of various crops under field conditions [18]. Algae are mainly phototrophic organisms; but several *Chlorella* spp. can grow under autotrophic and heterotrophic conditions [19, 20]. In heterotrophic and even mixotrophic conditions, higher specific growth rate and biomass yields can be achieved (with significant lipid and carbohydrate production) compared to photoautotrophic condition [21–23], contributing to their potential as biofertilizer. In addition, the mixotrophic growth can contribute to the settlement of the microalgae on plant leaves and soil [24], making them a viable option as a biofertilizer and biostimulant agent through their metabolites produced [17, 25, 26]. Kusvuran [17] found that the foliar application of microalgae reduced membrane damage and attenuated oxidative stress in leaves exposed to drought. The CO_2 sequestration provides organic matter that improves soil properties [25].

Most of the studies consider the effect of macro- and microalgal extracts on plant growth under laboratory and field conditions [17, 24, 25, 27]. However, the application of viable microorganisms as biofertilizer has a chance of survival under agricultural conditions, providing a continuous beneficial effect for plants through volatile and non-volatile metabolites. Our aim was to evaluate the PGP effect of two microalgae under mixotrophic conditions and three rhizobacteria (sourced from Witaria Culture Collection – Budapest, Hungary) on *Arabidopsis thaliana* growth and quality parameters including plant and root length and root architecture. For this purpose, three different co-cultivation systems were established: direct root colonization contact, contact with diffusible compounds, and contact with volatile compounds. The dose-response for the direct root colonization effect of bacteria and microalgae applied to the model plant *Arabidopsis thaliana* was determined. Our further aim was to identify the VOC profile of the strains studied and to reveal any strain-specific effect of the non-volatile, diffusible and volatile metabolites produced by the microorganisms on the model plant based on morphological characteristics.

2 Materials and methods

2.1 Plant material, growth conditions

The wild-type *Arabidopsis thaliana* Columbia (Col-0 accession) seeds were received from Budapest University of Technology and Economics (Budapest, Hungary). The surface of the seeds was sterilized by immersing them in 70% V/V ethanol for 2 min, followed by a 20 min immersion in 1% V/V sodium hypochlorite solution then they were rinsed with sterile distilled water three times. The surface sterilized seeds were stored in sterile tubes at 4 °C in the dark for 48 h, to ensure stratification. The seeds were sown Murashige and Skoog (MS) salt medium (Gibco, Thermo Fisher Scientific Inc. USA) with the modification in its agar (0.8%) and sucrose (1.5%) content called half-strength MS agar. The pH of the half-strength MS agar was adjusted to pH 5.7. The plates were incubated vertically, at 22 ± 2 °C with a 16 h light / 8 h dark photoperiod under blue light (350–500 nm).

2.2 Microorganism strains, growth conditions and identification

The applied bacterial strains used in this study were isolated from soil and after phylogenetic identification *Azotobacter* sp. WCC-IZA56, *Azospirillum* sp. WCC-ASP12, *Bacillus* sp. WCC-B36 strains were deposited in the culture

collection WCC (Witaria Culture Collection; Budapest, Hungary; https://witaria.weebly.com/). Two microalgae *Chlorella* spp. (*Chlorella* sp. CHL2 and *Chlorella* sp. CHL13) were obtained from the culture collection WCC.

All bacteria and microalgae strains were maintained on thioglycollate (TIO) medium agar (Oxoid Ltd., Thermo Fisher Scientific Inc., USA) at 28 °C. Prior to work, the identification of the bacteria applied were carried out for confirmation purposes. The bacterial genomic DNA was extracted using DNeasy PowerLyzer Microbial kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a modification to the physical cell disruption step. The samples were shaken in "Bead Solution tubes" (Qiagen) containing microbeads at 25 Hz for 2 min using mixer mill MM301 (Retsch, Haan, Germany). For the amplification of 16S rRNA gene primers 27f (AGAGTTTGATCMTGGCTCAG) and 1401r (5′-CGGTGTGTACAAGACCC-3′) [28, 29] were applied with the following thermal profiles of the PCR reactions: initial denaturation at 98 °C for 5 min, followed by 32 amplification cycles of 30 s at 94 °C, for 45 s at annealing temperature 52 °C, 1 min at 72 °C, followed by final extension at 72 °C for 10 min. For one sample the PCR reaction mixture contained 1U of LC Taq DNA Polymerase (Fermentas, Vilnius, Lithuania), 1x Taq buffer with $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate (Fermentas), 0.65 mM of each primer, 1–2 mL DNA template in final volume of 50 mL. Amplification was performed with GeneAmp PCR System (Model 2400, Applied Biosystems, Foster City, USA). PCR products were visualized by UV light on 1% stained agarose gel. The 16S rRNA gene fragments were sequenced by Sanger method using 27F [28] forward primers at Eurofins Biomi Ltd. (Gödöllő, Hungary). Prior to the sequencing reaction, PCR products were purified using GeneJet PCR purification kit (Thermo Fisher Scientific Inc.). Sequences were aligned by Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the GenBank nucleotide database (National Library of Medicine, The National Center for Biotechnology Information, USA), the identification of the obtained strains were carried out using the EzTaxon-e [30] database. According to Tindall et al. [31] microbial genera and species were assigned at 95% and 97% similarity threshold levels, respectively, sequences obtained in this study were submitted to European Nucleotide Archive (Accession numbers: *Azotobacter* sp. WCC-IZA56: PP396500; *Azospirillum* sp. WCC-ASP12: PP396501; *Bacillus* sp. WCC-B36: PP396502).

2.3 Experimental setup and plant measurement

The aim of our study was to the describe the PGP effect of the microorganisms such as *Azotobacter* sp. WCC-IZA56, *Azospirillum* sp. WCC-ASP12, *Bacillus* sp. WCC-B36 and two strains of *Chlorella* spp. (*Chlorella* sp. CHL2 and *Chlorella* sp. CHL13), which were selected for two commercial biofertilizer product BioFil Alga and Green Gold Rush.

To investigate the different effects of the metabolic products produced by the bacterial and microalgae strains of the biofertilizer products, three experimental designs were set up. For cultivation half-strength Murashige and Skoog (MS) salt medium (Gibco, Thermo Fisher Scientific Inc. USA) containing 0.8% agar and 1.5% sucrose were applied, the pH was adjusted to pH 5.7:

1. The direct effects of bacteria and microalgae on *Arabidopsis thaliana* plants were investigated by microbial root colonization. For this purpose, CFU (colony forming unit) of the microbial and microalgae suspensions were determined using the serial dilution and plating method under sterile conditions (Fig. 1). The diluted samples were plated on thioglycollate agar and incubated O/N (30 °C). After the incubation, colonies were counted, then CFUs were calculated. The microbial and microalgae suspensions were stored at 4 °C during the CFU determination process, then 1–1 mL of each liquid suspension of microbial and microalgae cultures with 10^1, 10^3, 10^5, 10^7 CFU mL^{-1} was added to 20 ml MS agar medium at 35–40 °C. Thereafter the culture medium was mixed homogenously with the applied bacteria strains which were poured in Petri-dishes. Five sterilized seeds per plate were sown onto the upper thirds of the Petri-dish (Fig. 1) ("upper third" means closer to the upper edge of the vertically placed plates) containing half-strength MS medium.
2. The effect of the diffusible compounds produced by bacteria and microalgae on *Arabidopsis thaliana* growth in vitro was also investigated. For this purpose, five sterilized seeds per plate were sown onto the upper thirds of Petri-dishes containing half-strength MS solid medium, then bacteria or microalgae were inoculated onto the lower third (closer to the bottom edge) of Petri-dishes (Fig. 1).
3. To detect the effect of the volatile organic compounds, centre-partitioned Petri-dishes were applied with MS solid medium, where five *A. thaliana* seeds per plate were sown to one side of the plate, while the other side of the plate was inoculated with the bacteria and microalgae strains (Fig. 1). All experimental

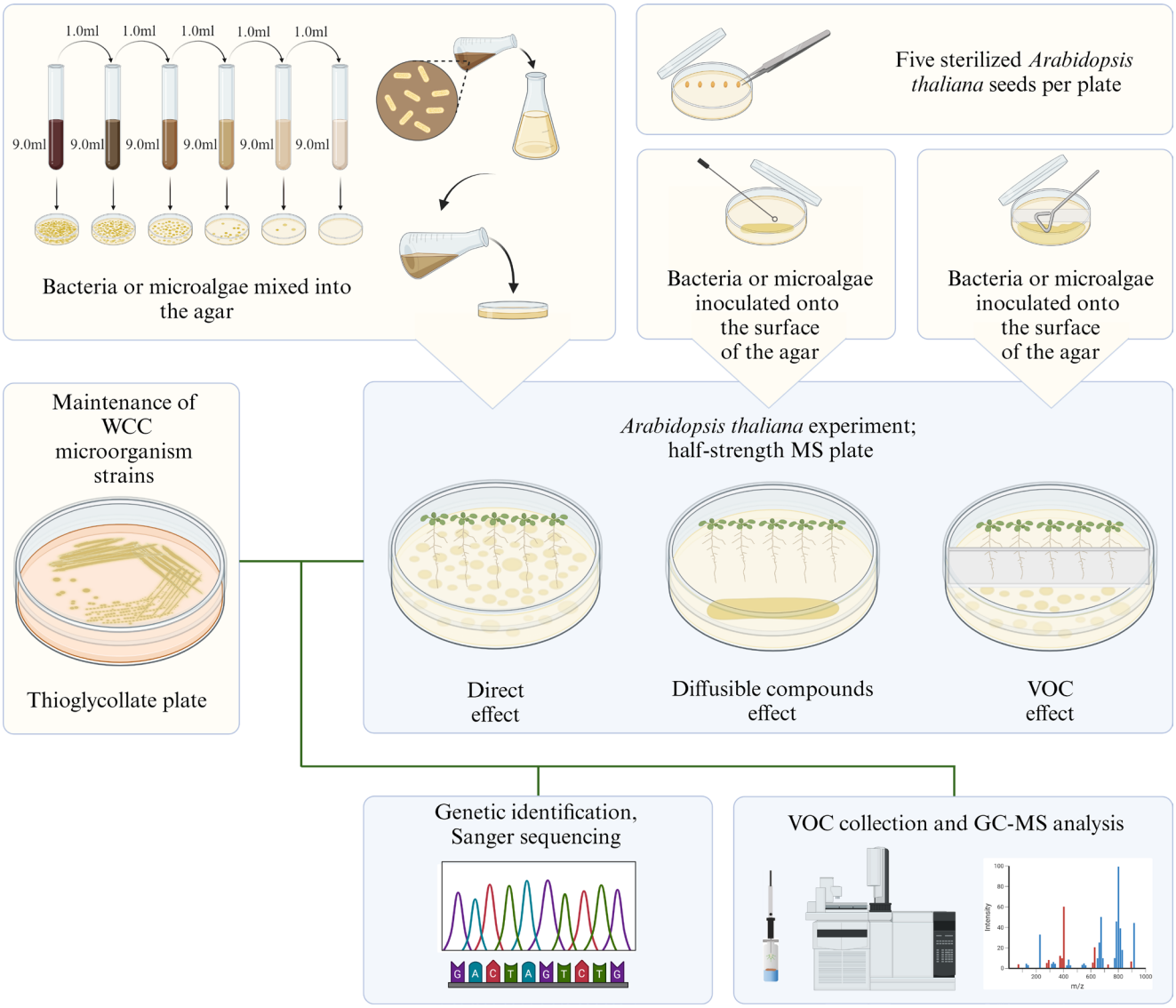

Fig. 1 Schematic representation of the experimental setup. Different inoculation methods were used to investigate the different effects (direct effects, effects of diffusible compounds, effects of volatile compounds) of bacterial and microalgae strains (Created with BioRender.com).

setups were performed in triplicate and *Arabidopsis thaliana* inoculated with sterile distilled water were cultivated on half-strength MS as a control. Considering the geotropic growth of the plants all Petri-dishes were incubated (on RT) in vertical position. To avoid release of the VOC products the Petri-dishes were sealed with Parafilm M sealing film (Amcor, Switzerland).

Evaluations began on the first days of germination (4^{th}–6^{th} days) and ended on the 15^{th} day of incubation. To monitor plant growth, plant height, number of leaves, branches and root length were measured every 5 days at a 15 days interval. Root length was determined manually on Petri-dishes, by measuring the primary root from the origin of the seed to the root apex without damaging the plants. First order lateral roots were counted and their length were also measured manually on Petri-dishes The photos were taken by Chameleon CMLN-13S2M CCD camera (Point Grey Research, Richmond Canada) with InfiniMite 2X Macro lens (Infinity Photo-Optical, Boulder, USA).

2.4 Volatile organic compounds collection and analysis by SPME/GC-MS method

The volatile organic compounds produced by the studied microorganisms were absorbed on solid phase micro extraction SPME fiber and analysed by Gas Chromatography/Mass Spectrometry (GC-MS) using a Shimadzu GCMS-QP2010SE (Shimadzu, Kyoto, Japan) gas chromatograph coupled with a Shimadzu AOC 6000

Plus autosampler unit (Shimadzu, Kyoto, Japan). At first, the microorganisms were inoculated onto 2 mL of half-strength MS agar medium (see Section. 2.1) in sterile 20 mL GC vials (ALWSCI, Shaoxing, China). The polytetrafluoroethylene (PTFE)-coated septa (Restek, Bellefonte, USA) sealed vials were incubated at 22 °C for 5 days. For VOCs extraction carboxen/polydimethylsiloxane (PDMS; 75 µm) SPME fiber (Supelco, Bellefonte, USA) was inserted into the headspace of the vials above the microorganisms' samples for 30 min at 30 °C. For GC-MS analysis, grade 5.0 helium (Messer, Bad Soden, Germany) was used as carrier gas at a flow rate of 0.7 mL min^{-1} in 30 m long, 0.25 mm inner diameter, 0.25 µm stationary phase capillary column (Phenomenex, Zebron ZB-SemiVolatiles, Torrance, USA). The volatile components were desorbed from the SPME fibers at 250 °C for 2 min, and the GC – MS run was 28 min long. The temperature protocol of the GC-MS run was as follows: the initial temperature was 30 °C for 10 min, 10 °C min^{-1} to 120 °C, then 30 °C min^{-1} to 240 °C, the final temperature was held for 5 min. The mass spectrometer was operated in electron ionization mode at 70 eV, the single quadrupole analyser continuously scanned the spectra from 30 m/z to 200 m/z. The volatile compounds were identified by comparing the mass spectra data with National Institute of Standards and Technology (NIST) Mass Spectrum Library. Identified components showed at least 90% mass spectra identity with the authentic standard from the NIST library.

2.5 Statistical analysis

Each experiment was prepared in three parallels, and the results presented are the average of the experiments in each case. Taking into account the different experimental setups, triplicate with five *Arabidopsis thaliana* replicate per treatment were performed. The mean values of the root length and plant height of five *Arabidopsis thaliana* per plate were calculated. Significances were calculated using Student's t-test, differences between means were considered significant for p-values <0.05 using PAST software system [32]. p-values are indicated in the figure captions with asterisk.

3 Results and discussion

3.1 Microorganisms strain identification

For phylogenetic identification, the isolated bacterial strains deposited in the WCC culture collection were characterized by partial 16S rRNA gene sequencing. According to the performed sequence analyses of the 16S rRNA genes of the three potential PGP rhizobacteria strains *Azotobacter* sp. WCC-IZA56, *Azospirillum* sp. WCC-ASP12 and *Bacillus* sp. WCC-B36 isolated from soil resulted in 100–98% sequence similarities to *Azotobacter vinelandii*, *Azospirillum canadense*, and *Bacillus proteolyticus*, respectively. We have to mention that the three isolates also showed 99–97% similarities with type strains of two or more species within the corresponding genera. The mentioned phenomenon is explained by the high sequence similarity of the 16S rRNA gene of several species within the genera. Accordingly, the proper species affiliation for the culture collection strains cannot be defined based on partial 16S rRNA gene. However, many organisms in these genera have a plant growth promoting effect, based on numerous literature data [33–38]. Kari et al. [39, 40] demonstrated that inoculation with *Azospirillum* spp., *Azotobacter* sp., and *Bacillus* sp. positively affected the development and yield of maize (Zea mays, L.) cultivated on degraded soils. The *Chlorella* spp. were acquired from WCC (Witaria Culture Collection), therefore phylogentic identification of the microalgae was not required. In addition, the exact species identification of *Chlorella* spp. cannot be disclosed for reasons of confidentiality.

3.2 The direct bacterization effect of bacteria on *Arabidopsis thaliana* plant and root growth

Plant growth promotion was tested by direct contact in root colonization assay with the application of potential PGP microorganisms. Considering the bacterization experimental design, effects of seed and root colonization were tested using half-strength MS agar inoculated with different densities of bacteria. The bacterial strains with different phylogenetic lineages: *Azospirillum* sp. WCC-ASP12 and *Bacillus* sp. WCC-B36 characterized with low 10^1 and 10^3 CFU mL^{-1} promoted significant ($p \leq 0.05$) shoot and root length growth compared to untreated plants during the entire 15-day period (Fig. 2). It should be noted that inoculation with *Azospirillum* sp. WCC-ASP12 at 10^3 CFU mL^{-1} induced the development of one of the longest root lengths at day 10. Treatment with higher bacterial density (< 10^7 CFU mL^{-1}) values caused significantly ($p \leq 0.05$) reduced plant and root length growth in comparison with untreated control plants regardless to the applied bacterial strain (Fig. 2). Méndez-Gómez et al. [41] found that the increased bacterium density (up to 10^6 CFU mL^{-1}) caused decreased root length, consistent with our results. Time course analysis revealed that the stimulatory effect of *Azospirillum* sp. WCC-ASP12 on the seedling and root length development increased over time. The plant

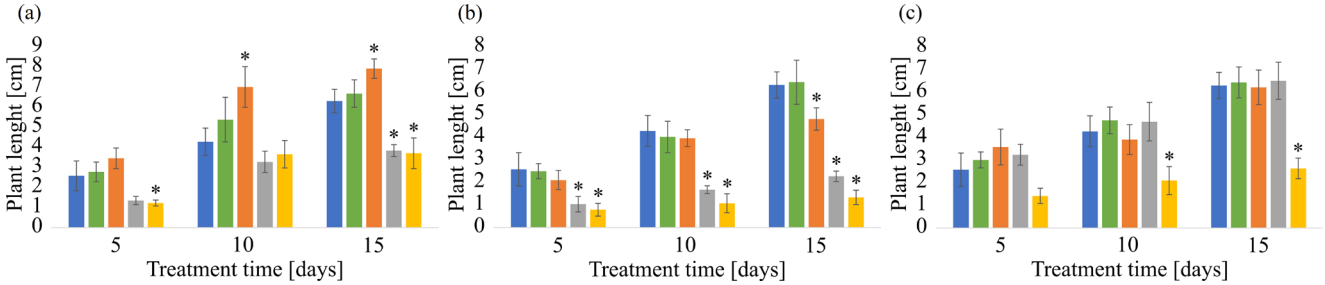

Fig. 2 The direct effect of the potential PGP bacteria on plant growth promotion on *A. thaliana*. Seedlings were cultivated with different bacterial strains with concentrations ranging 10^1 to 10^7 CFU mL^{-1} (Control: blue; 10^1 CFU mL^{-1}: green; 10^3 CFU mL^{-1}: orange; 10^5 CFU mL^{-1}: grey; 10^7 CFU mL^{-1}: dark yellow), growth parameters were measured at days 5, 10 and 15. A-C. Presents the plant height data cultivated with (a) *Azospirillum* sp. WCC-ASP12; (b) *Azotobacter* sp. WCC-IZA56; (c) *Bacillus* sp. WCC-B36, respectively. The asterisk above the bars indicates statistically significant differences $p < 0.05$, the error bars represent Standard deviations.

growth promoting effect of the *Bacillus* sp. WCC-B36 and *Azospirillum* sp. WCC-ASP12 was observed in the increased development of lateral root-system and root hairs (Fig. 3(b), Fig. 3(e)). The enhanced lateral root outgrowth and root hair formation, with inhibited primary root elongation could be triggered by the direct effect of the applied microorganisms, similarly to what was found during the investigation of *Bacillus amyloliquefaciens* UCMB5113 strain [43]. The positive effects on root morphology and plant growth such as the initiation, elongation

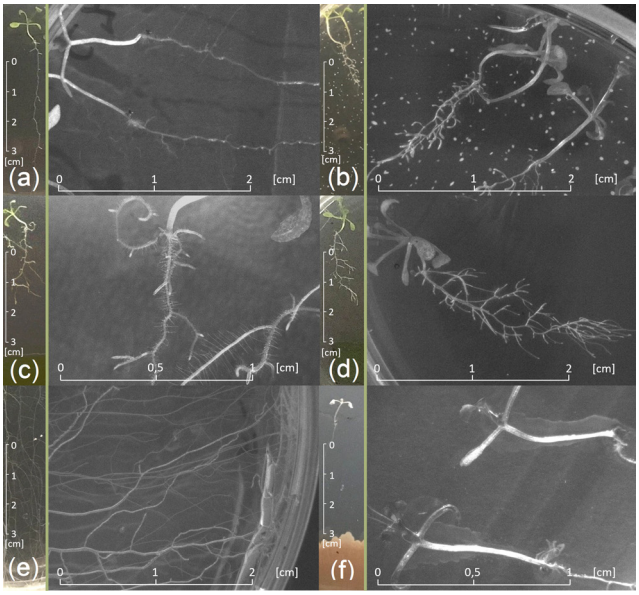

Fig. 3 Representative image of the growth of the model plant *A. thaliana* seedlings under different cultivation conditions on day 15, (a) axenically grown control plant (b) plants inoculated with *Azospirillum* sp. WCC-ASP12, representing the direct effect of the studied bacteria (c) the effect of VOC produced by *Chlorella* sp. CHL13 on the model plant (d) the effect of VOC produced by *Chlorella* sp. CHL2 on the model plant (e) plants inoculated with *Bacillus* sp. WCC-B36, representing the direct effect of the studied bacteria (f) the effect of diffusible compounds produced by *Azotobacter* sp. WCC-IZA56 on the model plant.

and development of lateral roots and leaf development was observed at lower bacterial density ($\leq 10^5$ CFU mL^{-1}). The well-developed root architecture (Fig. 3(b), 3(e)) can enhance nutrient uptake, e.g., phosphorus and iron solubilisation [42] and improve overall plant physiology through the increased relative root surface area that can be caused by the direct effect of PGP microorganisms [43]. Moreover, lateral root development and root growth increases the surface area of nutrient uptake and secretion, promoting microbial root colonization [43]. In addition, bacteria with plant root association can modulate root morphogenesis with enhanced lateral root and root hair development and stimulate root development through phytohormone (e.g., auxin, cytokinin, gibberellin) production [42–44]. It is noteworthy that *Bacillus proteolyticus* can activate induced systemic resistance of host plants against pathogens [45].

Inoculation with the known PGP bacteria *Azotobacter* sp. WCC-IZA56 at any CFU value significantly ($p \leq 0.05$) reduced primary root and shoot growth compared to the untreated plants throughout the 15-day period (Fig. 2). Considering the root system architecture *Azotobacter* sp. WCC-IZA56 inoculation caused less developed non-branching root architecture, and the leaf development was also reduced causing small two-leafed stages seedlings throughout the 15-day period. The reduced plant and root length compared with the control plants suggests that bacteria with a higher applied inoculation rate probably contributed to reduced growth of plants. *Azotobacter* is a well-known PGP rhizobacteria [10, 39, 40, 46], but Minut et al. [45], found that in sterile soil Azotobacter sp. with dilution 10^5 CFU mL^{-1} had inhibitory effect on plant growth in pot experiment. The plant growth stimulating effect of different *Azospirillum* [47] and *Azotobacter* [46] strains were tested, and according to these results, differences in plant growth stimulation among the tested strains

and among the plant cultivars were observed. The mentioned phenomenon could be explained by the differences in the produced growth regulators and the different sensitivity of the plant cultivars [47]. *Azotobacter* spp. are capable of producing large amounts of exopolysaccharides (EPS) [8, 33, 45] forming biofilms around the root as a compact mat-like structure [48]. In our case, the tested *Azotobacter* strain produced large amounts of EPS resulting in large, densely spread colonies even on the surface of the root, which probably had a negative effect on plant growth. In field conditions biofilm-forming ability is an added advantage for bacterial survival in the rhizosphere (Altaf 2017) and increases the resistance to water stress [8].

Considering the rapid growth of *Azotobacter* sp. WCC-IZA56 strain can contribute to the alteration of the microenvironment surrounded by the colonies. The high dosage of the microorganisms can contribute to competition for nutrients causing stress related growth reduction for the plant [49]. Weise et al. [50] found that the ammonia produced through metabolism led to an increase in pH, which contributed to the inhibition of plant growth, with the most significant inhibition effect occurring between day 1 and day 5. In our study, more pronounced plant growth inhibition was also observed at earlier stages of the development (day 5 and day 10), in line with the findings of Weise and co-workers [50].

3.3 The direct effect of microalgae on *Arabidopsis thaliana* plant and root growth

In our study, we focused on direct plant growth promotion effect of viable microalgae *Chlorella* sp. CHL2 and *Chlorella* sp. CHL13 under mixotrophic conditions, supplemented by the dose effect of these organisms. Chlorella sp. CHL2 and *Chlorella* sp. CHL13 caused significantly ($p \leq 0.05$) shortened shoot and primary root growth compared to untreated plants over the entire 15-day period, regardless of the applied density (Fig. 4). Considering the root-architecture of the model plant, inoculation with 10^7 CFU ml^{-1} value of *Chlorella* sp. CHL13 caused significant growth inhibition with less developed root-architecture on *Arabidopsis thaliana*. However, the root-system and the leaf architecture of the *Arabidopsis thaliana* inoculated with 10^1 and 10^3 CFU ml^{-1} of *Chlorella* sp. CHL13 resulted in short but well-developed branching root architecture, and the leaf development was enhanced, resulting in four-leafed stages seedlings after day 10. Moreover, the most pronounced root length retardation was observed after day 10 (Fig. 4). Based on the results of a study

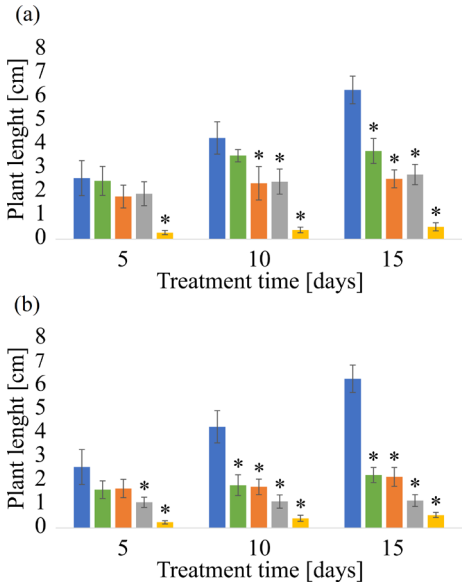

Fig. 4 The direct effect of two mixotrophic microalgae on plant growth promotion in *A. thaliana*. Seedlings were cultivated with different microalgae concentrations ranging 10^1 to 10^7 CFU mL^{-1} (Control: blue; 10^1 CFU mL^{-1}: green; 10^3 CFU mL^{-1}: orange; 10^5 CFU mL^{-1}: grey; 10^7 CFU mL^{-1}: dark yellow), growth parameters were measured at days 5, 10 and 15. A-B Presents the plant height data cultivation with (a) *Chlorella* sp. CHL13 and (b) *Chlorella* sp. CHL2, respectively. The asterisk above the bars indicates statistically significant differences $p < 0.05$, the error bars represent Standard deviations.

investigating the effect of the microalgae *Chlorella sorokiniana* on the development of seedlings, the metabolic substrates (soluble carbohydrates, proteins, phosphorus, auxins, gibberellins and cytokine) produced by the *Chlorella* sp. CHL13 microalgae in the growth media can enhance germination [51].

In our experiments, higher growth rate and well-developed plantlet architecture was observed. According to the inoculation with *Chlorella* sp. CHL2 at different rates resulted insignificantly ($p \leq 0.05$) shortened shoot and root growth (Fig. 4) with less developed architecture. According to the literature data, *Chlorella* spp. also have beneficial direct effect on plant germination, growth and well-developed root-architecture [23, 51]. Martini et al. [52] found that the microalgae treatment has a more pronounced effect on seedling growth at low nitrogen (0.1 mM) content. Considering the high nitrogen content (30 mM) applied, it could have an inhibitory effect on plant growth. Moreover, microalgae such as *Chlorella* spp. are capable of accumulating nutrients such as phosphorus [24]. Probably, the alteration of the microenvironment (e.g., pH, available nitrogen-forms, phosphorus etc.) surrounding the microalgae colonies, and the competition for nutrients, similar to plant root colonization, played a role

in the observed inhibitory effect of direct contact with *Chlorella* sp. CHL2 on plant growth.

3.4 Effect of VOC and diffusible compounds produced by bacteria and microalgae on Arabidopsis thaliana plant and root growth

Excluding the direct effect of bacteria and microalgae, the growth promotion in Arabidopsis thaliana plants by diffusible excreted substances and volatile organic chemicals produced was also investigated. The SPME-GC/MS method was applied to analyse and identify the VOCs produced by the selected microorganism strains. The identified VOCs based on more than 90% similarity belonged mainly to ketones, esters, furan and alcohol (Table 1) released at different quantities. Regarding the core VOCs produced, the microorganisms showed considerable differences, as each strain were characterized with unique VOC profile (Table 1). *Chlorella* sp. CHL2 showed the most diverse VOC profiles with main components belonging to ester group (methyl 2,2-dimethylpropanoate, methyl-2-ethylhexanoate, methyl-2-methylbutanoate, methyl 3,5,5-trimethylhexanoate) and an alcohol (hexan-1-ol) (Table 1). While the other *Chlorella* sp. CHL13 microalgae produced only one identified volatile component 2-pentylfuran with reported plant growth promoting effect [53]. Considering the effect of the VOCs and diffusible compounds produced by microalgae strains applied, pronounced and significantly ($p \leq 0.05$) shortened shoot and root length was observed compared to untreated control plants (Fig. 5). Considering the root-system and leaf architecture of *Arabidopsis thaliana*, short but well-developed branching root architecture, and four-leafed seedlings were developed, which could be a beneficial effect of the volatile (Fig. 3 (c) and (d)) and diffusible compounds produced by microalgae. The alteration in seedling development and root architecture suggested that the produced metabolites induced premature root hair differentiation and lateral root formation, similar to the findings of Asari et al. [42].

Zou et al. [53] found that 2-pentylfuran shows plant-growth inhibition at a concentration of ≥1690 μg L^{-1}. According to our plant development results (Fig. 4),

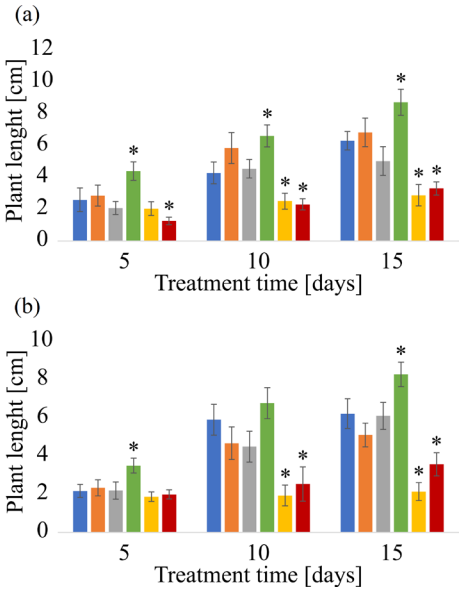

Fig. 5 The effect of the rhizobacteria and microalgae (Control: blue; *Azospirillum* sp. WCC-ASP12: orange; *Azotobacter* sp. WCC-IZA56: grey; WCC-*Bacillus* sp. B36: green; *Chlorella* sp. CHL13: dark yellow; *Chlorella* sp. CHL2: dark red) produced (a) diffusible substances and (b) volatile compounds on the model plant *Arabidopsis thaliana*. The asterisk above the bars indicates statistically significant differences $p < 0.05$, the error bars represent Standard deviations.

Table 1 The VOCs produced by the studied microbial strains and detected by SPME/GC-MS with >90% mass spectra identity based on the NIST library standard

Compound groups	Identified components	CAS No.	Microorganism strains				
			Azospirillum sp. WCC-ASP12	*Azotobacter* sp. WCC-IZA56	*Bacillus* sp. WCC-B36	*Chlorella* sp. CHL2	*Chlorella* sp. CHL13
Ketone	2-Heptanone	110-43-0		×			
	2-Nonanone	821-55-6		×			
	Propan-2-one (Acetone)	67-64-1	×				
	Butane-2,3-dione	431-03-8			×		
Ester	Methyl 2,2-dimethylpropanoate	598-98-1				×	
	Methyl 2-ethylhexanoate	816-19-3				×	
	Methyl 2-methylbutanoate	868-57-5				×	
	Methyl 3,5,5-trimethylhexanoate	71500-39-5				×	
Alcohol	Hexan-1-ol	111-27-3				×	
Furan	2-Pentylfuran	3777-69-3					×

the concentration of the 2-pentylfuran was below the reported inhibitory concentration.

VOC compounds identified as ketones such as acetone and butane-2,3-dione were released by *Azospirillum* sp. WCC-ASP12 and *Bacillus* sp. WCC-B36, respectively (Table 1). *Bacillus* sp. WCC-B36 volatiles and diffusible substances significantly ($p \leq 0.05$) initiated the growth of seedlings and primary roots (Fig. 5), but structurally less developed root-system architecture and two-leafed stages seedlings were developed. Based on our results, the produced volatile and diffusible substances had less influence on the architecture of the seedlings than the direct effect of the microorganisms. The diffusible microbial products excreted by *Azospirillum* sp. WCC-ASP12 promoted primary root and seedling length growth with the most pronounced effect of the diffusible substances at day 10 (Fig. 5). Méndez-Gómez et al. [40] found that volatile compounds produced by *Azospirillum* sp. caused increased primary root length, lateral root number and density, moreover the shoot and fresh weight of the plants also increased.

The diffusible and volatile compounds produced by *Azotobacter* sp. WCC-IZA56 induced variable effects on plant growth during the 15-day period of the study. Significant seedling growth was observed which was promoted by VOC at day 10 (Fig. 5). Later stages of the plant development, seedling and primary root growth were reduced (Fig. 3(f)) which was caused by VOCs and diffusible compounds, respectively (Fig. 5). The observed growth promotion versus inhibition probably indicates changes in the composition and even concentration values of VOCs and diffusible compounds over time. Studies have shown that VOC concentrations and the composition of volatile profiles can vary significantly depending on the microbial cell count and growth stage of the culture [54–56]. In addition, the observed different response to the bacterial substances produced could depend on the growth state of the plant [23]. Considering the identified volatile compounds, Azotobacter sp. WCC-IZA56 strain produced 2-heptanone and 2-nonanone witch reportedly have antifungal and antimicrobial effects [57], respectively. Based on our data the identified volatiles have a plant growth promoting effect improving the physiology of the plant.

4 Conclusion

In this study, we examined the PGP effects through root colonization and investigation of volatile and diffusible compounds produced by three potential PGP bacteria and two microalgae on the phenotypic properties (plant growth and root architecture) of axenically grown *Arabidopsis thaliana* Col-0. Positive growth promotion effect was observed on *Arabidopsis thaliana* cultivated with *Bacillus* sp. WCC-B36, *Azospirillum* sp. WCC-ASP12 and *Chlorella* sp. CHL13 altering the root system development causing shortened but well-developed branching root architecture. Root and shoot growth inhibitory effect was observed at a higher microbial density (above 10^5 CFU mL^{-1}) suggesting the alteration of the microenvironment surrounding the colonies, and/or competition for nutrients.

The volatile and diffusible substances of the *Chlorella* sp. CHL13 and *Bacillus* sp. WCC-B36 had the most significant effect on the growth of seedlings and primary root. The combined effect of VOCs and diffusible compounds should also be considered, since the different types of interactions affect the plant morphology and growth. It is worth to note that the biostimulant properties of the studied bacteria and microalgae strains should be investigated in combination and in field experiments in order to develop and propose their appropriate application in agriculture.

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