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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences

Master of Biomedical Sciences

Master's thesis

Evaluating the effects of aqueous extracts from blueberry leaves, berries, and biochar on seedling growth of Arabidopsis thaliana and crop species

Fien Vandenberghe

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Ann CUYPERS

MENTOR :

De heer Seb TOMBEUR

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*Running title: *Effects of blueberry extracts on seedling growth*

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ABSTRACT

Plant residues, including those from blueberry cultivation, are often discarded despite containing valuable compounds such as phenolics and flavonoids that may promote plant growth, yet their potential as biostimulants remains unexplored. This study investigated the growth-promoting effects of three aqueous blueberry residue extracts— biochar extract (BCE), leaf extract (LE), and unripe berry extract (UBE) on seedling development using three bioassays: (1) a 96-well plate assay with Arabidopsis thaliana for fundamental screening, (2) a petri dish assay with lettuce and radish using seed priming and continuous exposure methods, and (3) a preliminary pot experiment with radish to assess the effects over an extended growth period. In the 96-well assay, Arabidopsis seeds were treated with 0.5–2% BCE. Results showed a dose-dependent stimulation of fresh weight and root length with an optimal concentration between 0.5 and 1%. The petri dish assay exposed lettuce and radish seedlings to BCE (0.5–1.5%), unripe berry extract (UBE), and leaf extract (LE) at 0.0025–0.01%. Seed priming increased fresh weight in lettuce and radish, whereas continuous exposure had neutral or negative effects. In the preliminary pot experiment, radish seeds were sown into miscanthus substrate and treated twice weekly with BCE (0.5%, 1%) or LE/UBE (0.01%, 0.05%). No significant growth differences were observed, due to suboptimal experimental conditions. These results suggest BCE, LE, and UBE have potential as biostimulants, with effects dependent on concentration and exposure method. Further research is needed to optimize exposure conditions, clarify underlying mechanisms, and assess long-term effects on growth and crop quality.

INTRODUCTION

One of the major issues the world is facing today is food security for the growing population, which is projected to reach 9.7 billion people by 2050. This rise in population will require a 70% increase in food production (1). With the food production facing difficulties already, such as limited land, climate change, and resource depletion, there is a need for a sustainable way to boost crop growth and quality (2).

Although chemical fertilizers and pesticides are heavily used to boost food production, their harmful effects are becoming non-negligible. The excessive use of chemical fertilizers has numerous harmful effects on both human health and the environment (3). It contributes to groundwater and air pollution, lowers soil and food quality, and increases production costs. In addition, excessive use of fertilizers increases ecosystem vulnerability to environmental stresses. For example, nutrient pollution from agricultural runoff can cause eutrophication, leading to oxygen depletion and harmful algal blooms in aquatic ecosystems (4-7). These impacts emphasize the importance to develop sustainable alternatives that boost plant growth and yield while safeguarding the environment and human health.

Biostimulants are products that, when applied to plants or their rhizosphere, stimulate plant nutrition processes independently of nutrient content, aiming to improve nutrient use efficiency, abiotic stress tolerance, crop quality, or nutrient availability in soil (8-10). Among the various types of biostimulants, plant-derived extracts have gained increasing attention for their multifunctional roles in agriculture. For instance, seaweed extracts have been shown to modulate hormonal pathways by upregulating genes involved in auxin, gibberellin, and cytokinin biosynthesis, leading to improved plant growth. In addition, these extracts enhance the plant's defense mechanisms by increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase, thereby contributing to increased resilience under abiotic stress conditions (11).

Plant extracts such as *Moringa oleifera* leaf extract (MLE) have also demonstrated strong biostimulant potential. MLE is particularly rich in phytohormones, including cytokinins, auxins, and gibberellins, which are known to regulate key physiological processes such as cell division, chlorophyll synthesis, and biomass synthesis, supporting enhanced plant growth and productivity (12). Furthermore, a recent study assessing the biostimulant activity of 25 herbal extracts on wheatgrass through seed priming identified four extracts that significantly increased germination rates, seedling length and weight, and overall biomass. These effects were accompanied by elevated levels of total phenolics, flavonoids, and antioxidant enzyme activity, indicating a role in both growth promotion and stress mitigation (13).

A plant with strong potential as biostimulants is the blueberry (*Vaccinium corymbosum*). Known for their many health benefits, including their high antioxidant capacity, blueberries have become increasingly popular and are consumed worldwide (14). Between 2010 and 2023, global blueberry production more than quadrupled from 439 thousand tonnes to nearly 1.78 million tonnes (14, 15). Nonetheless, the cultivation of this crop and their by-products results in the generation of significant amounts of waste, such as blueberry pomace, unripe and damaged berries, and pruning materials (16). As a result, there is growing interest in valorising this waste to extract bioactive compounds suitable for developing biostimulants. While most research focuses on blueberry pomace waste, pruning waste, although less appealing, can be a promising source of biostimulatory substances, as noted by Dorosh et al. (2024) (16). The value of pruning waste has previously been recognized in a variety of crops, including apple, olive and grapevine pruning waste (17-19).

Among the approaches to valorize pruning waste, aqueous extraction from fresh biomass represents a cost-effective method. Blueberry pruning extracts have been found to be rich in polyphenols, particularly flavonoids and procyanidins (20, 21). The effects of polyphenols on plant growth are complex and multidimensional, influenced both by concentration and species-specific responses. At low concentrations, polyphenolic compounds support plant development by modulating phytohormone levels, reducing growth inhibitors like abscisic acid, and enhancing antioxidant enzyme activity to mitigate oxidative stress (22, 23). They also improve nutrient uptake and induce systemic resistance, increasing resilience to biotic stress. However, at higher concentrations, polyphenols may inhibit growth (24). This concentration-dependent behavior is well illustrated by resveratrol, a widely studied polyphenol; at concentrations below 50 μM, it enhances cellular antioxidant defenses and supports mitochondrial network formation, whereas concentrations above 50 μM can disrupt mitochondrial membrane potential and induce apoptosis in cancer cells (25). Besides being concentration-dependent, the effects of polyphenols also vary between plant species. Certain phenolic compounds can be classified as allelochemicals, meaning that different plant species can respond differently to the same polyphenol, by a process called allelopathy (26). Negative allelopathy is a process where plants release secondary metabolites that inhibit the growth of other plants, including their own species. These compounds can affect key physiological processes such as seed germination, cell division, and photosynthesis, serving as a strategy for plants to manage competition both within and between species (27, 28).

Another valorization strategy involves the conversion of pruning residues into biochar through pyrolysis, a thermochemical process conducted at 350–700 °C under low-oxygen conditions (29). The resulting biochar contains essential macro- and micronutrients, including N, P, K, Ca, Mg, Fe, Mn, Cu, and Zn (30). Pyrolysis could also lead to the formation of bioactive molecules such as karrikins, compounds with hormone-like properties that stimulate germination and growth (30). These benefits can be captured in aqueous biochar extracts, which allow for flexible application methods, such as foliar sprays and irrigation, and provide an immediately available nutrient source (31, 32). Previous research has shown that biochar extracts can influence seed germination and seedling growth, but the effects are often inconsistent and highly dependent on the type of biochar, extraction method, concentration, and plant species. For example, Hille et al. (2005) (33) observed that certain pine biochar extracts stimulated seed germination at specific toxin concentrations, while other studies reported no effect of corn stalk and wood biochar extracts on corn seed germination (34, 35). Similarly, the study by Ma et al. (2022) demonstrated that biochar extracts from rice straw, cotton stalk, wheat straw, and Spartina alterniflora had variable effects on corn and rice seed germination and growth, with some extracts enhancing corn seedling growth but inhibiting rice seedling development (36). Because the effects of biochar extracts vary and can sometimes be contradictory depending on the type of biochar and the plant species, more detailed and controlled studies are needed.

Although blueberry extracts have been widely investigated for their pharmacological and nutritional benefits, their potential as plant biostimulants in agriculture remains relatively underexplored. Notably, two studies have demonstrated the positive effects of blueberry fruit extracts on maize growth and metabolism. For instance, Ertani et al. (2016) showed that phenol-rich extracts, including those from blueberry fruit, can stimulate plant metabolic pathways and enhance biomass accumulation in maize (37). Similarly, Ertani et al. (2011) reported that blueberry extracts promote phenolic compound accumulation and improved plant physiological responses such as enhanced biomass production in both roots and leaves, increased chlorophyll content, and improved nitrogen uptake and assimilation (38). These findings provide a strong basis to hypothesize that blueberry extracts may have broader applications across different plant species. However, the effects of extracts made from pruning residues, rather than from fruits, have not been thoroughly studied under controlled conditions, especially during early seedling development and on various crops.

We hypothesize that extracts derived from blueberry residues or biochar stimulate seedling growth. Therefore this study investigates the effects of different blueberry-derived aqueous extracts on early plant development in *Arabidopsis thaliana*, lettuce (*Lactuca sativa*), and radish (*Raphanus sativus*). *Arabidopsis thaliana* is a well-established model organism in plant biology and ecotoxicology, providing a sensitive system for detecting physiological responses to bioactive compounds. Lettuce and radish were selected as representative crops, with lettuce serving as a leafy vegetable and radish as a root vegetable, to assess the potential agronomic relevance of these extracts across crop types.

EXPERIMENTAL PROCEDURES

Extract preparation— Leaves and unripe berries of *Vaccinium corymbosum* (var. Valor) were obtained from Compas Agro (Venlo, The Netherlands) and stored at -80°C. Samples were oven-dried at 40°C for approximately 18 hours. To accelerate the drying process, the berries were cut in quarters. The dried plant material was ground to a fine powder using a Retsch MM 400 mixer mill (Retsch GmbH, Haan, Germany) at 25 Hz for 3 min with a stainless-steel bead (diameter 20 mm). The powder was suspended in Milli-Q water to prepare a1.5% (m/v) solution and shaken at 100 rpm for 24 hours at room temperature. The extracts were filtered through a 0.45 µm filter.

Winter prunings of a different cultivar (var. Duke) were shredded, sieved to ≤1 cm and oven-dried at 60°C to constant weight. Pyrolysis was carried out at 450°C in a pilot-scale reactor. The resulting biochar was ground using the same mixer mill conditions and extracted at 5% (m/v) in Milli-Q water by shaking for 24 h at room temperature (100 rpm).

The biochar extract was filtered through a 0.45 µm filter.

Extract characterization— All aqueous extracts were characterized by measuring pH and electrical conductivity (EC) at 25°C. Elemental analysis was performed on aqueous biochar extract by inductively coupled plasma atomic emission spectroscopy (ICP-AES) and the total polyphenolic content in the plant extracts was quantified using the Folin-Ciocalteu assay using the 96-well microplate method (39).

Arabidopsis thaliana screening assay (SAFETY96)— The SAFETY96 high-throughput screening method was used in a 96-well plate system. Before sowing, *Arabidopsis thaliana* seeds were surface-sterilized with 70% ethanol and rinsed with sterile water. Subsequently, seeds were stratified at 4°C for 2 to 3 nights to promote water uptake and synchronize germination. The seeds were germinated in ¼ Murashige and Skoog (MS) medium supplemented with different concentrations of extracts. For biochar extract (BCE), the concentrations ranged from 0.05% to 2% (m/v); for leaf and unripe berry extracts (LE and UBE), the concentrations ranged from 0.001% to 0.01% (m/v). Each well was filled with ¼ MS medium containing the respective extract concentration and one seed was sown per well. The plates were incubated in a climatic chamber (65% relative humidity, 12 h light/12 h dark photoperiod, 170 µmol m^{-2} s^{-1} photosynthetically active radiation, 22°C/18°C day/night temperatures). From day 4 to day 7, multispectral imaging (MSI) was performed on dark-adapted seedlings (after 15 min in the dark) using the PlantExplorer XS system (PhenoVation, Wageningen, The Netherlands). Data analysis was conducted with the Data Analysis™ software version 5.8.4-64b. The maximum quantum efficiency of photosystem II photochemistry (Fv/Fm) was calculated via following formula:

$$\frac{Fv}{Fm} = \frac{Fm - F0}{Fm}$$

Where F_0 is the minimal fluorescence of the dark-adapted sample, and F_m is the maximal fluorescence measured after a saturating light pulse. On day 7, root length was measured. On day 10, both root length and fresh weight were measured, and samples for chlorophyll quantification were harvested. Six biological replicates, each consisting of six pooled seedlings, were harvested and snap-frozen in liquid nitrogen. The chlorophyll content was extracted using 80% acetone and quantified by spectrophotometry, according to the method described by Wellburn et al. (1984) (40). This method outlines the procedures for determining the concentrations of chlorophyll a, chlorophyll b and carotenoids.

Petri dish bioassay with radish and lettuce— Petri dish assays were used to evaluate both continuous exposure and priming effects of the extracts, following the U.S. EPA lettuce (*Lactuca Sativa*) root elongation assay (EPA Method 850.4200, U.S. EPA, 1996) with minor modifications. The Petri dish bioassay was first conducted on lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), spinach (*Spinacia oleracea*), and common beans (*Phaseolus vulgaris*). Lettuce and radish were selected for further experiments based on their suitability, while spinach and common bean were excluded due to their slower germination rates in Petri dish bioassays.

In the continuous exposure assay, 90 mm Petri dishes lined with Whatman filter paper were wetted with five mL of extract or milli-Q water (control). Twenty seeds were sown on each plate, and three plates were used for each treatment in total. Plates were sealed in plastic bags and incubated for five days under controlled conditions in the dark (65% relative humidity, 22°C/18°C day/night temperatures). Biochar extract was tested at concentrations of 0.5% to 1.5% (m/v) and LE and UBE at 0.0025% to 0.01% (m/v). Root length (n = 8) and fresh weight (n = 10) were measured five days after sowing (DAS).

For priming, 60 seeds were incubated in a two mL extract solution for 24 hours at room temperature before sowing on filter paper moistened with five mL Milli-Q water. The seeds were incubated under the same conditions as in the continuous exposure experiment described above, using the same concentrations. Combination treatments were tested using the priming method at the optimal concentrations: 1% BCE for both plant extracts; 0.0025% LE and 0.0025% UBE for radish; and 0.01% LE and 0.0025% UBE for lettuce. Mixtures in the

ratios 25:75, 50:50 and 75:25 (v/v) were prepared, as well as single extracts.

Pot experiment with radish— Radish (*Raphanus sativus*) was selected for this experiment due to its shorter growth cycle compared to lettuce, making it more suitable given the time constraints of the study. Thirty grams of dried miscanthus fibers (<4 mm)was soaked in 150 mL of deionised water and spread evenly across four compartments of black plastic containers. Four seeds were sown on each side of the compartment, totaling eight seeds per compartment. One week after sowing, the seedlings were thinned to two plants per compartment. During the first week, the pots were watered twice with Hoagland solution, prepared according to the modified formulation described by Smeets et al. (2008) (41), and three times with deionized water until the initial weight was restored.

From ten DAS, each compartment received 10 ml of extract as treatment or Milli-Q water (control) twice a week , for a total of 20 ml per week. On the remaining weekdays, compartments were irrigated twice with Hoagland's nutrient solution and once with deionized water to restore the initial container weight and maintain consistent moisture levels.

Statistics— All statistical analyses were performed using R Studio (version 2024.12.1+563). The normality of the data was assessed using the Shapiro–Wilk test and homogeneity of variances (homoscedasticity) was evaluated using Bartlett's test. When both assumptions were met, the data were analyzed using a one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) post hoc test for pairwise comparisons. If either assumption was violated, the data were subjected to appropriate transformations (square root, logarithmic, exponential or inverse) until the assumptions were met. If transformation did not correct the violations of the assumptions, a non-parametric Kruskal–Wallis test was applied, followed by Wilcoxon rank-sum tests for post hoc comparisons. Statistical significance was defined as $p < 0.05$.

RESULTS

Characterization of the extracts – The physicochemical characteristics of the extracts were determined (Table 1). BCE showed a near-neutral pH, while both plant-based extracts (UBE and LE) were more acidic, with UBE being the most acidic. UBE exhibited the highest EC among the extracts, despite being more diluted than BCE (1.5% vs 5% m/v, respectively), indicating a relatively high ion content. LE had the lowest measured EC in this comparison, though at equal concentrations it would likely exceed that of BCE.
While a higher ion concentration was observed in UBE, LE contained nearly four times more polyphenols.

The elemental composition of biochar and its aqueous extract was identified (Table 2)**.** The solid biochar contained significantly higher levels of all measured elements compared to the extract. However, the leached fraction in the extract reflects the bioavailable portion that is directly accessible to plants.
Among the measured elements, potassium (K) and phosphorus (P) were the most effectively leached macronutrients into the aqueous extract. Despite magnesium (Mg) being the most abundant element in the solid biochar, only a minimal fraction leached into the water phase. Most micronutrients, including zinc (Zn), copper (Cu), and barium (Ba), were not detected in the extract, suggesting that their concentrations were below the detection limit and their mobility extremely low under the tested conditions.

Effect of biochar and plant extracts on A. thaliana seedling growth parameters— To assess the effect of biochar extract (BCE) on plant growth, two biometrical parameters, i.e. root length (measured 7 and 10 DAS) and fresh weight (measured 10 DAS) were determined in *A. thaliana* seedlings grown in 96-well plates. Exposing *A. thaliana* seedlings to BCE at various concentrations influenced root development and biomass of the seedlings (Fig. 1A-C). Seven DAS, the BCE concentrations of 1% and 1.5% (m/v) led to significantly increased root lengths compared to the reference condition. While 0.5% (m/v) also tended to increase root growth, the difference was no longer statistically significant. Similarly, 2% (m/v) BCE showed no significant effect on root length. Ten days after sowing (10 DAS) the concentration-dependent increase in root elongation in response to BCE was still observed, but with a shift to 0.5% BCE showing

the highest and only significant increase in root length as compared to the control.

At 10 days after sowing (DAS), seedlings treated with 0.5% and 1.5% BCE showed the

Table 1 – Physicochemical characteristics of aqueous extracts derived from blueberry pruning biochar (5% m/v), unripe berries, and leaves (1.5% m/v). Data are presented as mean (± SE). (Abbreviations: BCE = biochar extract, UBE = unripe berry extract, LE = leaf extract, TPC = total polyphenol content, EC = electric conductivity, GAE = gallic acid equivalents, DW = dry weight)

Extract type (concentration)	pH	EC (µS/cm)	TPC (µmol GAE/g DW)
BCE (5% m/v)	7.68 (± 0.015)	656 (± 6.66)	/
UBE (1.5% m/v)	2.72 (± 0.024)	1172.33 (± 7.51)	55.51 (± 2.06)
LE (1.5% m/v)	3.22 (± 0.012)	581.33 (± 7.09)	205.81 (± 12.46)

Table 2 – Elemental composition analysis of blueberry pruning biochar and its aqueous extract (5% m/v). Data are presented as mean (± SE). (Abbreviations: DW = dry weight, PTEs = potential toxic elements)

Element	Blueberry pruning biochar	Aqueous blueberry pruning biochar extract
Macronutrients	**(mg/kg DW)**	**(mg/L)**
Ca	6684 (± 140.789)	11.76 (± 0.105)
K	7195 (± 49.285)	151.4 (± 1.3)
Mg	150601.5 (± 214.025)	11.80 (± 0.075)
P	4410 (± 32.44)	50.45 (± 0.2)
S	1462 (± 27.188)	30.08 (± 1.595)
Micronutrients		
Cu	44.99 (± 1.434)	< 0.25
Fe	167.0 (± 4.003)	0.463 (± 0.0075)
Mn	217.7 (± 2.588)	0.725 (± 0.005)
Na	957.8 (± 28.285)	8.908 (± 0.0125)
Ni	< 10.0	< 0.25
Zn	57.51 (± 1.131)	< 0.25
PTEs for plants		
Cd	< 2.0	< 0.05
Cr	14.25 (± 0.614)	< 0.25
Hg	< 20.0	< 0.5
Pb	< 10.0	< 0.25
Other elements		
Ag	< 10.0	< 0.25
Al	227.9 (± 30.276)	< 0.25
Ba	26.97 (± 0.5143)	< 0.25
Co	< 10.0	< 0.25
Li	170.0 (± 24.598)	< 0.025
Sr	28.30 (± 0.413)	< 0.25

highest average fresh weight per seedling, both significantly greater than the control. However, the fresh weight at 1% BCE did not differ significantly from the control and was characterized by higher variability (standard error). Similarly to the root lengths, the stimulating effect of BCE on seedling growth disappeared after application of 2% BCE.

A similar experiment was conducted using blueberry-derived extracts from unripe berries (UBE) and leaves (LE) using a concentration range from 0.001% to 0.01%. Contrary to BCE, these plant extracts had no beneficial effect and even showed a negative effect at the highest concentration (0.01%) compared to the control seedlings (Fig. S1).

imaging (MSI) from four to seven DAS and by analyzing pigment concentrations at 10 DAS.

Chlorophyll *a* levels were similar between the control, 0.5% BCE and 1% BCE, but dropped significantly from 1.5% BCE onwards. A similar pattern was observed for carotenoids and chlorophyll *b* levels (Fig. 2B). For the latter, only the difference between the control (95.4 ± 7.8 µg g^{-1} FW) and 1.5% BCE-exposed seedlings (75.0 ± 3.8 µg g^{-1} FW) was statistically significant, however, the effect of 2% BCE was borderline not significant (76.9 ± 2.1 µg g^{-1} FW, $p = 0.052$). Multispectral imaging (MSI) from four to seven DAS was used to investigate the photosynthetic performance of seedlings exposed to the different BCE concentrations.

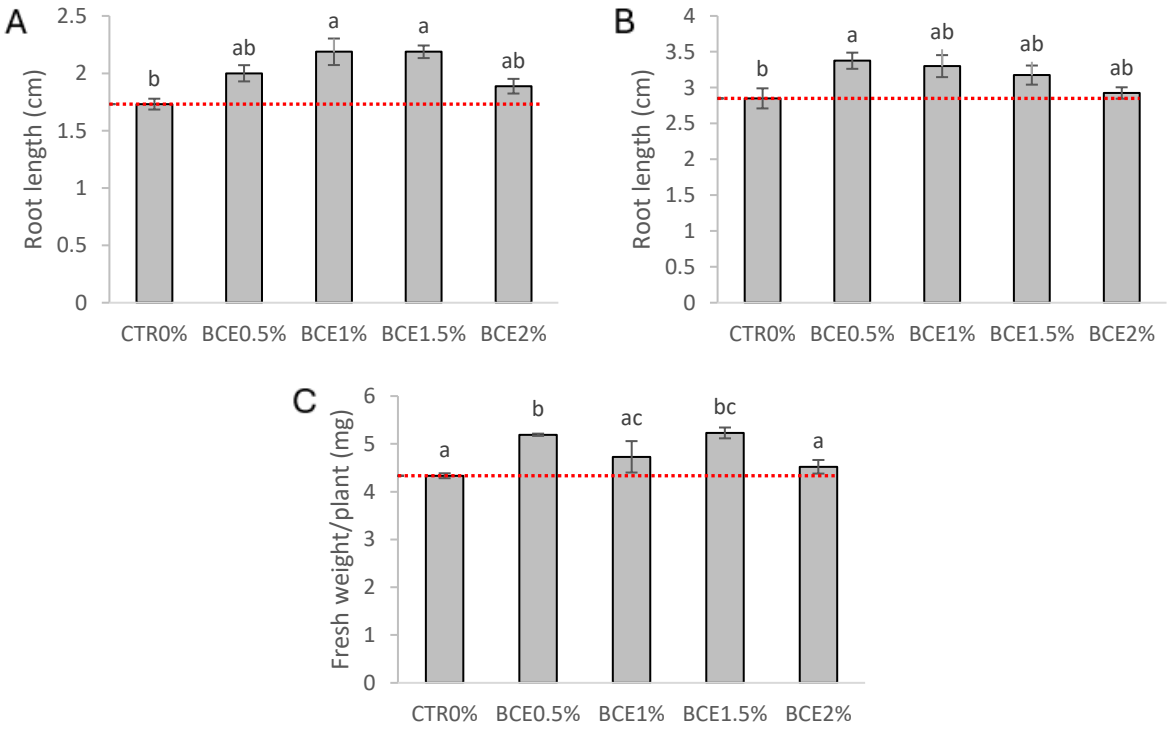

Fig. 1. Effects of BCE application on growth parameters, in *A. thaliana* seedlings using a 96-well screening system. Root length (n = 8) at 7 DAS (A) and 10 days (B), and fresh weight (n = 10) at 10 DAS (C) in response to increasing concentrations of BCE (0–2% m/v). Data are presented as mean ± SE. Different letters indicate significant differences between treatments (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test for root length; Kruskal-Wallis, followed by a Wilcoxon rank-sum test for FW, $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, BCE = biochar extract)

Influence of biochar and plant extracts on photosynthesis of A. thaliana seedlings— The effect of BCE on the photosynthesis of *A. thaliana* seedlings was assessed by measuring photosynthetic parameters using multispectral

This was assessed by analyzing the photosynthetic efficiency of photosystem II (Fv/Fm) in dark-adapted *A. thaliana* seedlings. No differences in Fv/Fm values between the different BCE concentrations and the control were observed on day 4. However on day 5,

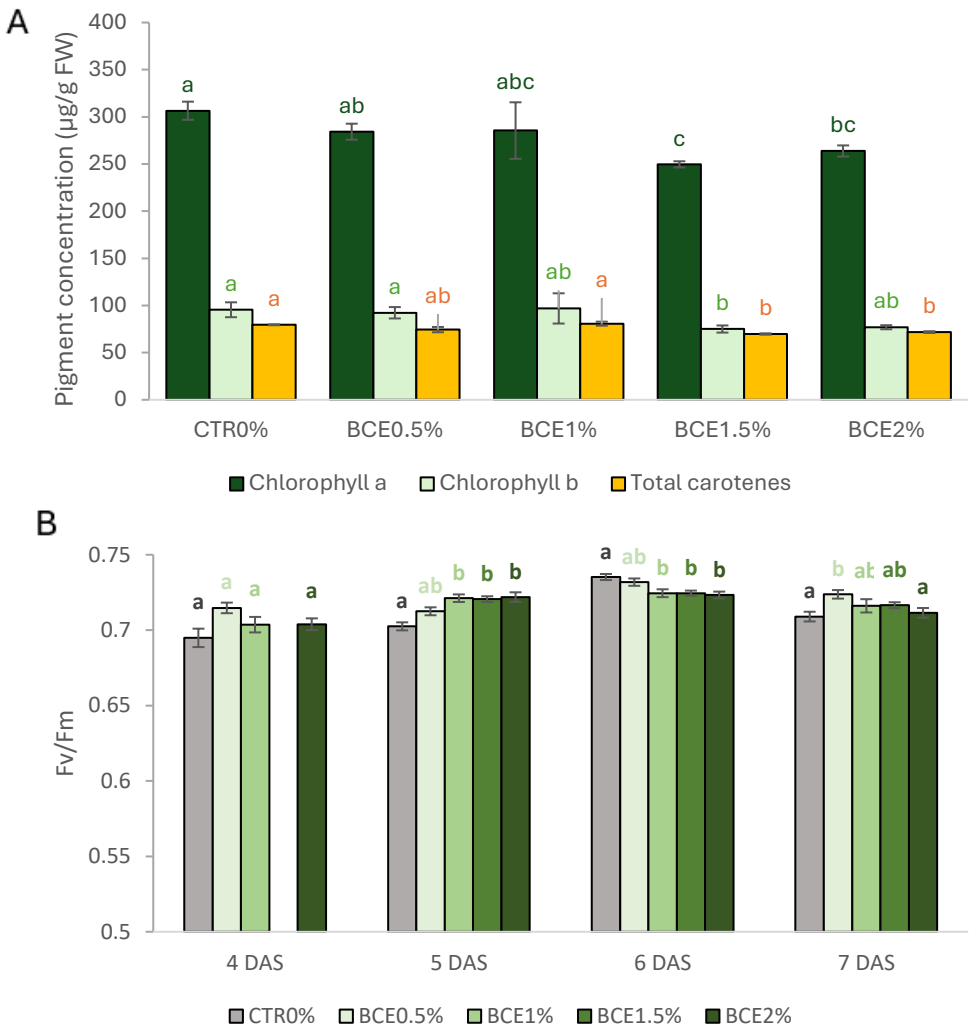

Fig. 2. Effects of BCE application on pigment concentration (A) and photosynthetic efficiency (B) in A. thaliana seedlings using the SAFETY96 screening system. (A) Chlorophyll a, chlorophyll b, and total carotene concentrations (µg g^{-1} FW) in *A. thaliana* seedlings were measured at 10 DAS (n = 10); (B) Changes in the maximum quantum yield of PSII (Fv/Fm) in *A. thaliana* seedlings were measured from 4 to 7 DAS using multispectral imaging (n ≈ 32 for 4 DAS, n > 59 for 5 till 7 DAS). The missing Fv/Fm data for the 1.5% BCE treatment at 4 DAS is due to a measurement error that resulted in data loss. Data are presented as mean ± SE. Different letters indicate significant differences between treatments at each timepoint (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test for Fv/Fm; Kruskal-Wallis, followed by a Wilcoxon rank-sum test for chlorophyll a, b and total carotenoids, $p < 0.05$). (Abbreviations: CTR = control, BCE = biochar extract, FW = fresh weight, PSII = photosystem II, Fv = variable fluorescence, Fm = maximum fluorescence)

seedlings treated with 1%, 1.5%, and 2% BCE had significantly higher Fv/Fm values compared to control seedlings. On day 6, the reverse pattern was observed on the efficiency of photosystem II (Fv/Fm), except for 0.5% BCE as compared to the control seedlings. Seven DAS, 0.5% BCE treatment was the only concentration that significantly increased the Fv/Fm values in comparison to the control seedlings.

In a similar experiment using UBE and LE using a concentration range from 0.001% to 0.01%, Fv/Fm values remained unchanged, except for a higher value in 0.1% LE-treated seedlings compared to the control at six DAS. Pigment levels in UBE-treated seedlings were similar to the control, while LE treatment significantly increased chlorophyll *a* at all concentrations and chlorophyll *b* at every dose except 0.01%. Carotenoid levels remained

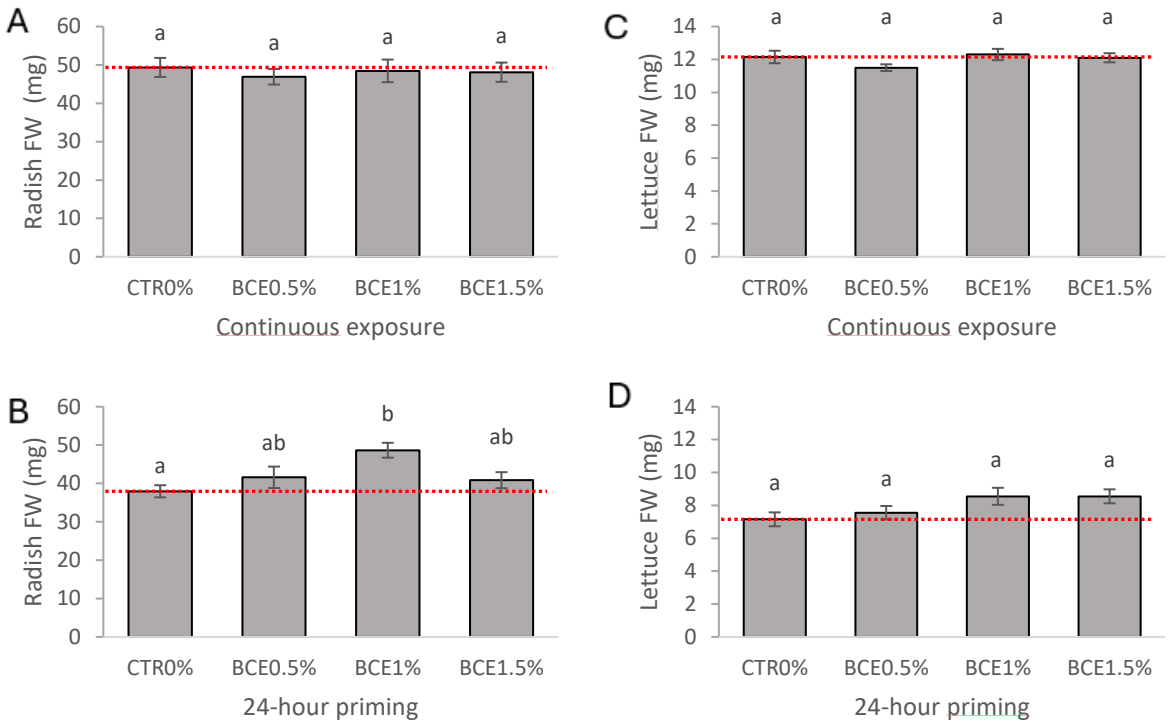

Fig. 3 Effect of BCE application (0.5%, 1%, and 1.5% m/v) on the fresh weight of radish (A, B) and lettuce (C, D) seedlings under continuous exposure (A, C) and 24-hour priming (B, D). Bars represent mean ± SE. Different letters indicate statistically significant differences between treatments (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test , $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, BCE = biochar extract)

unchanged (Fig. S2).*Fresh weight response to blueberry biochar extract (BCE) in radish and lettuce* – In the next phase of the research, the effects of BCE were assessed on crops. Specifically, the fresh weight of radish and lettuce seedlings grown in Petri dishes varied depending on the exposure method (continuous versus priming) and BCE concentration.

In radish seedlings under continuous exposure, no significant differences in fresh weight were observed across treatments (Fig. 3A). All treatments maintained similar fresh weights, ranging from 46 (BCE 0.05%) to 49 mg (CTR

0%), indicating that continuous exposure to BCE did not enhance radish growth compared to the control. In contrast, primed radish seeds showed a concentration-dependent trend with a maximum at 1% BCE which significantly increased fresh weight compared to the control (Fig. 3B). Biochar extract at 0.5% and 1.5% showed intermediate values but these were not significantly different from the control.

In lettuce seedlings, continuous exposure to BCE also showed no significant differences between treatments (Fig. 3C). All treatments, including the control, averaged fresh weights around 12 mg, indicating that lettuce growth was not notably affected by BCE during continuous exposure.

Lettuce priming with BCE (Fig. 3D) did not produce statistically significant differences either in fresh weight across treatments. Nonetheless, a subtle upward trend was observed, with BCE 1% and 1.5% producing slightly higher mean fresh weights compared to the control.

Fresh weight response to plant extracts from unripe berries (UBE) and leaves (LE) in radish and lettuce— The effects of UBE and LE at different concentrations (0.0025%, 0.005%, and 0.01%) on the fresh weight of radish and lettuce seedlings grown in Petri dishes varied according to the exposure method (continuous vs. priming) and plant species.

Under continuous exposure, radish seedlings showed a subtle dose-dependent trend with a maximum at 0.005% for LE and 0.0025% for UBE (Fig. 4A). However, none of the

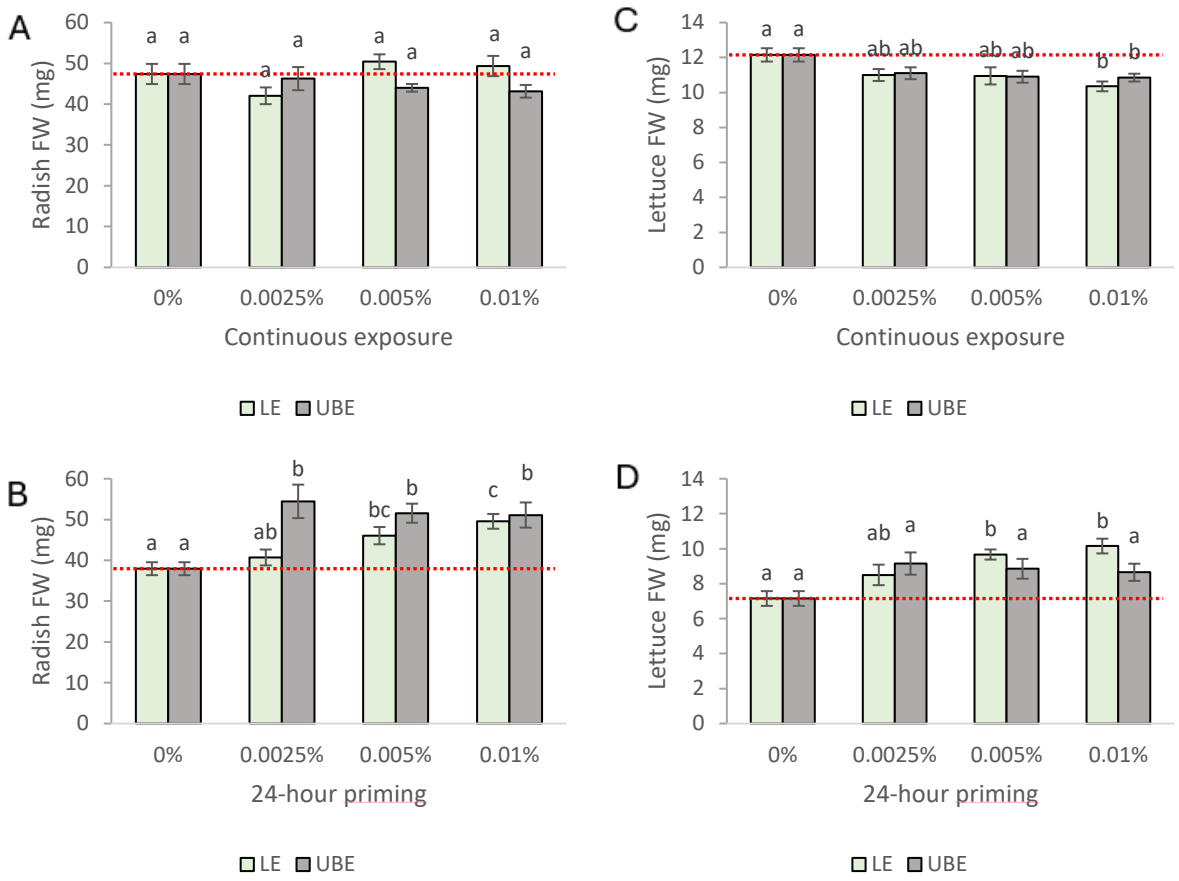

Fig. 4. Effect of plant extract (UBE and LE) application (0.0025%, 0.005%, and 0.01% m/v) on the fresh weight of radish (A, B) and lettuce (C, D) seedlings under continuous exposure (A, C) and 24-hour priming (B, D), compared to the control. Bars represent mean ± SE. Different letters indicate significant differences between the control and concentrations of the same extract type (UBE or LE) (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test , $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, UBE = unripe berry extract, LE = leaf extract)

concentrations tested showed a significant difference in fresh weight compared to the control. In contrast, radish seedlings subjected to 24 h priming showed a more pronounced response to the plant extracts (Fig. 4B). Leaf extract showed an increasing trend over the concentration range with an optimum at the highest concentration (0.01%), with a significantly higher fresh weight than the control seedlings. The optimum for UBE was reached at the lowest concentration (0.0025%), after which a slight decrease in fresh weight can be observed, although all the concentrations tested had a significantly higher fresh weight than the control seedlings.

For lettuce under continuous exposure (Fig. 4C), a modest decrease in fresh weight was observed over the concentration range for both LE and UBE, with significantly lower fresh weight at the highest concentration (0.01%) compared to the control. Interestingly, a similar trend was observed in *A. thaliana* seedlings exposed to the same extract concentrations in a 96-well system (Fig. S1).

For lettuce priming, a similar positive trend as for radish was observed, although less pronounced (Fig. 4D). For UBE-treated seedlings, the fresh weight increased significantly starting from 0.005% ($p < 0.05$), reaching 10 mg compared to 7 mg in the control. However, differences between UBE-treated seedlings and the control did not differ significantly.

Root length was measured in both Petri dish experiments with BCE and the plant extracts (UBE and LE) (Fig. S3, S4). No significant differences were observed in radish or lettuce seedlings treated with BCE

(0.5–1.5%), under either continuous exposure or 24-hour priming. For continuous exposure to UBE and LE (0.0025–0.01%), no significant effects were found. However, in 24-hour priming, UBE significantly increased root length in radish (0.0025%) and lettuce (0.0025% and 0.01%). Leaf extract increased radish root length at 0.005% and 0.01%, but had no significant effect on lettuce, although a positive trend was observed.

The effect of combinations made from biochar and plant extracts on radish early development — To assess the impact of the combination of biochar- and plant extracts on early radish seedling development, root length and fresh weight were measured after 5 days of growth in Petri dishes under various treatments (Fig. 5). The treatments included a control, BCE 1%, LE 0.01%, and UBE 0.0025%, as well as mixtures of BCE with either LE or UBE at varying volumetric ratios (25:75, 50:50, and 75:25). These concentrations were selected based on the optimal levels identified in the previous Petri dish experiments for each extract type. Overall, most treatments appeared to increase root length compared to the control, with the exception of the LE treatment alone, which showed no improvement (Fig. 5A).

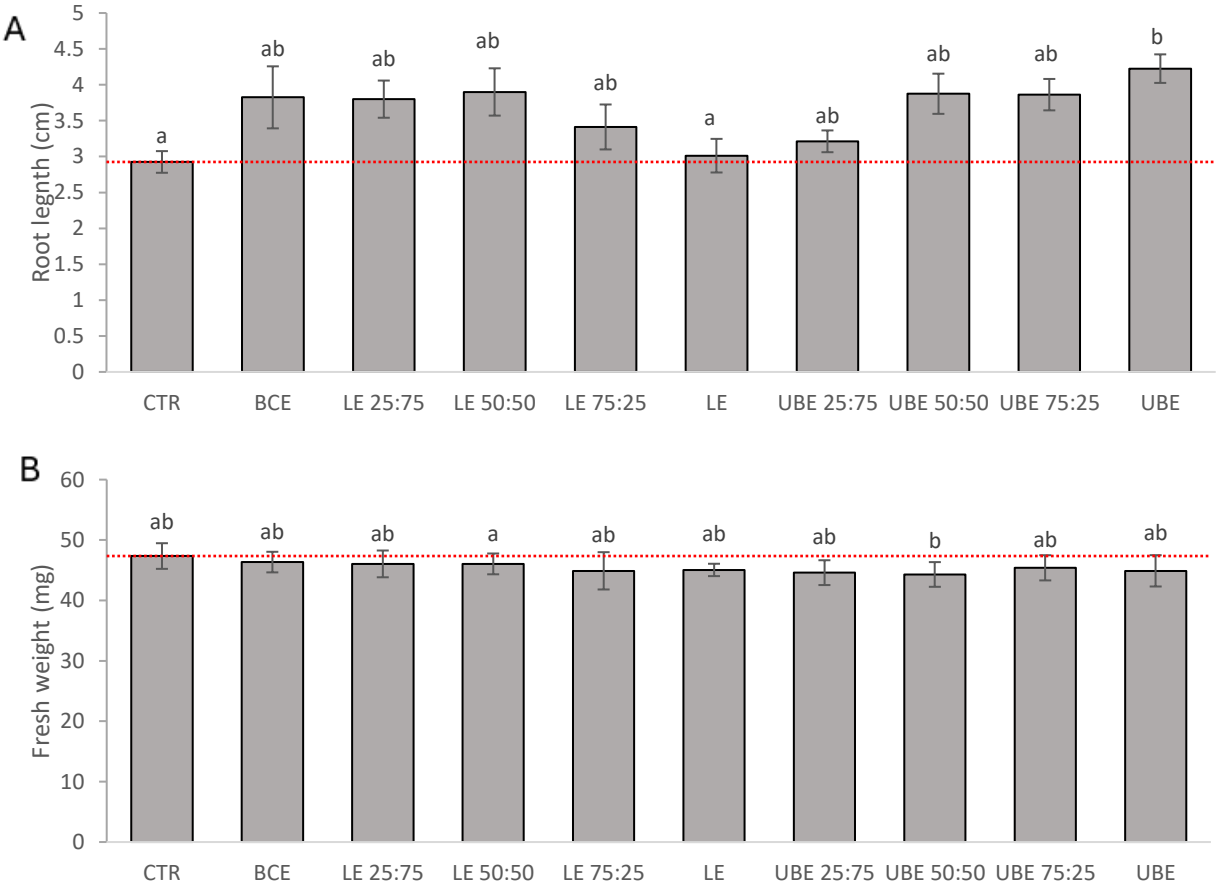

Fig. 5. Effect of combinations made from biochar and plant extracts on (A) root length and (B) fresh weight of radish seedlings at 5 DAS. Treatments include control, BCE 1% (m/v), LE 0.01% and UBE 0.0025%, as well as mixtures of LE or UBE with BCE in volumetric ratios of 25:75, 50:50, and 75:25, where the first number represents the plant extract and the second number BCE. Bars represent means ± SE (root length; n = 8, fresh weight; n = 10). Different letters indicate statistically significant differences between treatments (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test, $p < 0.05$). The red dashed line represents the mean value of the control group in each panel. (Abbreviations: DAS = days after sowing, LE = leaf extract, UBE = unripe berry extract, BCE = biochar extract, CTR = control)

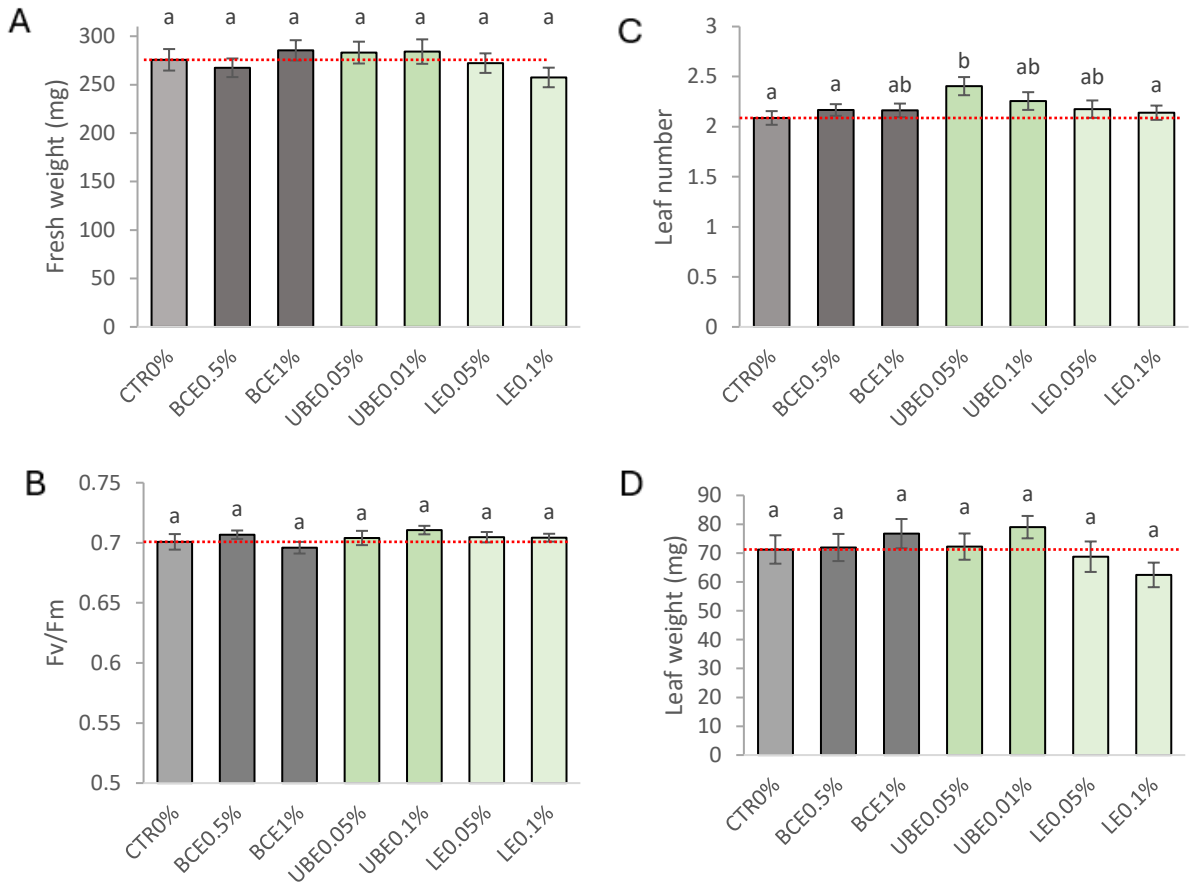

Fig. 6. Effects of blueberry-derived extracts on radish morphological and physiological parameters at 32 DAS. (A) Above-ground fresh weight; (B) maximum quantum yield of PSII (Fv/Fm); (C) total leaf number and (D) total leaf weight per plant. Treatments included control, BCE0.5%, BCE1%, UBE0.05%, UBE0.1%, LE0.05% and LE0.1%. Data are presented as mean ± SE (Fresh weight; n = 30, Fv/Fm; n = 28, leaf number; n > 40, leaf weight; n = 16). Different letters indicate significant differences between treatments (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test, $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, UBE = unripe berry extract, LE = leaf extract, DAS = days after sowing)

Combinations of BCE and LE consistently outperformed LE on its own, particularly as the proportion of BCE increased. In contrast, the opposite trend was observed for combinations of BCE and UBE, where higher ratios of UBE were associated with greater root growth. Notably, the UBE-alone treatment resulted in the longest roots and was the only treatment to show a statistically significant increase in root length compared to the control. All other treatments, while showing slight improvements, did not differ significantly from the control. Differences in fresh weight among treatments were less pronounced. The control seedlings exhibited the highest mean fresh weight, while all other treatments showed slightly lower values. However, none of these differences were statistically significant compared to the control (Fig. 5B).

Effect of blueberry-derived extracts on radish plants' morphological and physiological parameters— Whereas previous experiments focused on seedlings, this experiment aimed to investigate the long-term effects of blueberry-derived extracts on plant growth and photosynthetic performance in radish. The above-ground fresh weight of radish plants did not differ significantly between treatment groups and the control (Fig. 6A). Photosystem II efficiency (Fv/Fm), measured on day 32 using MSI, remained consistent across all treatments (Fig. 6B), with no significant differences observed. All groups exhibited values around 0.70. Among the parameters measured, leaf number was the only trait that showed a significant response to treatment (Fig. 6C). Plants treated with UBE0.05% had a significantly higher leaf number than the

control group, but this did not correspond to an increase in total leaf weight per plant (Fig. 6D). For all other treatments, both leaf number and total leaf weight remained comparable to the control.

DISCUSSION

The results of the first part of the research demonstrate that BCE can act as an effective biostimulant, enhancing *Arabidopsis thaliana* seedling growth in a concentration-dependent manner. Specifically, BCE significantly increased both root length and fresh weight at moderate concentrations (0.5%–1.5% m/v), while no further benefits were observed at the highest tested concentration (2% m/v). Although no literature is available about similar experiments using biochar extracts, a comparable dose-response outcome was reported in the study by Kunnen et al. (2024), exposing *Arabidopsis thaliana* to wheat-derived biochar in hydroponic systems. Low doses (0.25 and 0.5 mg/mL) significantly enhanced root length and fresh weight, whereas the highest concentration (1 mg/mL) did not yield further growth benefits (42). This suggests a concentration-dependent response, where moderate biochar levels promote growth, but higher concentrations may not provide additional advantages. The observed dose-dependent pattern is likely attributable to the complex chemical composition of BCE, which includes dissolved organic carbon (DOC), humic substances (HS), organic nitrogen (ON), phenolics, and a wide variety of essential minerals such as Ca, K, Mg, P, Fe, Mn, Cu, Zn, and B (31). These compounds are known to influence plant physiology through multiple mechanisms. For example, HS improve nutrient uptake by stimulating plasma membrane H^+-ATPase activity and modulating the expression of ATP-binding cassette (ABC) transporters important for auxin transport (43, 44). These actions can activate auxin-responsive genes in *Arabidopsis thaliana*, thereby promoting root elongation and biomass accumulation (45). Moreover, micronutrients such as Fe and Zn play essential roles in chlorophyll synthesis and enzyme activation, contributing to improved growth (46). Furthermore, ON, particularly in the form of amino acids, can be directly absorbed by plants, providing a more carbon-efficient nitrogen source, improving nitrogen use efficiency (NUE), and promoting root growth (47-49). Additionally, biochar can contain a range of phenolic compounds formed during the pyrolysis process. While certain phenolics such as 2,4-di-tert-butylphenol and benzoic acid have been reported to demonstrate phytotoxic effects (50, 51), others like karrikinolide (KAR1) are known to stimulate seed germination and early seedling development in select species (52). Essential mineral elements, including macronutrients (N, P, K, Ca, Mg, S) and micronutrients (Fe, Mn, Cu, Zn, B, Cl, Mo, Ni), are critical for plant metabolism, supporting enzymatic activities, maintaining structural integrity, and regulating key physiological functions (53, 54). However, while these elements are crucial in appropriate concentrations, their excess can become toxic. For example, excessive accumulation of certain metals can impair nutrient balance and cause oxidative damage within plant tissues. Iron (Fe) toxicity can increase reactive oxygen species (ROS), damaging lipids, proteins, and DNA, and impairing plant function (55). Similarly, excess levels of metals like Mn, Cu, Zn, Se, Co, Ni, and Cd disrupt cellular homeostasis, inhibit growth, and promote oxidative stress through lipid peroxidation (56). Therefore, the absence of a growth-promoting effect at 2% BCE suggests that this concentration may have exceeded the optimal threshold, resulting in micronutrient toxicity and oxidative stress.

In the second part of this study, the biostimulant potential of the aqueous extracts was evaluated on the crop species lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*), representing leafy and root vegetables, respectively. Both seed priming and continuous exposure approaches were assessed for their effects on seedling growth. While the *Arabidopsis thaliana* screening assay showed a positive effect on root length and fresh weight, the same concentrations of BCE had no noticeable effect on these parameters in lettuce and radish petri dish bioassays. Comparable findings were reported by Oh et al. (2012), who observed that residual wood biochar extracts slightly inhibited lettuce germination and root growth at a concentration of 3%, while lower concentrations (1%) had neutral to positive effects (57). As demonstrated in the A. *thaliana* screening assay, the growth-promoting effect of biochar extract is highly concentration-dependent, also supported by the literature. These findings suggest several possible explanations for the absence of a growth-promoting effect in lettuce and radish. One

possibility is that the higher concentrations of biochar extract used in the experiment exceeded the optimal threshold, resulting in mild phytotoxic effects due to excessive nutrient levels, as supported by both our A. *thaliana* data and previous studies (42, 57, 58). Alternatively, it is also possible that the concentrations applied were insufficient to trigger a physiological response in these particular crops.

The petri dish bioassay was also conducted with LE and UBE. And similar to biochar extracts, aqueous extracts from blueberry plant residues also contain valuable bioactive compounds, particularly polyphenols such as anthocyanins, known for their strong antioxidant activity (16, 59). Blueberry leaves generally contain higher levels of polyphenols and exhibit stronger antioxidant activity than berry extracts (60, 61), as also reflected in the higher polyphenol content for LE compared to UBE found in this study. Berry extracts, though lower in total polyphenols, are particularly rich in anthocyanins. These compounds, primarily located in the blueberry skin, can make up 35–74% of the total phenolic content in highbush varieties (60, 62). These compounds play diverse protective roles in plants, contributing to both abiotic and biotic stress tolerance. For example, anthocyanins act as antioxidants, scavenging free radicals and reactive oxygen species, and they reduce photoinhibition and photobleaching (63, 64).

Interestingly, neither of the plant extracts (LE and UBE) showed a growth-promoting effect on lettuce and radish seedlings. In fact, at the highest tested concentration (0.01% m/v), both extracts significantly reduced fresh weight in lettuce. Literature shows that the effects of plant extracts, similar to biochar extracts, are dose-dependent and can lead to growth promotion or inhibition depending on the concentration used (65, 66). For example, Wang et al. (2022) observed that aqueous extracts from *Artemisia frigida*, *Stellera chamaejasme*, and *Achnatherum splendens* inhibited lettuce germination and root growth at 5% (m/v), while lower concentrations (0.5% m/v) elicited hormetic responses, enhancing shoot development and biomass (67). Similarly, Możdżeń et al. (2021) demonstrated that high concentrations (5% m/v) of *Lapsana communis* shoot extract significantly impaired radish germination, reduced biomass, and caused membrane damage, while lower doses (1% m/v) resulted in milder or slightly stimulatory effects on seedling parameters (68). One possible explanation is that the concentrations used in this experiment were too high for the continuous exposure method, potentially leading to toxicity rather than beneficial effects on lettuce and radish seedling growth. Continuous exposure, especially at higher concentrations, may introduce allelopathic stress. It is known that specific phenolic compounds and flavonoids, although beneficial in small quantities, can interfere with root architecture and hormonal balance at elevated levels (69, 70).

In contrast, seed priming with the same concentrations of these extracts (LE, UBE and BCE) yielded positive results on seedling growth, particularly in enhancing fresh weight and root length. Seed priming is a cost-effective technique that enhances seed performance through controlled hydration, allowing pre-germinative metabolic processes to initiate before root emergence (71). This approach not only enhances seedling strength and uniformity but may also improve tolerance to abiotic and biotic stresses by activating stress-responsive genes and inducing epigenetic modifications such as DNA methylation and histone changes (72, 73). The positive effects observed in this study could be linked to such physiological and molecular changes initiated during priming. Supporting this, several studies have demonstrated the value of seed priming in enhancing stress resistance and plant performance (71, 74-78). Saxena et al. (2021) emphasized its role in promoting faster germination and improving seedling vigor (71). Botanical extracts like *Ascophyllum nodosum* (seaweed) and *Moringa oleifera* (leaf) have been shown to enhance crop tolerance to stress when used in priming (75). Priming can also upregulate antioxidant enzymes and repair membrane damage, further boosting stress resilience (76). Phytohormone-based priming has been shown to improve plant stress tolerance (77), and enhanced radish germination has also been reported following priming with tomato leaf extract (78). These findings suggest a valuable direction for future research: exploring whether seed priming with UBE, LE, and BCE can strengthen plant tolerance to abiotic stress.

Overall, the contrasting outcomes between continuous exposure and seed priming highlight the critical role of extract concentration and exposure method.

In the final phase of this study, a short-term pot experiment was conducted using radish and 100% miscanthus as substrate. Due to time limitations, the trial lasted only 32 days and served as a preliminary exploration. No significant effects were observed, suggesting either suboptimal concentrations, nutrient override from the applied Hoagland solution, or limitations related to the substrate itself.

Although miscanthus is a sustainable, low-cost substrate with potential in horticulture (79), previous studies report negative effects at high concentrations. For instance, Altland et al. (2011) found that 80% miscanthus reduced root coverage in *Hibiscus moscheutos* (80), while Pancerz et al. (2023) reported inhibited growth in *Sedum spectabile* and *Hydrangea arborescens* under similar conditions (81). These effects are likely due to the low water-holding capacity, nutrient leaching, high pH, or the high C:N ratio of raw miscanthus, which can lead to nitrogen immobilization. In this process, soil microbes consume available nitrogen from the soil to decompose carbon-rich organic material, such as miscanthus (82). Blending miscanthus with other substrates (e.g., peat moss) (81), or chemically modifying it (e.g., acidification) could mitigate these issues (82). Furthermore, foliar application may bypass substrate-related constraints, offering a promising alternative for delivering biostimulants directly to plant tissues (83). Seed priming, which enhanced seedling growth in this study, could be further explored as an exposure method, either on its own or in combination with foliar application or irrigation.

CONCLUSION

While the blueberry-derived biochar extract (BCE) significantly promoted *Arabidopsis thaliana* growth at optimal concentrations, higher doses reduced growth, likely due to micronutrient toxicity and oxidative stress. Similarly, blueberry leaf (LE) and unripe berry (UBE) extracts improved seedling fresh weight when applied via seed priming but showed neutral or negative effects under continuous exposure, reinforcing the importance of application method.

These results align with literature emphasizing the dose-dependent nature of plant- and biochar extracts, where phenolic compounds and nutrients may promote or inhibit growth depending on concentration. Future research should focus on further characterizing the polyphenolic profiles of these extracts using LC-MS, as current assays may be confounded by other antioxidant compounds (e.g., vitamin C, amino acids). In addition, the ongoing nutrient analysis for LE and UBE will help clarify whether the observed effects are due to bioactive compounds or simply nutrient input. Including treatments with the main nutrients alone could help distinguish between biostimulant and fertilizing effects.

Overall, this research lays a foundation for developing cost-effective biostimulants that promote circular economy. Further optimization of extract concentrations, application methods, and substrate blends is needed to fully harness the growth-promoting potential of BCE, LE, and UBE across different crop systems.

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SUPPLEMENTARY

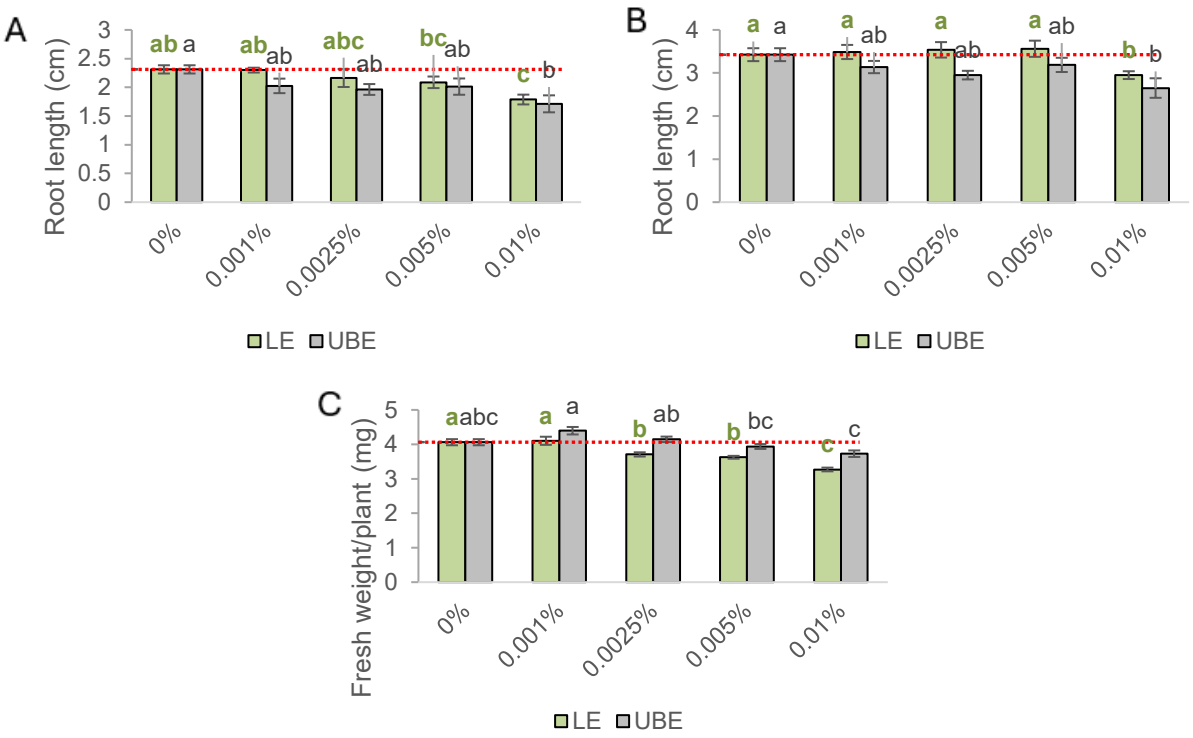

Fig. S1. Effects of LE and UBE application on growth parameters in *A. thaliana* seedlings using a 96-well growth system. Root length (n = 8) at 7 DAS (A) and 10 DAS(B), and fresh weight (n = 10) at 10 DAS (C) in response to increasing concentrations of the extract (0–0.01% m/v). Data are presented as mean ± SE. Different letters indicate significant differences between treatments for both extracts (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test for all variables except LE root length at 7 and 10 DAS, for which Kruskal–Wallis, followed by a Wilcoxon rank-sum test were used; $p < 0.05$). The red dashed line represents the mean value of the control group (0%) in each panel. (Abbreviations: CTR = control, LE = leaf extract, UBE = unripe berry extract)

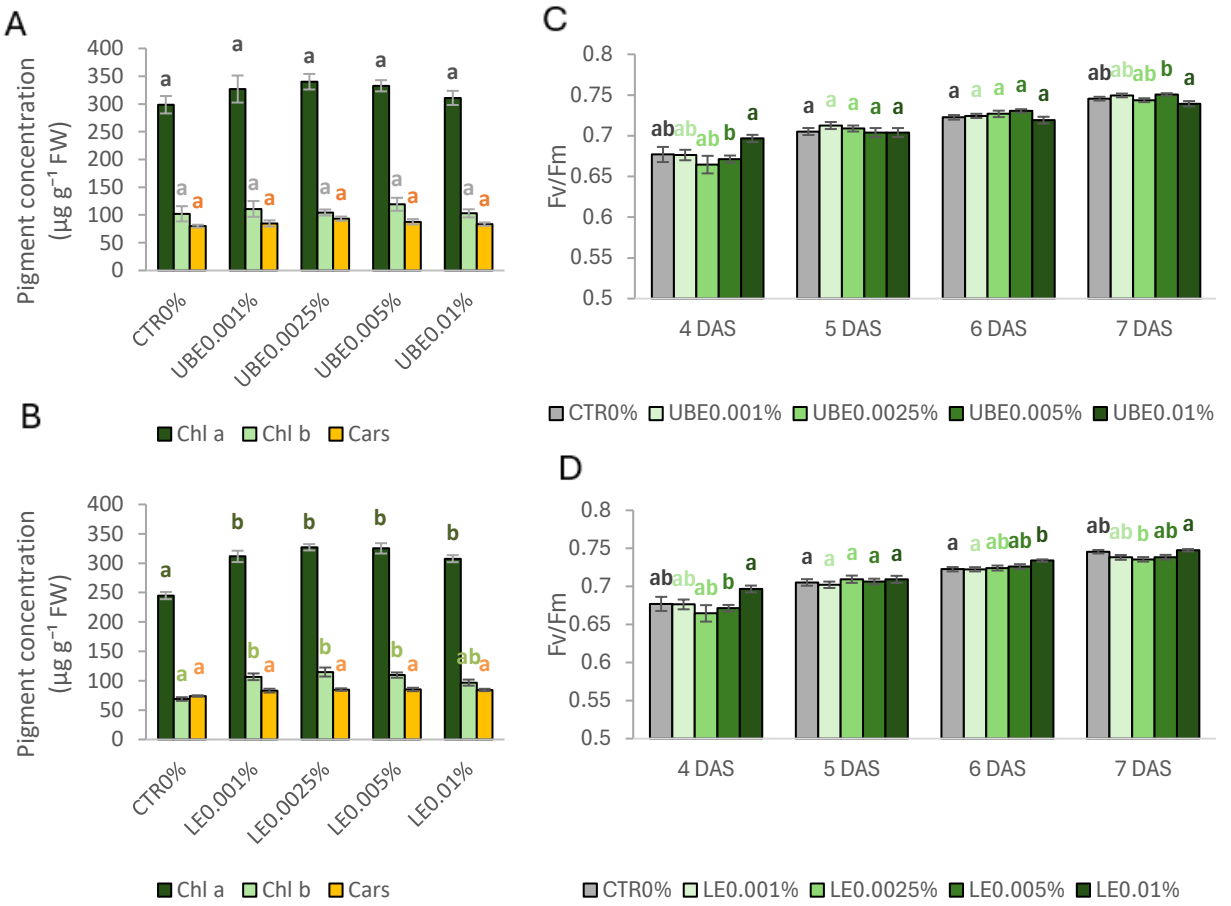

Fig. S2. Effects of LE (leaf extract) and UBE (unripe berry extract) application on pigment concentration (A & B) and photosynthetic efficiency (C & D) in *A. thaliana* seedlings using the SAFETY96 screening system. Chlorophyll *a*, chlorophyll *b*, and total carotene concentrations (µg g^{-1} FW) in *A. thaliana* seedlings exposed to UBE (A) and LE (B) were measured at 10 DAS (n = 10). Changes in the maximum quantum yield of PSII (Fv/Fm) in *A.* thaliana seedlings exposed to UBE (C) and LE (D) were measured from 4 to 7 DAS using multispectral imaging (n ≈ 32 for 4 DAS; n > 59 for 5 till 7 DAS,). Data are presented as mean ± SE. Different letters indicate significant differences between treatments at each timepoint (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test for all variables except chlorophyll *a* (UBE), for which Kruskal–Wallis, followed by a Wilcoxon rank-sum test were used; $p < 0.05$). (Abbreviations: CTR = control, LE = leaf extract, UBE = unripe berry extract, FW = fresh weight, Fv = variable fluorescence, Fm = maximum fluorescence, Chl a = chlorophyll a, Chl b = chlorophyll b, Cars = carotenes)

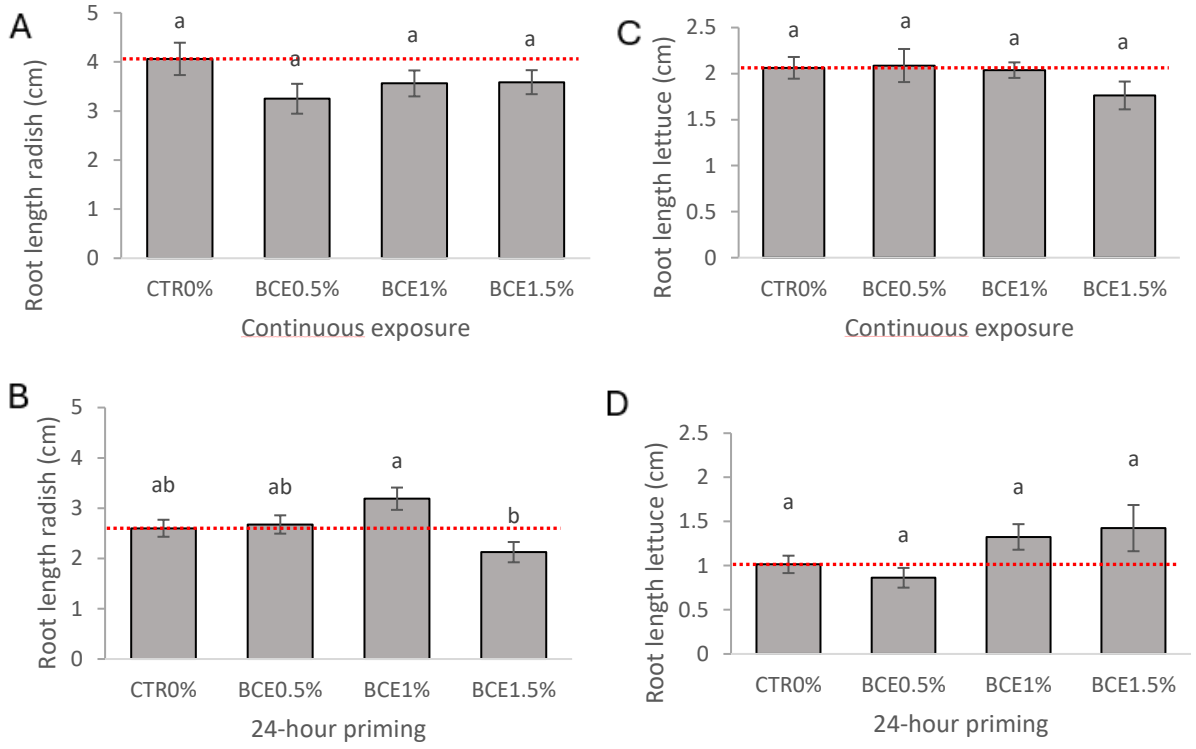

Fig. S3. Effect of BCE application (0.5%, 1%, and 1.5% m/v) on the root length of radish (A, B) and lettuce (C, D) seedlings under continuous exposure (A, C) and 24-hour priming (B, D). Bars represent mean ± SE. Different letters indicate statistically significant differences between treatments (one-way ANOVA followed by a Tukey's Honestly Significant Difference (HSD) test, $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, BCE = biochar extract)

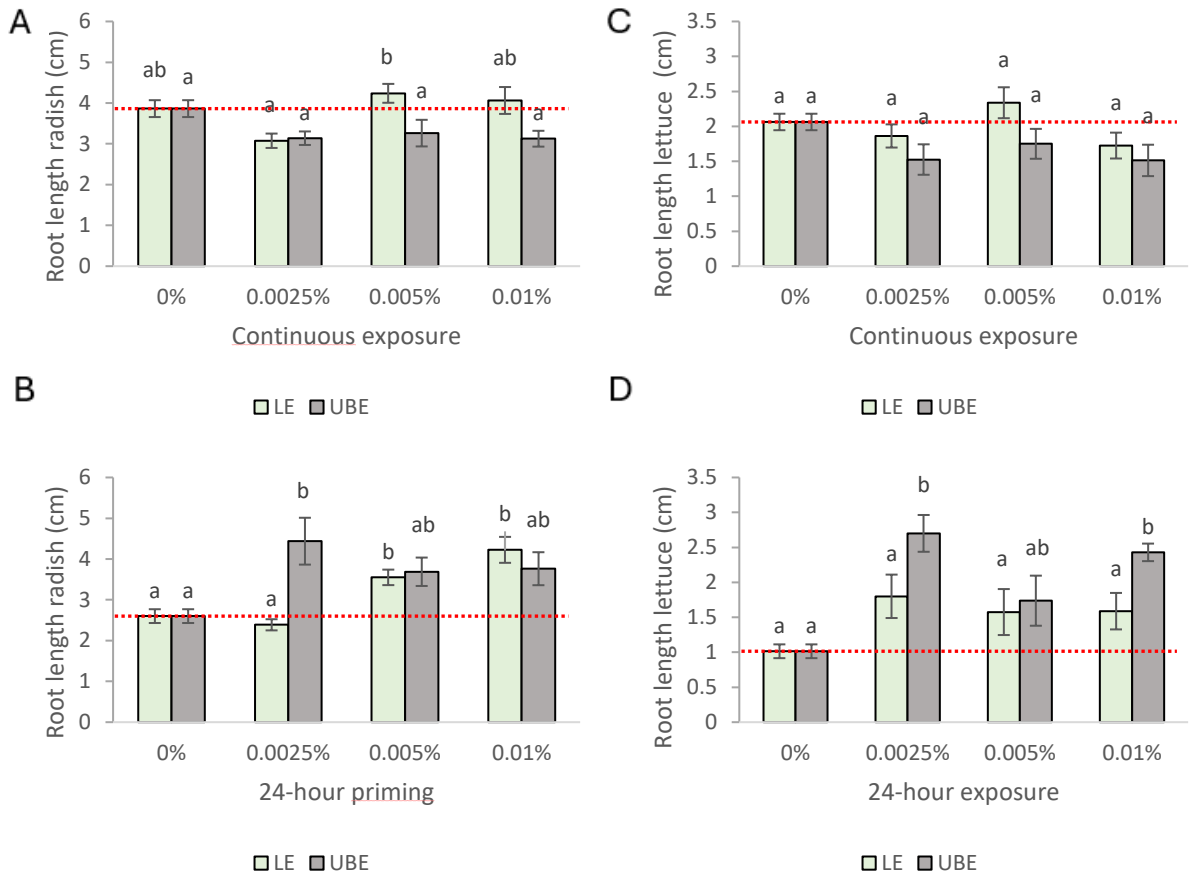

Fig. S4. Effect of plant extract (UBE and LE) application (0.0025%, 0.005%, and 0.01% m/v) on the root length of radish (A, B) and lettuce (C, D) seedlings under continuous exposure (A, C) and 24-hour priming (B, D), compared to the control. Bars represent mean ± SE. Different letters indicate significant differences between the control and concentrations of the same extract type (UBE or LE) (one-way ANOVA, followed by a Tukey's Honestly Significant Difference (HSD) test , $p < 0.05$). The red dashed line represents the mean value of the control group (CTR 0%) in each panel. (Abbreviations: CTR = control, UBE = unripe berry extract, LE = leaf extract)