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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Grapevine pruning-derived biochar as a tool to enhance seedling growth and heat stress resilience

Zara Entjes

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

SUPERVISOR :

Prof. dr. Ann CUYPERS

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De heer Seb TOMBEUR

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ABSTRACT

Viticulture generates large amounts of grapevine pruning waste, raising environmental concerns. Converting this biomass into biochar (BC) offers a sustainable waste management strategy. Biochar is a soil amendment that can improve plant growth and heat stress resilience, which is critical for maintaining agricultural productivity under climate change. However, the mechanisms behind these effects remain unclear, and biochar aqueous extract (BCE) may serve as a tool to investigate them. This study evaluated the effects of grapevine pruning-derived BC and BCE on seedling growth and heat stress resilience in *Arabidopsis thaliana*. Seedlings were treated with different concentrations of BC and BCE under control and heat stress conditions. Plant growth parameters, photosynthetic efficiency, and expression of photosynthesis- and stress-related genes were assessed. To enhance agricultural relevance, findings were validated in broccoli microgreens. Biochar enhanced root length and fresh weight in *A. thaliana* in a concentration-dependent manner, likely due to increased macronutrient availability and improved photosynthetic performance (F_v/F_m). Biochar also upregulated genes related to photosynthesis, suggesting karrikin signaling involvement. Under heat stress, BC further increased the expression of antioxidant and heat shock-related genes, indicating improved oxidative stress defense. In broccoli microgreens, BC also promoted fresh weight, supporting its broader applicability. In contrast, BCE had no significant effects on *A. thaliana* but enhanced fresh weight in broccoli microgreens, suggesting substrate-dependent responses. These findings demonstrate that grapevine pruning-derived BC can promote seedling growth and heat stress resilience while presenting an environmentally friendly solution for managing grapevine waste. Further research is needed to clarify the mechanisms involved and validate the findings.

INTRODUCTION

Viticulture represents a significant sector within the global food and beverage industry, with vineyards producing approximately 77.8 million tons of grapes annually (1). This large-scale production generates a considerable amount of by-products, estimated to range between 15 and 37 million tons per year (2). These organic residues, which arise during various stages of grape cultivation and winemaking, include grapevine stalks, pomace, and prunings. Among these, grapevine prunings represent a significant source of biomass, as they result from essential vineyard management practices aimed at maintaining vine health and optimizing grape quality and yield (1, 2). Pruning is performed twice annually, in winter and summer, and is crucial for balancing vegetative growth and fruit production. This process generates approximately two to five tons of pruning residues per hectare, depending on factors such as grape variety, climate, and plantation density (3, 4). Specifically, winter pruning results in the generation of large quantities of woody residues, including stems, shoots, and canes (4, 5). Traditionally, these residues are either left in the field or incinerated, which entails high costs and presents serious environmental and public health concerns (2).

These practices contribute to greenhouse gas emissions, degradation of soil and water quality, and the spread of disease-carrying vectors (1, 2, 6). Given these concerns, there is an urgent need to explore sustainable alternatives for managing grapevine pruning waste.

A promising approach for managing woody grapevine prunings is their conversion into biochar (BC) (6). Biochar is a carbon-rich material that is produced through pyrolysis, which is a thermal decomposition process that occurs in an oxygen-limited environment (2). Woody grapevine prunings contain lignocellulosic compounds, including lignin, cellulose, and hemicellulose. The pyrolysis of these compounds results in the formation of BC, along with bio-oil and non-condensable gases (7). This process offers a cost-effective and environmentally sustainable waste management solution, aligning with circular economy principles by converting organic waste to valuable products that can be reintegrated into crop cultivation systems (2, 6, 8).

Biochar is widely used as a soil amendment due to its unique physicochemical properties, including high porosity, large surface area, and strong metal sorption capacity. These characteristics contribute to improved soil structure, enhanced water and nutrient retention, increased aeration, and greater microbial activity, ultimately supporting plant growth and increasing crop yields (8, 9). Additionally, BC reduces the reliance on chemical fertilizers and pesticides, thereby promoting more sustainable agricultural practices (10). Despite its benefits, the exact mechanisms by which BC enhances plant growth are not yet fully understood due to the complexity of its physicochemical properties (11).

In addition to enhancing soil quality, BC can contribute to positive environmental outcomes. As a stable form of carbon, BC can sequester carbon dioxide (CO_2) in soils for centuries, thereby serving as a valuable tool for climate change mitigation (6, 12). Additionally, BC's high adsorption capacity enables the capture of pollutants, such as heavy metals and organic contaminants, thereby contributing to the remediation of soil and water (13). However, the physicochemical properties of BC, which determine its effectiveness, vary depending on several factors, including feedstock type and pyrolysis conditions, such as temperature and residence time. For instance, BC produced at lower temperatures has been shown to retain more nutrients and organic compounds, while BC produced at higher temperatures exhibits greater stability and surface area (2, 8).

Several mechanisms have been proposed to elucidate the plant growth-promoting effects of BC. One hypothesis suggests that BC releases hormone-like organic compounds formed during pyrolysis, which may activate signaling pathways associated with plant growth (11, 14, 15). Among these compounds, karrikins have recently been identified as chemical signals that stimulate seed germination and regulate plant development (16). However, further research is necessary to fully elucidate the effects of BC on plant growth signaling pathways and its broader impact on plant performance.

Another potential mechanism through which BC exerts its effects involves improved photosynthetic efficiency (11). By enhancing soil water retention, nutrient availability, and root development, BC can contribute to increased stomatal conductance, transpiration, and chlorophyll content, ultimately boosting photosynthetic rates (17). Several studies indicate that BC application positively influences photosynthetic parameters such as chlorophyll fluorescence, stomatal conductance, and quantum efficiency (17, 18). However, some studies report neutral or negative effects, underscoring the need for further research to clarify BC's role in photosynthesis and plant growth (17).

Beyond its role in promoting plant growth, BC has the potential to improve plant tolerance to abiotic stressors that are exacerbated by climate change (8). Climate change presents a significant global challenge, as it poses severe threats to agricultural productivity and food security. These threats are a result of rising atmospheric CO_2 levels, increasing temperatures, shifting precipitation patterns, and a higher frequency of extreme weather events (8, 19). Among these factors, rising global temperatures, projected to increase by approximately 0.3 °C per decade, represent a major concern, as this leads to more frequent and intense heat waves that place pressure on agricultural resilience (19). As a result, heat stress has emerged as a critical abiotic stressor that disrupts physiological processes essential for plant growth and development. Given their dependence on ambient conditions, plants are

especially vulnerable to such temperature extremes, highlighting the urgency of strategies that improve heat stress tolerance (20, 21).

Heat stress occurs when plants are exposed to elevated temperatures for extended periods, impairing essential physiological functions. A primary consequence of heat stress is the excessive production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radicals ($^{\bullet}OH$) (21-23). The generation of these molecules occurs through various cellular pathways, including NADPH oxidases (22). While ROS function as signaling molecules in stress response pathways when present in moderate concentrations, their excessive accumulation induces oxidative stress, leading to damage of critical cellular components such as DNA, proteins, and lipids (21, 22). Heat stress-induced ROS accumulation also activates heat shock transcription factors (HSFs), which regulate the production of heat shock proteins (HSPs). These proteins are critical for protecting and stabilizing cellular proteins during stress (22). To counteract oxidative stress, plants have evolved antioxidant defense systems. These include enzymatic antioxidants, such as superoxide dismutase, catalase, and peroxidase, as well as non-enzymatic antioxidants, such as glutathione (20, 21). These defense systems scavenge ROS and help to restore cellular redox homeostasis. However, prolonged or intensified heat stress can overwhelm these defense systems, leading to severe physiological damage and significant yield losses. This is an increasingly critical issue for modern agricultural systems (21, 22).

Biochar has emerged as a promising strategy to enhance plant resilience against heat stress by modulating antioxidant responses. Studies indicate that BC amendments can stimulate the activity of key antioxidant enzymes, thereby enabling plants to regulate ROS levels more effectively under high-temperature conditions (12, 24). For instance, studies on *Arabidopsis thaliana* indicate that the application of BC induces early acclimation responses, thereby improving heat tolerance (24). Similarly, rice (*Oryza sativa*) plants supplemented with BC exhibited increased expression of antioxidant enzymes, which mitigated the detrimental effects of heat stress and supported overall plant growth (12). Despite these promising findings, the exact mechanisms underlying BC-induced heat stress tolerance remain largely unexplored.

Although BC offers numerous advantages, its application can also have adverse effects on plant growth under certain conditions. Biochar may contain toxic compounds, such as polycyclic aromatic hydrocarbons (PAHs), which can accumulate on the surface of BC during pyrolysis (25). Furthermore, certain BCs have been found to release harmful substances, including heavy metals and organic pollutants, into the soil, potentially inhibiting plant development (26, 27). Additionally, BC's alkaline nature can disrupt soil chemistry, leading to osmotic stress and nutrient imbalances in acidophilic plant species. In order to mitigate the aforementioned risks, a proposed solution involves the leaching of the pristine BC with water as a pre-treatment. This process is intended to lower the pH of BC and remove harmful compounds, thereby improving its safety and efficacy as a soil amendment (27).

Despite the potential presence of harmful substances, biochar aqueous extract (BCE) emerges as a valuable by-product arising from this pretreatment process. This extract contains the water-soluble fraction of BC, including hormone-like substances and nutrients, which can directly affect plant physiology (11). However, it is often discarded as a waste product. Recent studies suggest that BCE is one of the most promising BC-derived fractions for promoting plant growth due to its direct biological effects on plants. For instance, the application of straw-derived BCE has been demonstrated to stimulate the growth of maize (*Zea mays*) (11), and in another study, BCE derived from residual wood was found to stimulate seed germination in lettuce (*Lactuca sativa*) (28). Furthermore, the utilization of BCE facilitates the investigation of BC's biological effects without interference from its complex physicochemical properties. Following the leaching process, the washed BC remains a valuable soil amendment, and the resulting aqueous extract offers additional potential as a plant growth stimulator. The utilization of both BC and BCE is an effective strategy for reducing waste, thereby aligning with the principles of a circular economy.

Although BCE has demonstrated the potential to promote plant growth, no studies have investigated its ability to improve heat stress resilience in plants, regardless of the type of BC feedstock used. A study reported that the

application of rice husk-derived BCE enhanced the cold stress tolerance of rice seedlings (29), suggesting that BCE may contribute to increased resilience under other abiotic stress conditions as well. Several studies have examined the positive effects of BC on plant growth and heat stress resilience. However, to the best of our knowledge, no research has examined the effects of BC derived specifically from grapevine prunings. Additionally, the mechanisms through which BC promotes plant growth and enhances heat stress tolerance are not fully understood, particularly with regard to the modulation of photosynthesis and antioxidant responses. Therefore, this study aims to investigate whether and how grapevine pruning-derived BC and its aqueous extract promote plant growth and alleviate heat stress in *Arabidopsis thaliana* seedlings.

We hypothesize that BC and BCE enhance seedling growth by improving photosynthetic efficiency and increase seedling resilience under heat stress by activating antioxidant defense mechanisms in *Arabidopsis thaliana*.

To test this hypothesis, different concentrations of BC and BCE are applied separately to *A. thaliana* seedlings grown in a 96-well growth system under both control and heat stress conditions. The effects on plant growth are evaluated by measuring biometric growth parameters, analyzing cell cycle dynamics, monitoring photosynthetic performance through non-destructive imaging and chlorophyll measurements, and assessing the expression of photosynthesis- and oxidative stress-related genes. In addition, alterations in the physicochemical properties of the plant growth medium following BC application are assessed. This approach enables us to gain insight into the underlying physiological and molecular responses under both control and heat stress conditions. To validate these findings in an economically relevant crop, we also examined the plant growth-promoting potential of BC and BCE in broccoli microgreens (*Brassica oleracea var. cymose*).

EXPERIMENTAL PROCEDURES

BC production and characterization – Woody winter prunings from Chardonnay grapevines were shredded in an SM100 mill (Retsch) over a 1 x 1 cm sieve and subsequently oven-dried at 60 °C until constant weight. The dried material underwent pyrolysis in a pilot-scale rotary kiln reactor at 450 °C. Following pyrolysis, the BC was outgassed at 105 °C for 24 hours to remove volatile PAHs. The resulting biochar was ground into a fine powder using a Mixer Mill MM 400 (Retsch). Biochar extract [5% (w/v)] was prepared by shaking BC for 24 hours at 100 rpm in sterile Milli-Q water, followed by filtration through a 0.45 µm membrane filter. Biochar was characterized in terms of its elemental composition, ash content, and yield. The pH and electrical conductivity (EC) were measured in BCE, as BC has to undergo the same 24-hour shaking process at 100 rpm before pH and EC measurements. pH and EC measurements were performed using a 764 Multi-Calimatic pH meter (Knick) and a conductivity meter (Mettler Toledo), respectively. All analyses were performed in triplicate.

A. thaliana growth experiment – Wild-type *A. thaliana* seeds were surface-sterilized with 70% ethanol and stratified at 4 °C in the dark for three nights. The seeds were subsequently sown in 96-well plates containing ¼ Murashige and Skoog (MS) medium supplemented with BC or BCE at concentrations of 0%, 0.05%, 0.1%, 0.25%, or 0.5% (w/v). Afterward, the plates were placed in a climate-controlled chamber with a 12h photoperiod, a 22/18 °C day/night temperature cycle, 65% relative humidity, and 170 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). In a separate experiment under identical growth conditions, the medium was supplemented with 0%, 0.05%, 0.1%, or 0.25% (w/v) BC or BCE, and seedlings were subjected to heat stress (40 °C for 2 hours) in a VWR Incu-Line Tower oven at 4 DAS. Control seedlings underwent a mock treatment at 22 °C for 2 hours. Seedlings were harvested at 7 and 10 days after sowing (DAS). Root length was measured at both 7 and 10 DAS, and fresh weight was recorded at 10 DAS. After harvest, seedlings were snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

Nutrient leaching experiment – To mimic BC's behavior during the growth experiment, ¼ MS medium was supplemented with 0%, 0.05%, 0.1%, 0.25%, or 0.5% (w/v) BC. The samples were placed in a climate-controlled chamber with identical environmental conditions as the *A. thaliana* growth experiment. After 7 days, the medium was filtered through a 0.2 µm membrane filter. The pH and EC were measured on the filtrate. A subsample of each filtrate was acidified with 2% (v/v) HCl, and nutrient concentrations were

determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer Optima 8300). All measurements were performed in triplicate.

Cell cycle analysis – To determine the extent of endoreplication, nuclear ploidy levels were measured using the CyStain® PI Absolute P kit (Sysmex Partec). Four pooled seedlings per biological replicate were chopped in 250 µL nuclei extraction buffer (CyStain® PI Absolute P kit, Sysmex Partec). The extract was filtered through a 50 µm nylon filter (CellTrics®, Sysmex Partec) and stained with a staining solution consisting of 1 mL staining buffer, 6 µL propidium iodide (PI), and 3 µL RNase A (CyStain® PI Absolute P kit). After staining, samples were incubated in the dark at 4 °C for a minimum of 1 hour. Ploidy levels (2C, 4C, 8C, 16C, and 32C, with C corresponding to the haploid DNA content) for a minimum of 8000 nuclei per sample were analyzed using a CyFlow® Cube 8 flow cytometer (Sysmex Partec). Nuclei were excited at 488 nm, and forward scatter and PI fluorescence intensity (FL-2 channel, 580/30 nm) were measured. DNA content was quantified using FCS Express 5 software (De Novo Software). The endoreplication index (EI_0), indicating the average number of endocycles per cell, was calculated as follows: $EI_0 = [(0\ x\ \%\ 2C) + (1\ x\ \%\ 4C) + (2\ x\ \%\ 8C) + (3\ x\ \%\ 16C) + (4\ x\ \%\ 32C)]\ /\ 100$ (30). Additional endoreplication indices for plant growth (EI_{growth}) and defense ($EI_{defense}$) were calculated according to Cuypers *et al*. (31). The extent of cell division was estimated based on the concentration of nuclei in the flow cytometric extracts.

Multispectral imaging – Multispectral imaging was performed daily using the PlantExplorer XS (PhenoVation) to monitor photosynthetic efficiency in real-time from 4 to 7 DAS. Seedlings were dark-adapted for 15 min before measurements. Minimal fluorescence (F_0) was recorded when all photosystem II (PSII) reaction centers were open, whereas maximal fluorescence (F_m) was measured following a saturating light pulse when reaction centers were closed. The difference between F_m and F_0 gives the variable fluorescence (F_v). The maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ (32). The photosynthetic parameter was quantified using Data Analysis™ software version 5.8.4-64b (PhenoVation). The mask level was set to 2000 at 4 DAS and to 3000 at 5 to 7 DAS based on chlorophyll fluorescence to eliminate background signal.

Pigment profiling – A pool of six seedlings (minimum 20 mg) per biological replicate was pulverized under frozen conditions using two stainless steel beads in a Mixer Mill MM 400 (Retsch). Photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenes) were extracted in 80% acetone. After centrifugation (6000 rpm, 5 min), the absorbance of the supernatant was measured spectrophotometrically at 470 nm, 646 nm, and 663 nm with a plate reader (FLUOstar Omega). Pigment concentrations were calculated according to the formulae of Wellburn and Lichtenthaler (33).

Gene expression analysis – A pool of ten seedlings was pulverized under frozen conditions using two stainless steel beads in a Mixer Mill MM 400 (Retsch). RNA extraction was performed according to Valledor *et al*. (34), with a few modifications. These included the addition of 100 µL of Pellet Solubilization Buffer to each sample and the adjustments to wash buffer 1 (1 M TrisHCl, 1 M NaCl, 10 mM EDTA, 90% ethanol) and wash buffer 2 (1 M TrisHCl, 1 M NaCl, 10 mM EDTA, 70% ethanol). RNA concentration and purity were determined using the NanoDrop® ND-1000 spectrophotometer (Ambion, Thermo Fisher Scientific). The TURBO DNA-free™ Kit (Thermo Fisher Scientific) was used to remove any residual genomic DNA. For cDNA synthesis using the PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara Bio Inc.), an equal input of 1 µg RNA was used for each sample. The resulting cDNA was diluted tenfold in 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C. The QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) was used to conduct quantitative real-time PCR (qPCR) with the QuantiNova SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The sequences of the used forward and reverse primers (300 nM final concentration) are listed in Table S1. Data were analyzed using the comparative Cq ($2^{-\Delta\Delta Cq}$) method. Normalization was performed with a normalization factor based on five stably expressed reference genes, selected using the GrayNorm algorithm (35). To correct for technical variation between runs, an inter-run calibration was performed according to the method of Hellemans *et al*. (36).

Broccoli microgreen experiment – Broccoli microgreen seeds (*Brassica oleracea*

var. cymosa) were sown at a density of 2.2 g seeds/132 cm^2 in trays on 20 g of substrate, with the seeds evenly distributed across nine equal sections within each tray. The used substrate consisted of dried miscanthus fibers (< 4 mm). Biochar or BCE was added to the substrate at concentrations of 0.5%, 1%, or 1.5% (w/v) by dissolving it in 100 mL of MilliQ water. The resulting solution was mixed with the substrate, and the wetted mixture was homogenized before sowing. Afterward, the sown trays were covered on top with trays that had a total weight of 300 g. This was done to stimulate germination under dark conditions in a climate-controlled chamber with identical environmental conditions as the *A. thaliana* growth experiment. The cover trays were removed at 3 DAS. From 3 DAS onward, the trays were watered daily back to their initial weight. At 6 DAS, seedling fresh weight was measured by cutting the germinated microgreens right above the substrate surface.

Statistical analysis – All data were normalized using a normalization factor to align baseline values between the control and heat stress experiments. Statistical analyses were conducted using RStudio version 2024.04.02+764 (R Core Team). Significant outliers were identified using Grubb's test (GraphPad Software) and removed from the analysis. Normality and homoscedasticity were checked using the Shapiro-Wilk and Bartlett's tests, respectively. A one- or two-way analysis of variance (ANOVA) was used to compare conditions, followed by Tukey's honestly significant difference (HSD) post-hoc test for pairwise comparisons. If the assumptions of normality and homoscedasticity were not met after data transformation (logarithmic, square root, inverse, or exponential), a non-parametric Kruskal-Wallis test was used instead, followed by the Wilcoxon rank sum test for pairwise comparisons. Gene expression data were log-transformed by default. The significance level was set at 0.05 for all tests.

RESULTS

Differences in nutrient content between BC and BCE – The yield of BC was 26%, with an ash content of 11% (Table S2). Elemental analysis revealed that BC exhibited a high carbon content (71.7% ± 1.0). It also contained hydrogen (3.10% ± 0.04) and nitrogen (1.19% ± 0.02). Biochar was abundant in the macronutrients magnesium, calcium, and potassium, while phosphorus and sulfur were present in lower concentrations (Table S3). Among micronutrients, sodium, iron, and zinc were most abundant, whereas manganese and copper were present in smaller amounts. Levels of potential toxic elements (PTEs) were below the detection limit, except for chromium (13.72 ± 0.12).

BCE (5% w/v) contained 183 mg/L of non-purgeable organic carbon and 2.1 mg/L of total nitrogen (Table S2). Compared to BC, BCE contained lower concentrations of extracted nutrients. Potassium, sulfur, calcium, and magnesium were the most prevalent macronutrients (Table S4). Sodium was the most abundant micronutrient, while iron was detected in smaller amounts. All other micronutrients, as well as PTE levels, were below detection limits. Biochar and BCE had a pH of 9.03 (± 0.28) and an EC of 1608 (± 20) µS/cm (Table S2).

BC alters physicochemical properties of the growth medium – A BC leaching analysis in ¼ MS medium was conducted to assess the impact of BC on nutrient availability. Potassium was the most abundant macronutrient, increasing from 155.24 (± 0.27) mg/L in the control medium to 223.12 (± 0.37) mg/L at 0.5% BC (Table 1). Concentration-dependent increases in other macronutrients, including calcium, sulfur, phosphorus, and magnesium, were also observed, following potassium in order of abundance. Among the micronutrients, sodium exhibited the highest concentration, increasing from 1.75 (± 0.01) mg/L in the control medium to 4.05 (± 0.03) mg/L at 0.5% BC. In contrast, concentrations of manganese, iron, and zinc decreased with increasing BC concentrations. Copper levels remained below detection limits across all growth media. With respect to PTEs, cadmium was detected in low concentrations, increasing from 0.003 (± 0.001) mg/L in the control medium to 0.015 (± 0.001) mg/L at 0.5% BC. In addition to alterations in nutrient composition, both the EC and pH of the medium increased with higher BC concentrations. The EC increased from 1629.0 (± 0.9) µS/cm in the control to 1846.4 (± 3.2) µS/cm at 0.5% BC, while the pH increased from 5.31 (± 0.06) to 8.42 (± 0.01).

BC stimulates seedling growth in a concentration-dependent manner – To evaluate the effects of BC and BCE on seedling growth,

Table 1 – Nutrient concentrations of BC in the growth media after 7 days of leaching.

	BC concentration (%)				
Element	**0**	**0.05**	**0.10**	**0.25**	**0.50**
Macronutrients					
Ca	22.68 ± 0.07	29.91 ± 0.40	33.87 ± 0.56	34.48 ± 1.87	36.28 ± 1.50
K	155.24 ± 0.27	160.67 ± 0.13	168.43 ± 0.59	189.40 ± 0.71	223.12 ± 0.37
Mg	6.48 ± 0.02	7.25 ± 0.02	7.96 ± 0.07	9.79 ± 0.34	11.42 ± 0.22
P	9.66 ± 0.08	9.94 ± 0.22	10.18 ± 0.64	12.82 ± 0.79	12.10 ± 0.50
S	11.01 ± 0.01	11.00 ± 0.07	11.12 ± 0.04	11.88 ± 0.09	12.75 ± 0.02
Micronutrients					
Fe	0.87 ± 0.01	0.79 ± 0.02	0.72 ± 0.01	0.51 ± 0.08	0.25 ± 0.01
Mn	1.042 ± 0.003	0.964 ± 0.020	0.800 ± 0.029	0.598 ± 0.078	0.378 ± 0.006
Na	1.75 ± 0.01	2.04 ± 0.01	2.31 ± 0.02	2.99 ± 0.02	4.05 ± 0.03
Zn	0.343 ± 0.002	0.315 ± 0.001	0.279 ± 0.002	0.270 ± 0.022	0.226 ± 0.007
PTEs					
Cd	0.003 ± 0.001	0.004 ± 0.001	0.009 ± 0.001	0.014 ± 0.001	0.015 ± 0.001
Characteristics					
EC (µS/cm)	1629.0 ± 0.9	1664.7 ± 5.4	1691.0 ± 3.7	1762.0 ± 3.7	1846.4 ± 3.2
pH	5.31 ± 0.06	6.97 ± 0.05	7.52 ± 0.02	8.01 ± 0.05	8.42 ± 0.01

Data are presented as mean ± SE (mg/L) of at least 3 biological replicates. Abbreviations: Ca, calcium; Cd, cadmium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; Na, sodium; P, phosphorus; S, sulfur; Zn, zinc; BC, biochar; EC: electrical conductivity; PTEs: potential toxic elements.

A. thaliana seeds were subjected to varying concentrations of BC and BCE under both control and heat stress conditions. At 7 DAS, BC induced a concentration-dependent increase in root length. Under control conditions, significant increases in root length were observed at 0.05% and 0.10% BC compared to 0% BC under control conditions (hereafter referred to as NHC) (Fig. 1A). This growth-promoting effect declined at 0.25% BC but remained above NHC. Under heat stress conditions, a similar pattern was observed. Root length was significantly increased at 0.05% and 0.10% BC compared to 0% BC under heat stress conditions (hereafter referred to as HC). The effect diminished at 0.25% BC but remained above HC. Root length was reduced under heat stress when compared to control conditions at the same BC concentration. However, these differences were not statistically significant. Furthermore, a significant overall effect of heat stress was observed on root length ($p < 0.001$). In contrast, BCE had a neutral or slightly negative effect on root length under control conditions (Fig. 1B), with no significant differences observed at any concentration compared to NHC. Under heat stress, BCE exhibited neutral or slightly positive effects on root length compared to HC, but none of the BCE concentrations induced significant changes. Furthermore, no overall effect of heat stress was observed in seedlings treated with BCE ($p = 0.35$).

At 10 DAS, the concentration-dependent stimulatory effect of BC on root growth persisted (Fig. 1C). Under control conditions, seedlings treated with all BC concentrations exhibited significantly longer roots compared to NHC. Under heat stress, a similar concentration-dependent increase in root length was observed compared to HC. However, root length under heat stress was lower than under control conditions at the same BC concentrations, with a significant reduction observed at 0.10% BC. Furthermore, a significant overall heat stress effect was detected ($p < 0.001$). Conversely, at 10 DAS, BCE continued to have no significant effect on root length under either control or heat stress conditions, with no BCE concentration showing significant differences compared to 0% BCE (Fig. 1D).

In addition to root length, seedling fresh weight increased in a concentration-dependent manner following BC exposure under both control and heat stress conditions, with a maximum increase observed at 0.25% BC (Fig. 1E). Under control conditions, fresh weight increased significantly at 0.10% and 0.25% BC

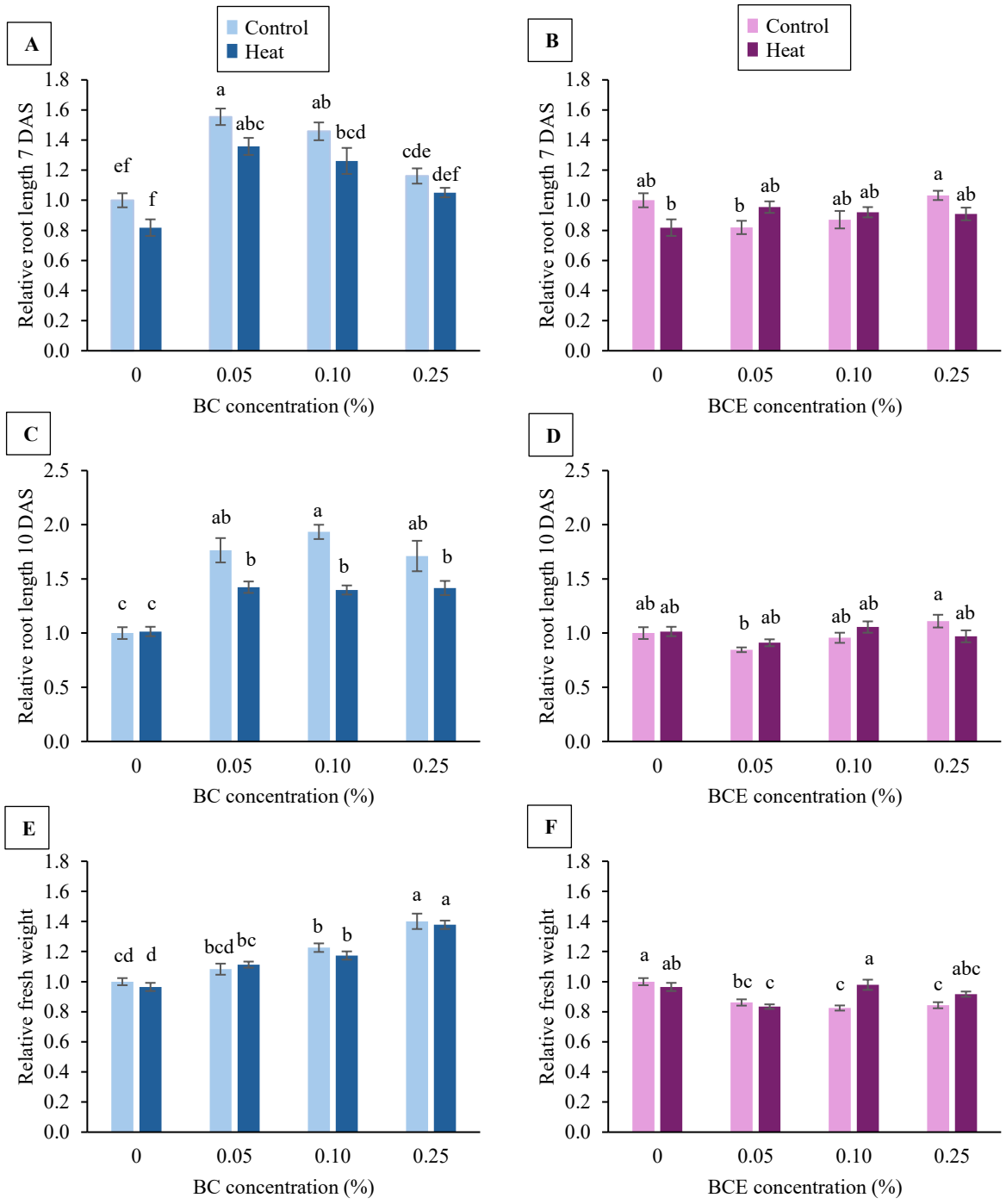

Fig. 1 – Relative root length and fresh weight of *Arabidopsis thaliana* seedlings. Seedlings were grown under control conditions or subjected to heat stress (40 °C for 2 h at 4 DAS) in 96-well plates containing ¼ MS medium supplemented with different concentrations of BC or BCE (0%, 0.05%, 0.10%, or 0.25%). Relative root length of seedlings exposed to BC and BCE was measured at 7 DAS **(A-B)** and 10 DAS **(C-D)**, and relative fresh weight at 10 DAS **(E-F)**. Data are presented as mean ± SE of at least 7 biological replicates relative to 0% BC under control conditions (set to 1.00). Different letters denote statistically significant differences (two-way ANOVA; $p < 0.05$). Abbreviations: BC, biochar; BCE, biochar extract; DAS, days after sowing.

compared to NHC. However, the stimulatory effect on fresh weight declined at 0.5% BC but remained above NHC (data not shown). Under heat stress conditions, fresh weight was significantly increased at all BC concentrations compared to HC. Furthermore, no overall effect of heat stress was observed (p = 0.36). In contrast, BCE showed neutral or negative effects on fresh weight (Fig. 1F). Under control conditions, all BCE caused a significant decrease in fresh weight compared to NHC. Under heat stress, only 0.05% BCE caused a

significant reduction in fresh weight compared to HC, whereas 0.10% and 0.25% BCE had no statistically significant effect. At higher BCE concentrations, fresh weights were slightly increased under heat stress compared to control conditions, with a significant increase observed at 0.10% BCE.

Cell division and endoreplication are minimally affected by BC – Flow cytometric analysis of nuclear DNA content was conducted to investigate the cellular mechanisms underlying BC-induced growth responses, with a focus on cell division and endoreplication. Under control conditions, increasing BC concentrations resulted in a gradual decrease in the number of nuclei per µL of flow cytometric extract compared to NHC (Fig. 2A). However, this decrease was not statistically significant except at 0.25% BC, which showed a significant reduction compared to NHC. Under heat stress conditions, an increase in the number of nuclei per µL was observed at 0.05% BC compared to HC, but this effect was not statistically significant and diminished at higher BC concentrations. Additionally, a significant decrease in nuclei numbers per µL extract was observed at 0% BC under heat stress compared to NHC.

The endoreplication index (EI_0) was significantly reduced following exposure to 0.05% and 0.10% BC compared to NHC (Fig. 2B). At 0.25% BC under control conditions, EI_0 was not statistically different compared to NHC. Under heat stress, a decline in EI_0 was observed at all BC concentrations compared to

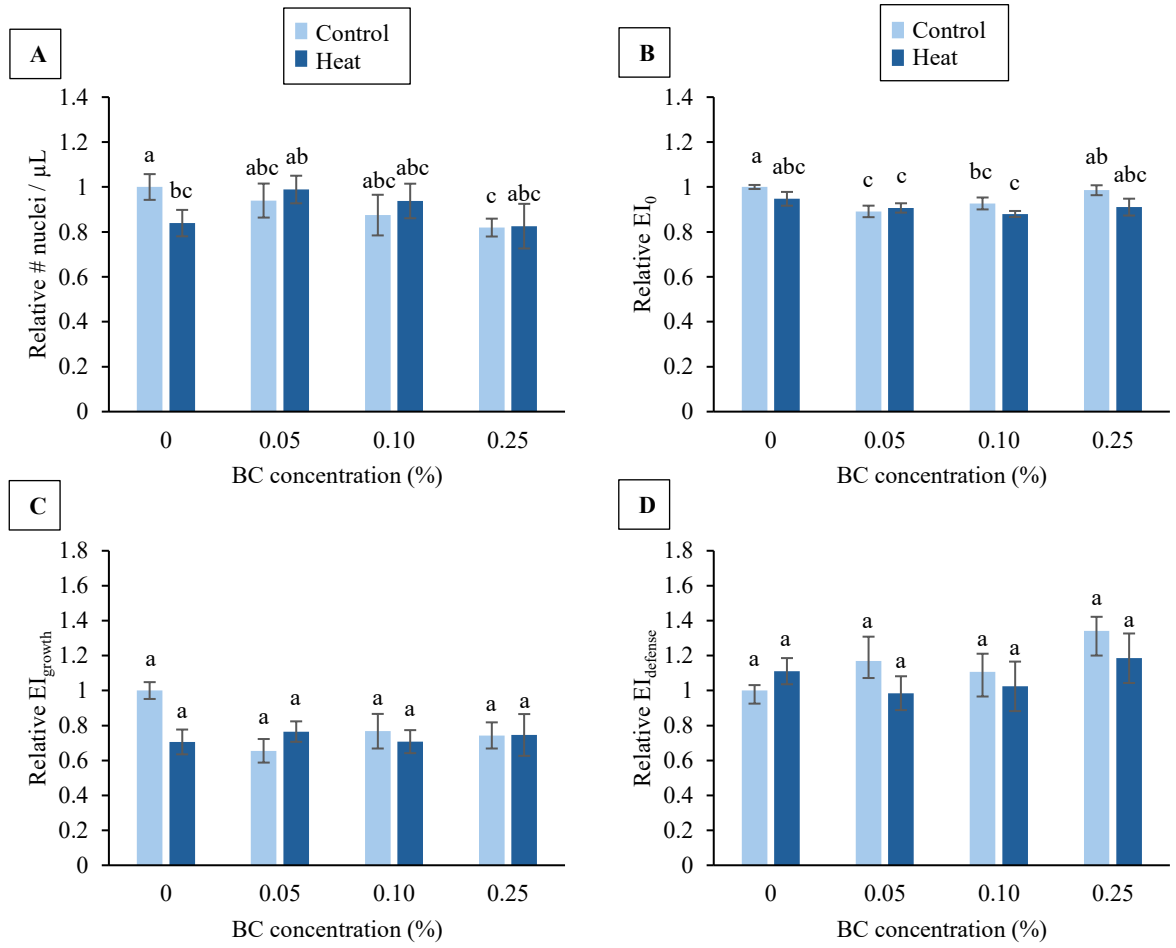

Fig. 2 – Relative endoreplication indices and nuclei number per µL of flow cytometry extracts from *Arabidopsis thaliana* seedlings at 7 DAS. Seedlings were grown under control conditions or subjected to heat stress (40 °C for 2 h at 4 DAS) in 96-well plates containing ¼ MS medium supplemented with different BC concentrations (0%, 0.05%, 0.10%, or 0.25%). Relative nuclei number per µL **(A),** endoreplication factor **(B)**, growth index **(C)**, and defense index **(D)** of seedlings exposed to BC. Data are presented as mean ± SE of at least 6 biological replicates relative to 0% BC under control conditions (set to 1.00). Different letters denote statistically significant differences (two-way ANOVA, Kruskal-Wallis test **(A, B)**; $p < 0.05$). Abbreviations: BC, biochar; DAS, days after sowing; EI, endoreplication index.

HC, although these changes were not statistically significant. Furthermore, a significant interaction effect between BC concentration and temperature condition on endoreplication was detected ($p = 0.007$).

To further elucidate these dynamics, the growth index (EI_{growth}) and defense index ($EI_{defense}$) were analyzed under both control and heat stress conditions. The growth and defense indices showed opposite trends in response to BC under both control and heat stress conditions. However, no statistically significant effects were detected. Under control conditions, EI_{growth} showed a decrease at all BC concentrations compared to NHC, with the strongest reduction observed at 0.05% BC, followed by 0.10% and 0.25% BC (Fig. 2C). In contrast, $EI_{defense}$ increased slightly at 0.05% and 0.25% BC compared to NHC while remaining unchanged at 0.10% BC (Fig. 2D). Under heat stress, EI_{growth} remained stable across all BC concentrations compared to HC. $EI_{defense}$ showed a slight decline at 0.05% and 0.10% BC compared to HC, followed by a minor increase at 0.25% BC. Additionally, a decrease in EI_{growth} and a slight increase in $EI_{defense}$ were observed at 0% BC under heat stress compared to NHC. However, these differences were not statistically significant.

BC enhances PSII efficiency with limited impact on pigment composition – The impact of BC on photosynthetic efficiency was assessed by measuring the maximum quantum yield of PSII (F_v/F_m) from 4 to 7 DAS using MSI. Under control conditions, F_v/F_m was significantly higher in all BC-treated seedlings compared to NHC on all measured days (Fig. 3). Under heat stress, F_v/F_m increased in a concentration-dependent manner compared to HC across all measured days, reaching a maximum at 0.25% BC. However, the increase observed at 0.05% BC was not statistically significant compared to HC. Under both control and heat stress conditions, the most pronounced increase in photosynthetic efficiency compared to the respective 0% BC was observed at 4 DAS.

The effects of BC concentrations on total chlorophyll (chlorophyll *a* + *b*) and carotene content in *A. thaliana* were evaluated under control conditions. Total chlorophyll content was not significantly affected by any BC concentration compared to 0% BC

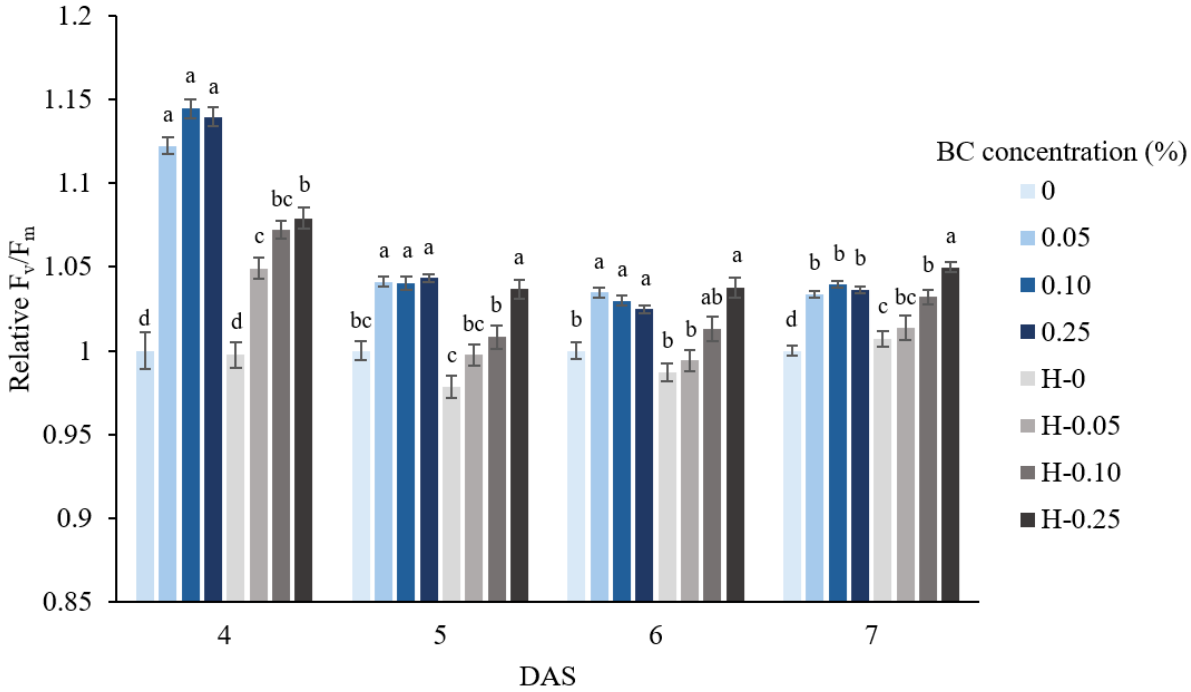

Fig. 3 – Relative maximum PSII quantum efficiency (F_v/F_m) of *Arabidopsis thaliana* seedlings from 4 to 7 DAS. Seedlings were grown under control conditions (blue bars) or subjected to heat stress (40 °C for 2 h at 4 DAS; grey bars) in 96-well plates containing ¼ MS medium supplemented with different BC concentrations (0%, 0.05%, 0.10%, or 0.25%). Data are presented as mean ± SE of at least 39 biological replicates for 4 DAS and at least 73 for 5 to 7 DAS, relative to 0% BC under control conditions (set to 1.00). Different letters denote statistically significant differences independently for each DAS (two-way ANOVA; $p < 0.05$). Abbreviations: BC, biochar; DAS, days after sowing.

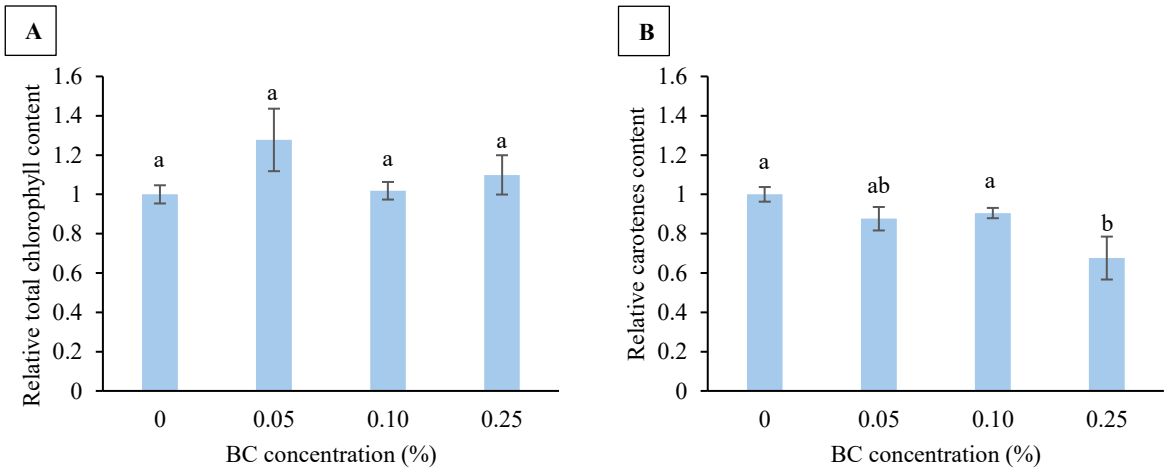

Fig. 4 – Relative photosynthetic pigment levels in *Arabidopsis thaliana* seedlings at 10 DAS. Seedlings were grown under control conditions in 96-well plates containing ¼ MS medium supplemented with different BC concentrations (0%, 0.05%, 0.10%, or 0.25%). Relative total chlorophyll **(A)** and carotene content **(B)** of seedlings exposed to BC. Data are presented as mean ± SE of at least 5 biological replicates relative to 0% BC (set to 1.00). Different letters denote statistically significant differences (one-way ANOVA **(A)**, Kruskal-Wallis test **(B)**; $p < 0.05$). Abbreviations: BC, biochar; DAS, days after sowing.

(Fig. 4A; chlorophyll *a* and *b* are shown in Fig. S1). Furthermore, a significant decrease in carotene content was observed at 0.25% BC, while no significant differences were detected at 0.05% and 0.10% BC compared to 0% BC (Fig. 4B).

Expression of photosynthesis-related and antioxidant genes altered by BC – To further elucidate the molecular responses underlying BC-induced growth effects, the expression of oxidative stress-related and photosynthesis-related genes was analyzed in *A. thaliana* seedlings under both control and heat stress conditions. Under control conditions, the photomorphogenesis-related gene *B-BOX DOMAIN PROTEIN 16 (BBX16)* and the photosynthetic genes *LIGHT-HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN 1.4 (LHCB1.4)* and *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 3B (RBCS3B)* were significantly upregulated at 0.05% and 0.25% BC compared to NHC (Table 2). Under heat stress conditions, all three genes were only significantly induced at 0.25% BC compared to HC. In addition, *LHCB1.4* and *RBCS3B* showed significantly lower expression levels at 0.05% BC under heat stress compared to the same BC concentration under control conditions.

Among the stress signaling genes, *HEAT SHOCK FACTOR A4A (HSFA4A)* and *WRKY DNA BINDING PROTEIN 33 (WRKY33)* were significantly upregulated at 0.25% BC compared to HC under heat stress conditions. Furthermore, *HSFA4A* also showed significantly higher expression at 0.25% BC under heat stress compared to the same BC concentration under control conditions. No significant changes were observed for *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 2 (ACS2)* and *OXIDATIVE SIGNAL-INDUCIBLE 1 (OXI1)* under either control or heat stress conditions.

Regarding ROS scavenging genes, *CATALASE 1 (CAT1)* was significantly downregulated at 0.05% and 0.25% BC compared to NHC under control conditions, but its expression remained unchanged under heat stress. In contrast, *CATALASE 3 (CAT3)* was significantly upregulated at 0.25% BC compared to NHC under control conditions. The superoxide dismutase genes *CU/ZN SUPEROXIDE DISMUTASE 1 (CSD1)* and *CU/ZN SUPEROXIDE DISMUTASE 2 (CSD2)* were significantly induced at 0.25% BC compared to 0% BC under both control and heat stress conditions. However, expression of *CSD2* at 0.25% BC was significantly lower under heat stress compared to control conditions. Under control conditions, the ROS-scavenging gene *ASCORBATE PEROXIDASE 2 (APX2)* showed a concentration-dependent decrease in expression at all BC concentrations compared to NHC. Under heat stress conditions, this declining trend was also observed, with a significant reduction at 0.25% BC compared to HC. Furthermore, *APX2* transcript levels were strongly upregulated in response to heat stress,

Table 2 – Expression patterns of photosynthesis- and oxidative stress-related genes in *A. thaliana* seedlings at 7 DAS. Seedlings were grown under control conditions or subjected to heat stress (40 °C for 2 h at 4 DAS) in 96-well plates containing ¼ MS medium supplemented with different BC concentrations (0%, 0.05%, or 0.25%).

		BC concentration (%)		
Gene	**Condition**	**0**	**0.05**	**0.25**
Photosynthesis- and photomorphogenesis-related genes				
BBX16	control	1.00 ± 0.04	1.57 ± 0.05	2.07 ± 0.11
	heat	1.19 ± 0.08	1.36 ± 0.06	1.83 ± 0.13
LHCB1.4	control	1.00 ± 0.09	2.41 ± 0.12	2.97 ± 0.06
	heat	1.11 ± 0.06	0.61 ± 0.01*	2.55 ± 0.26
RBCS3B	control	1.00 ± 0.07	1.54 ± 0.10	2.25 ± 0.13
	heat	1.21 ± 0.04	1.00 ± 0.04*	2.15 ± 0.05
Stress signaling genes				
ACS2	control	1.00 ± 0.02	0.90 ± 0.12	0.76 ± 0.07
	heat	0.80 ± 0.11	0.81 ± 0.04	0.93 ± 0.27
HSFA4A	control	1.00 ± 0.06	0.86 ± 0.05	0.74 ± 0.02
	heat	0.74 ± 0.02	0.59 ± 0.02	1.12 ± 0.18*
OXI1	control	1.00 ± 0.05	0.76 ± 0.01	0.69 ± 0.01
	heat	1.55 ± 0.61	0.90 ± 0.17	1.20 ± 0.15
WRKY33	control	1.00 ± 0.06	0.79 ± 0.03	1.22 ± 0.18
	heat	0.66 ± 0.08	0.59 ± 0.05	1.83 ± 0.60
ROS scavenging genes				
CAT1	control	1.00 ± 0.07	0.67 ± 0.06	0.55 ± 0.09
	heat	0.82 ± 0.07	0.93 ± 0.06	0.75 ± 0.01
CAT3	control	1.00 ± 0.10	1.24 ± 0.16	1.53 ± 0.15
	heat	0.83 ± 0.07	1.00 ± 0.05	1.08 ± 0.04
CSD1	control	1.00 ± 0.07	1.84 ± 0.12	4.07 ± 0.31
	heat	1.34 ± 0.07	1.90 ± 0.08	4.52 ± 0.31
CSD2	control	1.00 ± 0.07	1.57 ± 0.12	3.41 ± 0.26
	heat	1.29 ± 0.07	1.75 ± 0.01	2.69 ± 0.06*
APX2	control	1.00 ± 0.15	0.52 ± 0.04	0.33 ± 0.05
	heat	255.50 ± 9.76*	266.21 ± 18.88*	165.94 ± 8.82*
GSH1	control	1.00 ± 0.06	0.92 ± 0.08	0.92 ± 0.08
	heat	0.88 ± 0.09	0.93 ± 0.04	1.00 ± 0.03
GSH2	control	1.00 ± 0.01	0.89 ± 0.10	0.96 ± 0.08
	heat	0.86 ± 0.03	0.85 ± 0.02	1.14 ± 0.10
ROS producing genes				
RBOH-D	control	1.00 ± 0.06	0.85 ± 0.06	0.92 ± 0.10
	heat	0.69 ± 0.03	0.67 ± 0.03	0.96 ± 0.12
RBOH-F	control	1.00 ± 0.12	0.71 ± 0.06	0.76 ± 0.08
	heat	0.92 ± 0.03	0.82 ± 0.05	1.05 ± 0.03
Heat stress responsive genes				
HSP70-1	control	1.00 ± 0.04	–	–
	heat	0.74 ± 0.06*	0.93 ± 0.04	0.95 ± 0.04
HSP90-1	control	1.00 ± 0.03	–	–
	heat	1.53 ± 0.16*	3.04 ± 0.13	1.35 ± 0.18
HSP101	control	1.00 ± 0.02	–	–
	heat	1.63 ± 0.23*	3.85 ± 0.16	1.73 ± 0.19

Data are presented as mean normalized fold change ± SE of 4 biological replicates under control conditions and 3 under heat stress, relative to 0% BC under control conditions (set to 1.00). Colors indicate statistically significant differences between the 0% BC and BC-treated seedlings under the same condition: green denotes upregulation, and red denotes downregulation. A dash (-) indicates BC concentrations that were not measured. Statistically significant differences between control and heat stress conditions at the same BC concentration are indicated with an asterisk (*) (two-way ANOVA, one-way ANOVA (HSPs); $p < 0.05$). Abbreviations: *ACS2*, 1-aminocyclopropane-1-carboxylic acid synthase 2; *APX2*, ascorbate peroxidase 2; *BBX16*, B-box domain protein 16; *CAT1*, catalase 1; *CAT3*, catalase 3; *CSD1*, Cu/Zn superoxide dismutase 1; *CSD2*, Cu/Zn superoxide dismutase 2; *GSH1*, γ-glutamate-cysteine ligase; *GSH2*, glutathione synthetase; *HsfA4A*, heat shock factor A4A; *HSP101*, heat shock protein 101; *HSP70-1*, heat shock protein 70.1; *HSP90-1*, heat shock protein 90.1; *LHCB1.4*, light-harvesting chlorophyll a/b-binding protein 1.4; *OXI1*, oxidative signal-inducible kinase 1; *RBOHD*, respiratory burst oxidase homologue D; *RBOHF*, respiratory burst oxidase homologue F; *RBCS3B*, ribulose bisphosphate carboxylase small chain 3B; *WRKY33*, WRKY DNA binding protein 33; BC, biochar; DAS, days after sowing.

with an increase of 226-fold at HC compared to NHC. No significant changes in expression were detected in the glutathione-related genes *Γ-GLUTAMATE-CYSTEINE LIGASE (GSH1)* and *GLUTATHIONE SYNTHETASE (GSH2)* under either control or heat stress conditions. Similarly, the ROS-producing genes *RESPIRATORY BURST OXIDASE HOMOLOGUE F (RBOHF)* and *RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD)* were not significantly affected by BC under both conditions, although *RBOHD* showed slightly lower expression at 0% and 0.05% BC under heat stress compared to the same concentrations under control conditions.

Finally, the expression of genes involved in the heat shock response was modulated by BC under heat stress. *HEAT SHOCK PROTEIN 90.1 (HSP90-1)* and *HEAT SHOCK PROTEIN 101 (HSP101)* were significantly upregulated at 0.05% BC under heat stress compared to HC. Additionally, expression of these genes at 0% BC under heat stress was significantly higher compared to NHC. *HEAT SHOCK PROTEIN 70.1 (HSP70-1)* was significantly induced at 0.25% BC compared to HC under heat stress, although its expression at 0.25% BC remained lower compared to NHC.

BC and BCE increase fresh weight in broccoli microgreens – To validate the effects of BC and BCE on biometric growth parameters, broccoli microgreens were cultivated under control conditions, and fresh weight was measured at 6 DAS. Exposure to 1% BC resulted in a significantly higher fresh weight compared to 0% BC (Fig. 5A). For BCE, a significant increase in fresh weight was observed at 0.5% BCE compared to 0% BCE, while 1% BCE resulted in a non-significant upward trend compared to 0% BCE (Fig. 5B).

DISCUSSION

The current study investigated the effects of grapevine pruning-derived BC and BCE on seedling growth and heat stress resilience in *A. thaliana*, with the aim of elucidating underlying mechanisms behind these effects. Seedlings were cultivated in 96-well plates and treated with various concentrations of BC and BCE under both control and heat stress conditions. In addition to biometric growth measurements, mechanistic insight was obtained by analyzing BC-induced alterations in the physicochemical properties of the growth medium, cell cycle dynamics, photosynthetic efficiency, and the expression of photosynthesis- and oxidative stress-related genes.

BC promoted seedling growth under control conditions – Grapevine pruning-derived BC enhanced *A. thaliana* seedling growth in a concentration-dependent manner, with the highest root length and fresh weight observed at intermediate concentrations. This pattern is

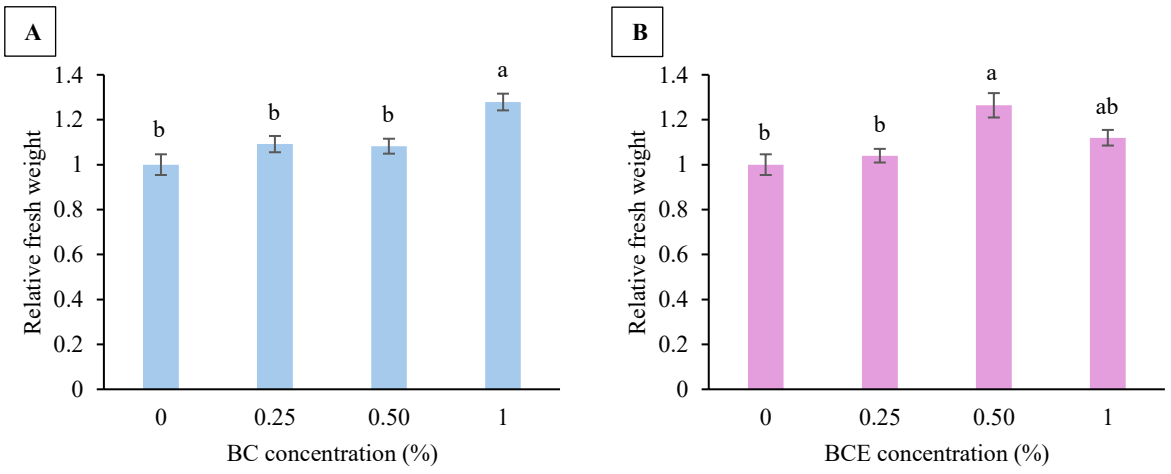

Fig. 5 – Relative fresh weight of broccoli microgreens (*Brassica oleracea var. cymosa*) at 6 DAS. Seedlings were grown under control conditions in trays containing miscanthus mixed with different BC or BCE concentrations (0%, 0.25%, 0.50%, or 1%). Relative fresh weight of seedlings exposed to BC **(A)** and BCE **(B)**. Data are presented as mean ± SE of at least 8 biological replicates relative to 0% BC (set to 1.00). Different letters denote statistically significant differences (one-way ANOVA; $p < 0.05$). Abbreviations: BC, biochar; BCE, biochar extract; DAS, days after sowing.

consistent with previous studies using BC from other feedstocks, which also reported the highest growth responses at intermediate concentrations in *A. thaliana* seedlings (10, 37). The concentration-dependent effect is likely at least partly mediated by BC-induced alterations to the physicochemical properties of the growth medium. The leaching experiment revealed increased EC, pH, and macronutrient levels, and reduced micronutrient levels with increasing BC concentrations. These findings are consistent with those reported by Kunnen *et al*. (37), in which similar shifts in the physicochemical properties of the growth medium were observed following the application of wheat-based BC. These alterations may offer a potential explanation for the diminishing growth-promoting effects observed at higher BC concentrations. Elevated EC and potassium levels have been shown to induce mild osmotic stress in plants (38, 39), and high pH can reduce nutrient solubility and bioavailability (40). Additionally, the depletion of essential micronutrients such as iron, zinc, and manganese, which are crucial for enzymatic activity and chlorophyll synthesis (41), could further restrict growth. Together, these factors may contribute to the decline in BC-induced growth stimulation at higher concentrations.

In contrast, BCE exhibited neutral to slightly negative effects on *A. thaliana* seedling growth. This aligns with findings by Oh *et al*. (28), in which wood-derived BCE had no growth-promoting effect on lettuce seedlings. However, their methodology differed from our experiment, as higher extract concentrations were applied and filter paper-based growing assays were conducted. A similar absence of growth-promoting effects was observed by French and Iyer-Pascuzzi (15), who reported that BCE derived from a woody feedstock had no effect on tomato (*Solanum pennellii*) seedling growth. In contrast, a second BCE derived from the same feedstock but produced under different pyrolysis conditions did stimulate growth (15), highlighting variability in BCE's effects. In our study, the absence of a growth-promoting effect is likely indicative of the absence of absorptive properties in BCE. In contrast to solid BC, which gradually releases nutrients over time, BCE contains only the water-soluble fraction of BC and releases its components immediately into the growth medium. This underscores the critical role of BC's absorptive characteristics in mediating its plant growth-promoting effects (9).

A subsequent analysis of nuclear DNA content revealed a decrease in the number of nuclei per µL flow cytometric extract at higher BC concentrations, suggesting a reduction in mitotic activity (30). Concurrently, lower BC concentrations led to a decrease in the endoreplication index, indicating that both cell division and endoreplication are modulated in a concentration-dependent manner. As endoreplication involves genome duplication without mitosis and leads to endopolyploidy, it is often used as a proxy for plant stress responses (30). Changes in the endoreplication index may therefore reflect BC-induced stress modulation. Together, these findings suggest that BC influences two nuclear processes essential for *A. thaliana* growth and development (42). Although growth and defense indices did not significantly differ across BC concentrations, subtle shifts suggest a minor reallocation of resources toward defense (43). This contrasts with observations by Lataf *et al.* (10), in which BC-induced shifts toward growth investment were reported in *A. thaliana* seedlings treated with manure- and wood-derived BC. Furthermore, in their study a clear alignment between biometric growth parameters and growth/defense indices was observed (10). In our study, such alignment between biometric growth parameters and cell cycle dynamics was not observed, which may be due to differences in BC feedstock. This absence of effect may also be indicative of tissue-specific responses to BC (44) that are obscured when entire seedlings are analyzed, as proposed by Kunnen *et al.* (37).

All BC concentrations enhanced F_v/F_m across all measured days, suggesting improved PSII quantum efficiency. These findings are consistent with those of previous studies conducted on safflower (*Carthamus tinctorius*) seedlings and mature alfalfa (*Medicago sativa*) leaves (45, 46). This may be explained by BC-induced stabilization of active PSII centers, thereby enabling more efficient light energy conversion by PSII (18, 47). However, chlorophyll concentrations remained unaffected, and carotene content decreased following BC addition. An increase in photosynthetic pigment levels following BC amendment was observed in mature basil (*Ocimum basilicum*) (48) and mature chickpea (*Cicer arietinum*) (49). However, studying the

effect of BC in lettuce, Christou *et al*. (50) observed no effects, consistent with our observations. These outcomes highlight the variability in BC's effects on photosynthesis (17).

Transcriptomic analysis revealed an upregulation of several genes in BC-exposed seedlings, including *BBX16*, *LHCB1.4*, and *RBCS3B*, which are associated with photomorphogenesis and photosynthesis. These three genes have been linked to karrikin signaling, which are smoke-derived compounds that can promote germination and photomorphogenesis and can be found in BC (16, 51). *BBX16* is influenced by the *KAI2* signaling pathway (unpublished data from our research group), which is essential for karrikin-induced responses such as seed germination (52). Furthermore, the *LHCB1* and *RBCS* genes have previously been demonstrated to respond to karrikin treatment in *A. thaliana* seedlings (51). These findings suggest that grapevine pruning-derived BC may release karrikins, which activate these pathways and contribute to growth promotion. However, the presence of karrikins in grapevine pruning-derived BC remains to be confirmed. In addition, BC modulated the expression of antioxidant genes. The upregulation of *CSD1*, *CSD2*, and *CAT3*, along with the downregulation of *CAT1*, indicates a shift in H_2O_2 homeostasis. Given the signaling role of H_2O_2 in plant development (53), these alterations are likely indicative of regulated redox signaling rather than oxidative stress. However, to confirm this, measurements of oxidative stress markers and quantification of H_2O_2 would be necessary.

In order to assess the translational potential of BC and BCE, their effects on broccoli microgreens were evaluated. The application of 1% BC led to a significant increase in fresh weight, thereby confirming that its growth-promoting effect is not limited to *A. thaliana* seedlings. Interestingly, BCE demonstrated a growth-promoting effect in microgreens, contrasting its neutral or slightly negative effects in *A. thaliana* seedlings. This contrast may be indicative of the role of the substrate. Broccoli microgreens were cultivated on shredded miscanthus, a fibrous material with water-retentive and absorptive properties (54). This substrate may have absorbed nutrients from BCE and released them gradually, mimicking the buffering capacity of solid BC. These findings suggest that the efficacy of BCE may depend on substrate characteristics.

BC enhanced seedling resilience to mild heat stress – Under heat stress conditions, BC maintained its growth-promoting effect in a concentration-dependent manner. This pattern was consistent with previous findings in which *Phragmites karka* plants also exhibited concentration-dependent responses to BC addition under drought stress (55). However, in our study, while an overall heat stress effect was observed in root length, this effect was not observed in fresh weight at 10 DAS. Furthermore, the negligible difference between 0% BC seedlings under control and heat stress conditions suggests partial recovery. Heat stress recovery is referred to as the ability of plants to re-establish homeostasis after sublethal heat exposure (56), which likely occurred following our short-term treatment. Consequently, only seedlings harvested at 7 DAS were used to evaluate the effects of BC under heat stress. In addition, BCE induced neutral to slightly negative effects on seedling growth under heat stress, similar to its effects under control conditions. These results are likely attributable to the lack of absorptive properties in BCE, as discussed above.

Flow cytometric analysis revealed no significant alterations in nuclear DNA content or growth-defense indices across all BC concentrations under heat stress conditions. This suggests that BC does not enhance heat stress resilience through alterations in cell division or endoreplication. However, the effects may again be obscured by measuring entire seedlings. Additionally, no significant differences were observed between control and heat stress conditions either. This contrasts with typical abiotic stress responses, in which shifts in resource allocation from growth to defense are frequently observed (30). Although heat stress has been shown to alter endoreplication during early kernel development in maize (57), Namgung *et al*. (58) reported no significant alterations in endoreplication in *A. thaliana* leaves during the recovery phase following a similar heat stress regime. This finding aligns with our observations and suggests that the seedlings may have already entered the recovery phase at 7 DAS.

Multispectral imaging revealed that increasing BC concentrations consistently led to higher F_v/F_m across all measured days under

heat stress. This trend aligns with findings reported by Chen *et al*. (59), in which BC amendment under salt stress led to similar concentration-dependent increases in F_v/F_m in cabbage (*Brassica oleracea*) seedlings. Under control conditions, no concentration-dependent differences were observed, as PSII efficiency was already near its physiological maximum (~0.83) across all BC concentrations (32). However, under heat stress conditions, this maximum is not reached, likely due to PSII becoming more susceptible to damage and inaction by ROS. These accumulate due to impaired electron transport and excess excitation energy, thereby reducing PSII efficiency (22). Although F_v/F_m did not decline under heat stress compared to control conditions in the absence of BC, the observed increase in F_v/F_m at higher BC concentrations may indicate a stabilizing effect on PSII (47). These results suggest a potential role for BC in maintaining PSII efficiency during mild heat stress conditions.

At the gene expression level, heat stress strongly induced *APX2*, a ROS-scavenging gene that protects cells from oxidative damage and enables effective recovery following heat stress (56). Concurrently, the heat shock protein genes *HSP90.1* and *HSP101* were upregulated, consistent with their known prolonged expression during the recovery phase (56). These transcriptional responses indicate that the seedlings experienced heat stress that was physiologically relevant. Furthermore, under heat stress conditions, BC also induced the expression of *BBX16*, *LHCB1.4*, and *RBCS3B*. As these genes are linked to karrikin signaling, as described above, and the *KAI2*-dependent pathway has been shown to enhance *A. thaliana* stress tolerance (60), it is plausible that this response may also be induced by karrikin, which may be present in grapevine pruning-derived BC. Furthermore, BC also increased the expression of *CSD1* and *CSD2*, consistent with previous studies reporting increased superoxide dismutase activity (61) and enhanced antioxidant enzyme activity (62) following BC addition under stress conditions. In addition, BC increased the expression of *HSP70.1*, *HSP90.1*, and *HSP101* under heat stress conditions. Similar effects were also reported by Kutlu (62), who found that urban waste- and manure-derived BCs enhanced *HSP70* and *HSP90* expression in maize plants, while pruning-derived BC did not. This suggests that the effect on heat-responsive gene expression may vary depending on the BC feedstock. Finally, the upregulation of *HSFA4A* and *WRKY33*, both downstream of *MAPK3/6* stress signaling (63, 64), suggests that BC may influence MAPK-mediated oxidative stress pathways, in addition to other stress-related and photosynthesis-related gene expression responses.

Limitations and future perspectives – It is important to note that some findings from the heat stress experiment should be interpreted with caution, as the control and heat stress experiments were conducted in separate cultivation batches. Although normalization was applied to enable comparison, residual batch effects may have influenced the results. Additionally, the extent of the applied heat stress may not have been sufficiently intense. It is plausible that seedlings had already entered the recovery phase at 7 DAS, when analyses were performed, thereby underestimating the true impact of BC under heat stress conditions. Furthermore, flow cytometric analysis revealed no substantial differences between treatments under either control or heat stress conditions. Although subtle changes in some cell cycle dynamics were detected, the extent of these effects was less pronounced than expected given the growth-promoting properties of BC. The absence of these alterations may indicate potential limitations or inconsistencies in the flow cytometric measurements or subsequent analysis. Assessing the expression of cell cycle-related genes in future studies could help clarify these observations.

To more accurately assess the effects of BC under heat stress, future experiments should apply more severe or repeated heat stress treatments to avoid analyzing seedlings during the recovery phase. Moreover, performing all experiments within the same cultivation batch will help minimize batch effects. Given the observed effects on PSII efficiency, a broader set of photosynthetic parameters and related gene markers should be included in follow-up studies to gain more mechanistic insight. Additionally, chemical characterization of grapevine pruning-derived BC may reveal organic compounds, such as karrikins or other hormone-like molecules, potentially responsible for the observed effects. If such compounds are identified, the use of signaling mutants could help clarify the underlying

pathways. Finally, BCE showed promising growth-promoting effects in the broccoli microgreen cultivation system, in contrast to its neutral or negative effects in *A. thaliana* seedlings. Future experiments using *A. thaliana* grown on solid or fibrous organic substrates may uncover conditions that supports BCE's positive effects. In addition, assessing the effects of BC and BCE on heat stress resilience in broccoli microgreens is essential to evaluate their translational potential to edible, commercially relevant crops.

CONCLUSION

In conclusion, this study demonstrated that grapevine pruning-derived BC promotes seedling growth and enhances heat stress resilience in *A. thaliana*. The consistent enhancement of photosynthetic performance following BC application suggests a key mechanistic basis for its growth-promoting effects. Under heat stress, BC further activated antioxidant and stress signaling pathways, indicating a role in redox-mediated stress mitigation. In contrast, BCE exhibited more variable effects, highlighting the importance of cultivation conditions such as substrate composition. Overall, these findings underscore the potential of grapevine pruning-derived BC as a sustainable tool to improve plant performance under both control and stress conditions, while offering mechanistic insight into its physiological effects.

REFERENCES

1. Baroi AM, Popitiu M, Fierascu I, Sărdărescu ID, Fierascu RC. Grapevine Wastes: A Rich Source of Antioxidants and Other Biologically Active Compounds. Antioxidants (Basel). 2022;11(2).
2. Prelac M, Palčić I, Cvitan D, Anđelini D, Repajić M, Ćurko J, et al. Biochar from Grapevine Pruning Residues as an Efficient Adsorbent of Polyphenolic Compounds. Materials (Basel). 2023;16(13).
3. Andelini D, Cvitan D, Prelac M, Paskovic I, Cerne M, Nemet I, et al. Biochar from Grapevine-Pruning Residues Is Affected by Grapevine Rootstock and Pyrolysis Temperature. Sustainability. 2023;15(6).
4. Sun X, Wei X, Zhang J, Ge Q, Liang Y, Ju Y, et al. Biomass estimation and physicochemical characterization of winter vine prunings in the Chinese and global grape and wine industries. Waste Manag. 2020;104:119-29.
5. Aliaño-González MJ, Richard T, Cantos-Villar E. Grapevine Cane Extracts: Raw Plant Material, Extraction Methods, Quantification, and Applications. Biomolecules. 2020;10(8).
6. Nunes LJR, Rodrigues AM, Matias JCO, Ferraz AI, Rodrigues AC. Production of Biochar from Vine Pruning: Waste Recovery in the Wine Industry. Agriculture-Basel. 2021;11(6).
7. Cabalová I, Krilek J, Kacík F, Lagana R, Jurczyková T. Valorization of Wood-Based Waste from Grapevine. Forests. 2023;14(3).
8. Hasnain M, Munir N, Abideen Z, Zulfiqar F, Koyro HW, El-Naggar A, et al. Biochar-plant interaction and detoxification strategies under abiotic stresses for achieving agricultural resilience: A critical review. Ecotoxicol Environ Saf. 2023;249:114408.
9. Chi W, Nan Q, Liu Y, Dong D, Qin Y, Li S, et al. Stress resistance enhancing with biochar application and promotion on crop growth. Biochar. 2024;6(1):43.
10. Lataf A, Pecqueur I, Huybrechts M, Carleer R, Rineau F, Yperman J, et al. Co-pyrolysis of chicken manure with tree bark for reduced biochar toxicity and enhanced plant growth in Arabidopsis thaliana. Sci Rep. 2024;14(1):13956.
11. Liu C, Sun BB, Zhang XH, Liu XY, Drosos M, Li LQ, et al. The Water-Soluble Pool in Biochar Dominates Maize Plant Growth Promotion Under Biochar Amendment. Journal of Plant Growth Regulation. 2021;40(4):1466-76.
12. Huang M, Yin XH, Chen JN, Cao FB. Biochar supplementation altered the expression of antioxidant proteins in rice leaf chloroplasts under high-temperature stress. Applied Biological Chemistry. 2024;67(1).
13. Brtnicky M, Datta R, Holatko J, Bielska L, Gusiatin ZM, Kucerik J, et al. A critical review of the possible adverse effects of biochar in the soil environment. Science of the Total Environment. 2021;796.
14. Viger M, Hancock RD, Miglietta F, Taylor G. More plant growth but less plant defence? First global gene expression data for plants grown in soil amended with biochar. Global Change Biology Bioenergy. 2015;7(4):658-72.
15. French E, Iyer-Pascuzzi AS. A role for the gibberellin pathway in biochar-mediated growth promotion. Sci Rep. 2018;8(1):5389.
16. Kochanek J, Long RL, Lisle AT, Flematti GR. Karrikins Identified in Biochars Indicate Post-Fire Chemical Cues Can Influence Community Diversity and Plant Development. PLoS One. 2016;11(8):e0161234.
17. He Y, Yao Y, Ji Y, Deng J, Zhou G, Liu R, et al. Biochar amendment boosts photosynthesis and biomass in C3 but not C4 plants: A global synthesis. GCB Bioenergy. 2020;12(8):605-17.
18. Liu XN, Zhang J, Wang Q, Chang TT, Shaghaleh H, Hamoud YA. Improvement of Photosynthesis by Biochar and Vermicompost to Enhance Tomato (Solanum lycopersicum L.) Yield under Greenhouse Conditions. Plants-Basel. 2022;11(23).
19. Duchenne-Moutien RA, Neetoo H. Climate Change and Emerging Food Safety Issues: A Review. Journal of Food Protection. 2021;84(11):1884-97.
20. Hendrix S, Dard A, Meyer AJ, Reichheld JP. Redox-mediated responses to high temperature in plants. Journal of Experimental Botany. 2023;74(8):2489-507.

21. Zeeshan M SA, Afridi MS, Jan M, Ullah A, Hu Y, Ammar M, Sajid M, Zhang Z. Biochar for Mitigation of Heat Stress in Crop Plants. Singapore: Springer.
22. Fortunato S, Lasorella C, Dipierro N, Vita F, de Pinto MC. Redox Signaling in Plant Heat Stress Response. Antioxidants. 2023;12(3).
23. Awasthi R, Bhandari K, Nayyar H. Temperature stress and redox homeostasis in agricultural crops. Frontiers in Environmental Science. 2015;3.
24. Kumar A, Friedman H, Tsechansky L, Graber ER. Distinctive in-planta acclimation responses to basal growth and acute heat stress were induced in Arabidopsis by cattle manure biochar. Scientific Reports. 2021;11(1).
25. Vercruysse W, Kunnen K, Gomes CL, Marchal W, Cuypers A, Vandamme D. Common Ivy (Hedera helix L.) Derived Biochar's Potential as a Substrate Amendment: Effects of Leached Nutrients on Arabidopsis thaliana Plant Development. Waste and Biomass Valorization. 2024;15(4):2071-82.
26. Zhang X, Zhao BW, Liu H, Zhao Y, Li LJ. Effects of pyrolysis temperature on biochar's characteristics and speciation and environmental risks of heavy metals in sewage sludge biochars. Environmental Technology & Innovation. 2022;26.
27. Carril P, Ghorbani M, Loppi S, Celletti S. Effect of Biochar Type, Concentration and Washing Conditions on the Germination Parameters of Three Model Crops. Plants-Basel. 2023;12(12).
28. Oh TK, Shinogi Y, Chikushi J, Lee YH, Choi B. Effect of Aqueous Extract of Biochar on Germination and Seedling Growth of Lettuce (Lactuca sativa L.). Journal of the Faculty of Agriculture Kyushu University. 2012;57(1):55-60.
29. Yuan J, Meng J, Liang X, Yang E, Yang X, Chen WF. Biochar's Leacheates Affect the Abscisic Acid Pathway in Rice Seedlings Under Low Temperature. Frontiers in Plant Science. 2021;12.
30. Hendrix S, Keunen E, Mertens AIG, Beemster GTS, Vangronsveld J, Cuypers A. Cell cycle regulation in different leaves of Arabidopsis thaliana plants grown under control and cadmium-exposed conditions. Environmental and Experimental Botany. 2018;155:441-52.
31. Cuypers A JM, Sophie H. Method for determining a toxicity and/or growth promotion effect of a treatment or compound. International: WIPO. 2022.
32. Baker NR. Chlorophyll fluorescence: A probe of photosynthesis in vivo. Annual Review of Plant Biology. 2008;59:89-113.
33. Wellburn AR, Lichtenthaler H. Formulae and Program to Determine Total Carotenoids and Chlorophylls A and B of Leaf Extracts in Different Solvents. In: Sybesma C, editor. Advances in Photosynthesis Research: Proceedings of the VIth International Congress on Photosynthesis, Brussels, Belgium, August 1–6, 1983 Volume 2. Dordrecht: Springer Netherlands; 1984. p. 9-12.
34. Valledor L, Escandón M, Meijón M, Nukarinen E, Cañal MJ, Weckwerth W. A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. Plant J. 2014;79(1):173-80.
35. Remans T, Keunen E, Bex GJ, Smeets K, Vangronsveld J, Cuypers A. Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. Plant Cell. 2014;26(10):3829-37.
36. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 2007;8(2):R19.
37. Kunnen K, Ali MM, Lataf A, Van Hees M, Nauts R, Horemans N, et al. From crop left-overs to nutrient resource: growth-stimulating potential of biochar in nutrient solutions for wheat soilless cultivation systems. Front Plant Sci. 2024;15:1414212.
38. Ding X, Jiang Y, Zhao H, Guo D, He L, Liu F, et al. Electrical conductivity of nutrient solution influenced photosynthesis, quality, and antioxidant enzyme activity of pakchoi (Brassica campestris L. ssp. Chinensis) in a hydroponic system. PLoS One. 2018;13(8):e0202090.
39. Pantha P, Oh DH, Longstreth D, Dassanayake M. Living with high potassium: Balance between nutrient acquisition and K-induced salt stress signaling. Plant Physiol. 2023;191(2):1102-21.

40. Langenfeld NJ, Pinto DF, Faust JE, Heins R, Bugbee B. Principles of Nutrient and Water Management for Indoor Agriculture. Sustainability. 2022;14(16).
41. Ahmed N, Zhang BG, Chachar Z, Li J, Xiao GS, Wang Q, et al. Micronutrients and their effects on Horticultural crop quality, productivity and sustainability. Scientia Horticulturae. 2024;323.
42. Sablowski R, Carnier Dornelas M. Interplay between cell growth and cell cycle in plants. J Exp Bot. 2014;65(10):2703-14.
43. Hendrix S, Alfano R, Plusquin M, Cuypers A. Comparing cadmium-induced effects on the regulation of the DNA damage response and cell cycle progression between entire rosettes and individual leaves of Arabidopsis thaliana. Plant Physiology and Biochemistry. 2023;204.
44. Solaiman ZM, Murphy DV, Abbott LK. Biochars influence seed germination and early growth of seedlings. Plant and Soil. 2012;353(1-2):273-87.
45. Yan XQ, Wang ZJ, Zhao MQ, Hao JF, Liu JY, Yan YT, et al. Hydrothermal biochar enhances the photosynthetic efficiency and yield of alfalfa by optimizing soil chemical properties and stimulating the activity of microbial communities. Scientific Reports. 2024;14(1).
46. Ghassemi-Golezani K, Farhangi-Abriz S. Biochar alleviates fluoride toxicity and oxidative stress in safflower (Carthamus tinctorius L.) seedlings. Chemosphere. 2019;223:406-15.
47. Kalaji HM, Baba W, Gediga K, Goltsev V, Samborska IA, Cetner MD, et al. Chlorophyll fluorescence as a tool for nutrient status identification in rapeseed plants. Photosynthesis Research. 2018;136(3):329-43.
48. Jabborova D, Ma H, Bellingrath-Kimura SD, Wirth S. Impacts of biochar on basil (Ocimum basilicum) growth, root morphological traits, plant biochemical and physiological properties and soil enzymatic activities. Scientia Horticulturae. 2021;290.
49. Hashem A, Kumar A, Al-Dbass AM, Alqarawi AA, Al-Arjani AF, Singh G, et al. Arbuscular mycorrhizal fungi and biochar improves drought tolerance in chickpea. Saudi Journal of Biological Sciences. 2019;26(3):614-24.
50. Christou A, Stylianou M, Georgiadou EC, Gedeon S, Ioannou A, Michael C, et al. Effects of biochar derived from the pyrolysis of either biosolids, manure or spent coffee grounds on the growth, physiology and quality attributes of field-grown lettuce plants. Environmental Technology & Innovation. 2022;26.
51. Thussagunpanit J, Nagai Y, Nagae M, Mashiguchi K, Mitsuda N, Ohme-Takagi M, et al. Involvement of STH7 in light-adapted development in Arabidopsis thaliana promoted by both strigolactone and karrikin. Bioscience Biotechnology and Biochemistry. 2017;81(2):292-301.
52. Waters MT, Nelson DC, Scaffidi A, Flematti GR, Sun YK, Dixon KW, et al. Specialisation within the DWARF14 protein family confers distinct responses to karrikins and strigolactones in Arabidopsis. Development. 2012;139(7):1285-95.
53. Nazir F, Fariduddin Q, Khan TA. Hydrogen peroxide as a signalling molecule in plants and its crosstalk with other plant growth regulators under heavy metal stress. Chemosphere. 2020;252.
54. Nguyen VH, Kraska T, Winkler W, Aydinlik S, Jackson BE, Pude R. Primary Mechanical Modification to Improve Performance of Miscanthus as Stand-Alone Growing Substrates. Agronomy-Basel. 2022;12(2).
55. Abideen Z, Koyro HW, Huchzermeyer B, Ansari R, Zulfiqar F, Gul B. Ameliorating effects of biochar on photosynthetic efficiency and antioxidant defence of Phragmites karka under drought stress. Plant Biology. 2020;22(2):259-66.
56. Oyoshi K, Katano K, Yunose M, Suzuki N. Memory of 5-min heat stress inArabidopsis thaliana. Plant Signaling & Behavior. 2020;15(8).
57. Monjardino P, Smith AG, Jones RJ. Zein transcription and endoreduplication in maize endosperm are differentially affected by heat stress. Crop Science. 2006;46(6):2581-9.
58. Namgung Y, Lee HG, Lee H, Seo PJ. Heat-induced leaf epidermal cell damage triggers autophagy-mediated mesophyll cell expansion in Arabidopsis. Plant Commun. 2024;5(3):100770.
59. Chen RX, Zheng LJ, Zhao JJ, Ma JJ, Li XF. Biochar Application Maintains Photosynthesis of Cabbage by Regulating Stomatal Parameters in Salt-Stressed Soil. Sustainability. 2023;15(5).

60. Wang L, Waters MT, Smith SM. Karrikin-KAI2 signalling provides Arabidopsis seeds with tolerance to abiotic stress and inhibits germination under conditions unfavourable to seedling establishment. New Phytol. 2018;219(2):605-18.
61. Ye Y, Qiuxue Z, Tongtong Y, Congcong C, Yue W, Chan Q, et al. Biochar modulates the antioxidant system and hormonal signaling in tobacco under continuous-cropping conditions. Journal of Plant Interactions. 2024;19(1).
62. Kutlu I. Biochar-mediated Stimulation of Antioxidant Defense and Heat Shock Protein Expression in Maize Under Extreme Temperature Stress. Journal of Plant Growth Regulation. 2024.
63. Pérez-Salamó I, Papdi C, Rigó G, Zsigmond L, Vilela B, Lumbreras V, et al. The Heat Shock Factor A4A Confers Salt Tolerance and Is Regulated by Oxidative Stress and the Mitogen-Activated Protein Kinases MPK3 and MPK6. Plant Physiology. 2014;165(1):319-34.
64. Mao GH, Meng XZ, Liu YD, Zheng ZY, Chen ZX, Zhang SQ. Phosphorylation of a WRKY Transcription Factor by Two Pathogen-Responsive MAPKs Drives Phytoalexin Biosynthesis in Arabidopsis. Plant Cell. 2011;23(4):1639-53.

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SUPPORTING INFORMATION

Table S1 – Sequences of forward and reverse primers used during qPCR.

Gene	Locus	Forward primer	Reverse primer
BBX16	AT1G73870	TGGGATAATCACGGTTCGCC	CGTACCCACCCACAACATGA
LHCB1.4	AT2G34430	CGTCCCCGGAAAGTGAGTT	TGCAACAAACCGGATACACAC
RBCS3B	AT5G38410	CCTATTGTCTGTGTTCTTTTTCTCTTTATG	TCAAGACGCACGGATATATAAATTACA
ACS2	AT1G01480	CATGTTCTGCCTTGCGGATC	ACCTGTCCGCCACCTCAAGT
HsfA4A	AT4G18880	GAGTTTTCTAGAGATCTTCTTCCGAGATTC	TCCCATTGCTCAGGATCAGC
OXI1	AT3G25250	TAGAGGATCGAACCGGAAAG	GACCCTTGATTTCCTCAACG
WRKY33	AT2G38470	TCATCGATTGTCAGCAGAGACG	CCATTCCCACCATTTGTTTCAT
CAT1	AT1G20630	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
CAT3	AT1G20620	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
CSD1	AT1G08830	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
CSD2	AT2G28190	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
APX2	AT3G09640	TTGCTGTTGAGATCACTGGAGGA	TGAGGCAGACGACCTTCAGG
GSH1	AT4G23100	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
GSH2	AT5G27380	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
RBOH-D	AT5G47910	AACTCTCCGCTGATTCCAACG	TGGTCAGCGAAGTCTTTAGATTCCT
RBOH-F	AT1G64060	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA
HSP70-1	AT5G02500	AAGGAAACAGAACCACGCCA	TGTCAGAGAAACGACGACCG
HSP90-1	AT5G52640	GGACAGCCTGAACTCTTCATTAGA	CGCCTCCATAAACTCTTTTGTTCC
HSP101	AT1G74310	ACAACACTCTGTCTCTCGCC	TGAAGACAGCAACATGAGCCT

Abbreviations: *ACS2*, 1-aminocyclopropane-1-carboxylic acid synthase 2; *APX2*, ascorbate peroxidase 2; *BBX16*, B-box domain protein 16; *CAT1*, catalase 1; *CAT3*, catalase 3; *CSD1*, Cu/Zn superoxide dismutase 1; *CSD2*, Cu/Zn superoxide dismutase 2; *GSH1*, γ-glutamate-cysteine ligase; *GSH2*, glutathione synthetase; *HsfA4A*, heat shock factor A4A; *HSP101*, heat shock protein 101; *HSP70-1*, heat shock protein 70.1; *HSP90-1*, heat shock protein 90.1; *LHCB1.4*, light-harvesting chlorophyll a/b-binding protein 1.4; *OXI1*, oxidative signal-inducible kinase 1; *RBOHD*, respiratory burst oxidase homologue D; *RBOHF*, respiratory burst oxidase homologue F; *RBCS3B*, ribulose bisphosphate carboxylase small chain 3B; *WRKY33*, WRKY DNA binding protein 33.

Table S2 – Physicochemical characteristics of BC and 5% (w/v) BCE.

Characteristic	Mean ± SE
BC	
C (%)	71.71 ± 1.01
H (%)	3.10 ± 0.04
N (%)	1.19 ± 0.02
Ash (%)	11.37 ± 0.05
BC yield (%)	25.87
BCE	
NPOC (mg/L)	182.60
TN (mg/L)	2.05
BC/BCE	
EC (µS/cm)	1608.3 ± 19.5
pH	9.03 ± 0.28

Data are presented as mean ± SE of at least 3 biological replicates. Abbreviations: C, carbon; H, hydrogen; N, nitrogen; NPOC, non-purgeable organic carbon; TN, total nitrogen; EC, electrical conductivity; BC, biochar; BCE, biochar extract.

Table S3 – Nutrient concentrations of BC.

Element	Mean (mg/kg DW) ± SE
Macronutrients	
Ca	23346 ± 4
K	14165 ± 220
Mg	154418 ± 55
P	4062 ± 20
S	1584 ± 87
Micronutrients	
Cu	58.3 ± 7.8
Fe	298.8 ± 4.8
Mn	65.4 ± 0.1
Na	1824.5 ± 27.8
Ni	< 10.0
Zn	167.0 ± 0.8
Potential toxic elements	
Cd	< 2.0
Cr	13.72 ± 0.12
Hg	< 20.0
Pb	< 10.0
Other elements	
Ag	< 10.0
Al	556.1 ± 56.3
Ba	18.8 ± 1.7
Co	< 10.0
Li	187.5 ± 77.8
Sr	82.5 ± 0.1

Data are presented as mean ± SE (mg/kg DW) of at least 3 biological replicates. Abbreviations: Ag, silver; Al, aluminum; Ba, barium; Ca, calcium; Cd, cadmium; Co, cobalt; Cr, chromium; Cu, copper; Fe, iron; Hg, mercury; K, potassium; Li, lithium; Mg, magnesium; Mn, manganese; Na, sodium; Ni, nickel; P, phosphorus; Pb, lead; S, sulfur; Sr, strontium; Zn, zinc; BC, biochar; DW, dry weight.

Table S4 – Nutrient concentrations of 5% (w/v) BCE.

Element	Mean (mg/L) ± SE
Macronutrients	
Ca	28.99 ± 0.26
K	341.55 ± 1.05
Mg	17.71 ± 0.03
P	1.818 ± 0.003
S	36.56 ± 0.17
Micronutrients	
Cu	< 0.25
Fe	0.42 ± 0.01
Mn	< 0.05
Na	25.51 ± 0.02
Ni	< 0.25
Zn	< 0.25
Potential toxic elements	
Cd	< 0.05
Cr	< 0.25
Pb	< 0.25
Hg	< 0.5
Other elements	
Ag	< 0.25
Al	< 0.25
Ba	< 0.25
Co	< 0.25
Li	< 0.025
Sr	< 0.25

Data are presented as mean ± SE (mg/L) of at least 3 biological replicates. Abbreviations: Ag, silver; Al, aluminum; Ba, barium; Ca, calcium; Cd, cadmium; Co, cobalt; Cr, chromium; Cu, copper; Fe, iron; Hg, mercury; K, potassium; Li, lithium; Mg, magnesium; Mn, manganese; Na, sodium; Ni, nickel; P, phosphorus; Pb, lead; S, sulfur; Sr, strontium; Zn, zinc; BCE, biochar extract.

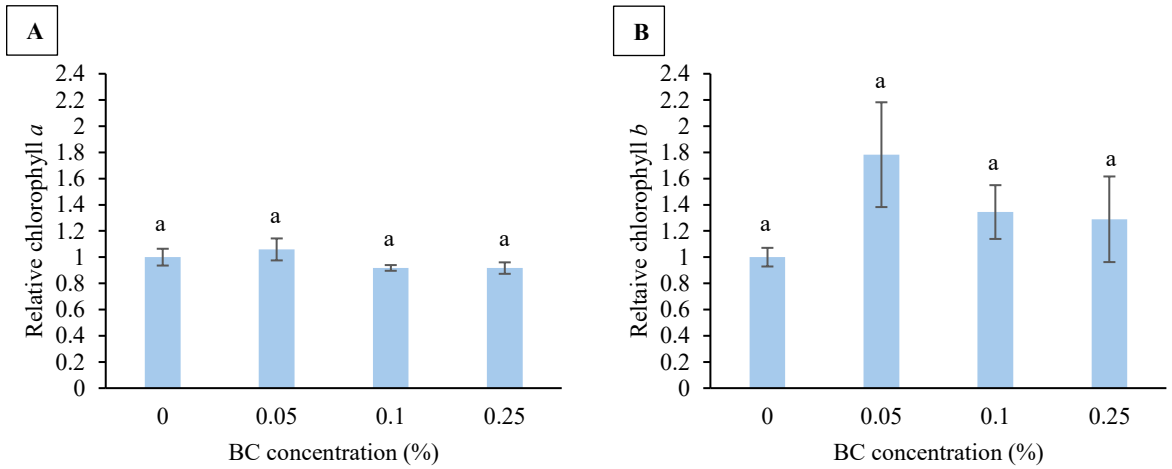

Fig. S1 – Relative photosynthetic pigment levels in *Arabidopsis thaliana* seedlings at 10 DAS. Seedlings were grown under control conditions in 96-well plates containing ¼ MS medium supplemented with different BC concentrations (0%, 0.05%, 0.10%, or 0.25%). Relative chlorophyll *a* **(A)** and b **(B)** content of seedlings exposed to BC. Data are presented as mean ± SE of at least 5 biological replicates relative to 0% BC (set to 1.00). Different letters denote statistically significant differences (one-way ANOVA **(A)**, Kruskal-Wallis test **(B)**; $p < 0.05$). Abbreviations: BC, biochar; DAS, days after sowing.