

Organ specific chrysanthemum biochar for circular waste reuse and sustainable plant production

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Abstract

Biochar (BC), a low-cost, carbon-rich material produced from a wide variety of biomass sources, is gaining increasing attention in horticultural practice. There is little research on the use of chrysanthemum residues in BC production, especially in the circular production of subsequent plant generations. This study aimed to compare the usefulness of BC derived from various organs of chrysanthemum waste during the acclimatization stage of new chrysanthemum plantlets. Roots, shoots, and flowers of glasshouse-grown chrysanthemums were pyrolyzed at 400 °C for 60 min. The obtained BCs were mixed with peat and perlite (2:1) at concentrations of 4%, 8%, and 12%. In vitro-derived plantlets were grown in the so-prepared substrate for 30 days and then subjected to biometric, biochemical, and physiological analyses. The composition of microorganisms in the growing substrates was compared, and the impact of the tested BCs on substrate utilization patterns of rhizosphere microbiome was evaluated. Both the concentration and source of biochar were found to influence its properties. The longest shoots, the highest number of leaves, and the highest chlorophyll content were obtained in the control, while high BC concentrations (12%, regardless of type) inhibited plant elongation. The application of BC, particularly from shoots or roots, at 4 or 8%, increased the fresh weight of shoots and roots, as well as leaf surface area. Biochar generally did not affect root architecture, but 12% flower-derived BC reduced root development. Biochar stimulated the synthesis of bioactive compounds and the activity of antioxidant enzymes, with the best results achieved when BC from flowers or shoots was applied at lower concentrations. Most BC combinations did not reduce plant photosynthetic parameters compared with the control, and some (e.g., 8% root BC) improved photosynthesis efficiency. Increasing carbon supplementation to plants reduced the functional diversity of root-associated bacterial isolates; however, the highest BC dose (12%) increased the numbers of bacteria and Actinobacteria in the growing substrate, without affecting filamentous fungi. Consequently, the use of shoot and flower BC (especially at 12%) increased the activity of most of the tested soil enzymes, while BC from roots showed a weaker or inhibitory effect. The obtained results highlight the utility of BC in chrysanthemum production.

Keywords Acclimatization · Enzymatic activity · Microorganisms · Plant physiology · Sustainable horticulture

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Introduction

Chrysanthemum (*Chrysanthemum* × *morifolium* /Ramat./ Hemsl.) of the Asteraceae family is among the most important species on the floristic market¹. Its popularity stems from its unique ornamental value and long flowering duration². *Chrysanthemum* inflorescences are also used in herbal medicine for their biologically active metabolites with cardiovascular, antimicrobial, anti-inflammatory, and antioxidative/anticancer/antitumor properties^{3,4}. Due to diverse applications in pharmaceuticals, food, and cosmetics, chrysanthemum cultivation is continually expanding, with annual production exceeding one million tons⁵. In 2022, the global chrysanthemum herbal tea market was valued at USD 153.1 million. This market is expected to grow by more than 50% by 2030⁶. Consequently, it is considered one of the most economically valuable edible/pharmaceutical flowers in the world⁴. This results in massive amounts of biowaste that have not been reused to date.

Like other herbaceous perennials, chrysanthemums are increasingly produced in vitro⁷. Micropropagation is a biotechnological method of vegetative propagation that uses laboratory facilities to rapidly obtain large numbers of genetically identical plants from a small amount of initial material, as each cell can give rise to a new plant. This method enables whole-year production, regardless of season or weather conditions, which is particularly useful with ornamental plants⁸. The technology has advanced significantly in recent decades, including the optimization of explant selection and disinfection, medium composition, the use of novel plant growth regulators (PGRs), and alternative light sources to overcome the shortcomings of conventional propagation techniques⁷. One of the main limitations of micropropagation is the difficulty in acclimatizing plantlets to nursery conditions. This is because in vitro-grown plants suffer from several morphophysiological disorders, such as dysfunctional stomata, a thin cuticle, and reduced photosynthetic capacity⁹. After ex vitro transplantation, these plants must adapt to entirely different, hazardous conditions. These include microbial infections, pest activity, temperature and lighting fluctuations, low humidity, and inadequate nutrition, which can lead to stress and reduced survival¹⁰. Furthermore, some cultivars have difficulty developing a functional root system, as adventitious shoots may lack vascular connections to the shoots¹¹. The use of biochar as a supplement to growing media may solve these problems.

Biochar (BC) is a product obtained in the pyrolysis process, i.e., anaerobic (or with minimal oxygen access) decomposition of biomass at temperatures ranging from 400 to 700 °C¹². It can be obtained from various types of biomass, e.g., algae, bark of woody plants, cellulose, fruit peels, hemicellulose, leaves of trees, lignin, straw, tea waste, and woody biomass (acacia, beech, eucalyptus, oak, pine, poplar, and other)¹³. Parameters of the initial feedstock directly affect the quality of the produced BC, even under similar conditions of pyrolysis. For example, Bednik et al.¹⁴ observed that BC from food waste had a less aromatic structure and higher content of labile carbon fractions, resulting in lower stability, compared to BC derived from high lignocellulose materials (grass, straw, and wood) that were strongly carbonized, with a persistent, aromatic structure.

Biochar is characterized by well-developed microporous and aromatic structure. These qualities make it useful in agriculture and animal husbandry, bioremediation (reduction of greenhouse gas emissions), and bioenergy (heat and power production)¹⁵. In horticulture, BC is used to improve soil structure, increase its ability to retain water and nutrients, and as a carrier of microorganisms that improve soil fertility and plant growth¹⁶. In the study on coral berry (*Ardisia crenata* Sims) cultivation, a fruit ornamental, the addition of BC increased soil nutrient content (14% rise in total nitrogen, 564% boost in total phosphorus, and 63% increase in total potassium). Moreover, it improved the physicochemical properties of the soil by reducing soil bulk density, increasing total porosity, and enhancing pore water. The growth of *A. crenata* was improved when BC was added at a concentration range of 30% to 50%, resulting in improved root parameters (root length, surface area, and volume by 49%, 62%, and 24%). Moreover, an increase in chlorophyll *a*, chlorophyll *b*, and the net photosynthetic rate was noted¹⁷. Biochar application at 3% increased plant height, fresh weight, number of leaves, as well as stem and flower diameter in garland chrysanthemum (*Chrysanthemum coronarium* L.)¹⁸. Zulfiqar et al.¹⁹ found that wheat straw-derived BC added at 20% volume concentration (alone or with compost) to peat-based substrate increased

growth, leaf NPK content, chlorophyll content, and photosynthetic rate in arrowhead plant (*Syngonium podophyllum* Schott).

Apart from plants, the use of biochar has a direct impact on the soil microbiome²⁰. compared the effect of BC derived from woodchips and bone-meat residues from a poultry slaughterhouse. They observed that both BC types notably affected microbial communities in the soil, rhizosphere, and roots of wheat (*Triticum aestivum* L.), although to different extents. In a sanqi (*Panax notoginseng* /Burkill/ F.H.Chen) production system, it stimulated the growth of beneficial bacteria *Bacillus* and *Lysobacter*, but suppressed pathogens *Fusarium* and *Ilyonectria*²¹. It was also found that the addition of BC increased the soil pH, electrical conductivity, organic matter, available P and K, and C/N ratio²¹. Jaiswal et al.²² observed significant differences in rhizosphere bacterial taxonomical composition between BC-amended and non-amended treatments in tomato (*Solanum lycopersicum* L.) cultivation. BC caused an increase in microbiome diversity and activity. At the same time, BC reduced *Fusarium* root colonization and root-rot, simultaneously improving plant growth and physiological parameters. The changes in microbiome functionality often correspond with altered soil enzymatic activity. Iturbe-Espinoza et al.²³ reported that BC amendment decreased the phosphomonoesterase activity, suggesting improved soil phosphate bioavailability, accompanied by an elevated prokaryotic diversity index. Pokharel et al.²⁴ analyzed 964 data points from 72 papers and found that BC either increased or had no negative effects on the activity of soil enzymes analyzed, such as urease, alkaline phosphatase, and dehydrogenase, which corresponded with increased microbial biomass. The effect of BC on the soil microbiome and rhizobiome was thoroughly reviewed by Samuditha et al.²⁵. In summary, biochar can have a beneficial impact on soil microorganisms if used in appropriate conditions²².

To date, there is little information on the use of chrysanthemum plant waste in the production of BC, although a large amount of flower waste is disposed of each year from cemeteries, religious rituals, and other festivities. Panditha et al.²⁶ used an aqueous extract of chrysanthemum waste-derived BC on the germination of Indian mustard (*Brassica juncea* /L./ Czern.) seeds. They reported that the application of BC resulted in similar or promoted germination, shoot, and root growth of Indian mustard compared to the control, depending on the pyrolysis conditions. Importantly, ornamental plant-derived BC has not been used in a closed cultivation cycle, i.e., for the production of subsequent plant generations of the same species, which could be valuable for horticulturalists. The effectiveness of BC pyrolyzed from different plant parts (roots, shoots, flowers) has not been compared. There is also no information on its use during the acclimatization phase of in vitro-produced plantlets.

This research aimed: (1) to produce and characterize biochars from various chrysanthemum plant parts (roots, shoots, and flowers), (2) to learn the effect of BC on the ex vitro growth of chrysanthemums in a circular cultivation system, their physiological properties and biochemical activity, and (3) to describe the effect of BC on the microorganisms of roots and growth substrate, as well as its enzymatic activity. None of these objectives were studied previously.

The research hypothesis assumed that the addition of biochar into the growing substrate would improve its structure and physicochemical properties, positively affect the substrate microorganisms, and promote the development of the plant root system. This would result in more ecological plant production with improved quality. Simultaneously, it was assumed that the efficiency of BC would vary, depending on the concentration and plant part from which it was produced (root, stem, or flower).

Results

Effect of biochar on chrysanthemum biometric traits

The longest shoots were produced by control plants (9.1 cm) and plants treated with 4% root BC (8.0 cm), whereas the shortest ones (5.8–5.9 cm) were found in the combinations with the highest (12%) BC content, regardless of its type (Table 2). The highest number of leaves per plant (12.1–12.3) was counted in the control and after the addition of 8% shoot BC, while the lowest was after the application of 12% flower BC. Plants cultivated

Table 1 Effect of biochar (BC) source and its concentration in the growing substrate on the mean shoot length, number of leaves per shoot, shoot and root fresh weight (FW), and root dry weight (DW), of chrysanthemum after 30 days of ex vitro cultivation.

Biochar type and concentration	Shoot length (cm)	Number of leaves	Shoot FW (g)	Root FW (mg)	Root DW (mg)
Control	9.1±0.9 a	12.1±0.6 a	1.58±0.15 a-c	300.6±40.0 bc	41.7±6.2 a
Root BC 4%	8.0±0.4 a	11.0±0.6 a-c	1.72±0.17 ab	320.3±49.0 a-c	34.1±5.1 a
Root BC 8%	7.8±0.5 ab	11.6±0.5 a-c	1.79±0.16 ab	338.4±63.7 a-c	40.7±4.4 a
Root BC 12%	5.9±0.4 c	11.0±0.7 a-c	1.31±0.10 cd	404.9±62.3 ab	41.4±6.0 a
Shoot BC 4%	7.6±0.6 ab	11.0±0.5 a-c	1.76±0.17 ab	363.8±38.2 a-c	44.8±5.8 a
Shoot BC 8%	7.9±0.7 ab	12.3±0.5 a	1.97±0.18 a	482.5±60.7 a	44.7±3.4 a
Shoot BC 12%	5.9±0.3 c	10.6±0.5 bc	1.61±1.42 a-c	373.1±61.5 ab	51.8±9.7 a
Flower BC 4%	6.5±0.3 bc	12.0±0.4 ab	1.37±0.05 b-d	421.6±105.1 ab	48.5±10.9 a
Flower BC 8%	7.7±0.4 ab	12.1±0.5 a	1.58±0.10 a-c	321.5±40.0 a-c	39.1±5.7 a
Flower BC 12%	5.8±0.5 c	10.5±0.5 c	1.17±0.12 d	199.2±39.4 c	23.4±5.2 b

Fisher's < 0.05; ± standard error.

Table 2 Effect of biochar (BC) source and its concentration in the growing substrate on the development of chrysanthemum leaves after 30 days of ex vitro cultivation.

Biochar type and concentration	Surface area (cm ²)	Perimeter (cm)	Length (cm)	Max. width (cm)	Mean width (cm)	Aspect ratio	Form coefficient
Control	6.28±0.45 b	13.65±0.52 ab	4.29±0.16 ab	2.83±0.10 b	1.436±0.051 bc	0.661±0.008 b-d	0.415±0.007 e
Root BC 4%	7.83±0.51 a	14.48±0.50 a	4.51±0.15 a	3.17±0.10 a	1.720±0.051 a	0.706±0.011 a	0.463±0.007 ab
Root BC 8%	6.29±0.33 b	12.99±0.36 bc	4.16±0.12 ab	2.85±0.08 b	1.497±0.044 bc	0.686±0.010 a-c	0.463±0.007 ab
Root BC 12%	5.10±0.29 c	11.56±0.29 de	3.67±0.09 cd	2.53±0.08 c	1.380±0.047 cd	0.691±0.013 ab	0.471±0.008 ab
Shoot BC 4%	6.37±0.37 b	13.19±0.26 b	4.22±0.12 ab	2.83±0.09 b	1.493±0.053 bc	0.670±0.012 bc	0.452±0.009 bc
Shoot BC 8%	6.35±0.43 b	13.37±0.47 b	4.25±0.15 ab	2.80±0.09 b	1.468±0.052 bc	0.660±0.008 b-d	0.438±0.009 cd
Shoot BC 12%	6.31±0.47 b	12.67±0.44 b-d	4.00±0.15 bc	2.82±0.10 b	1.541±0.056 b	0.707±0.010 a	0.481±0.006 a
Flower BC 4%	4.93±0.19 c	12.02±0.22 c-e	3.91±0.06 b-d	2.49±0.07 c	1.268±0.043 d	0.637±0.016 d	0.426±0.007 de
Flower BC 8%	5.64±0.30 bc	12.68±0.30 bc	4.09±0.09 b	2.68±0.08 bc	1.371±0.052 cd	0.655±0.014 cd	0.438±0.008 c-e
Flower BC 12%	5.10±0.35 c	11.56±0.39 e	3.67±0.13 d	2.52±0.09 c	1.369±0.050 cd	0.691±0.014 ab	0.470±0.008 ab

Fisher's < 0.05; ± standard error. Aspect ratio = leaf length ÷ leaf width; Form coefficient – expressing the relationship between the geometric dimensions of the leaf (area and perimeter).

in the substrate with 8% shoot BC had the highest shoot fresh weight (1.97 g; i.e., 24.7% more than the control group), while those grown with 12% root or flower BC had the lowest fresh weight (1.17–1.31 g; i.e., 25.9% less than the control group). Likewise, the highest and lowest root FW were found in the experimental combinations with 8% shoot (482.5 mg) and 12% flower BC (199.2 mg), respectively. This means a 60.5% and 33.7% increase/decreases, respectively, compared to the control. Most plants did not differ in root DW, although those from the 12% flower BC combination had significantly lower value of this trait (Table 1).

Leaves with the greatest area, perimeter, length, and maximal and mean width were found in plants grown in a substrate with 4% root BC (Table 2). Leaf area was therefore 24.7% larger than in the control. In contrast, leaves from experimental combinations containing root BC at 12% and flower BC, regardless of concentration, were usually the smallest. Likewise, root BC at 4% and shoot BC at 12% stimulated the development of leaves with the greatest length-to-width ratio, whereas flower BC at 4 and 8% the lowest. Consequently, biochar affected leaf shape. Leaves of chrysanthemums grown with 12% shoot BC had the highest form coefficients (0.481; i.e., they were more elongated), while the control had the lowest (0.415; i.e., more rounded) (Table 2).

Most experimental treatments did not differ statistically in root length, area, diameter, or volume. However, the lowest values of these parameters were observed in the root systems from the 12% flower BC treatment (Table 3). On the other hand, the highest number of shoot tips and bifurcations was found in treatments with 12% root BC and 4% shoot BC, respectively (an increase of 77.5% compared to the control group), whereas the lowest was observed after applying 12% flower BC (Table 3). Despite no statistical differences, applying 8% shoot

Table 3 Effect of biochar (BC) source and its concentration in the growing substrate on the architecture of the chrysanthemum root system after 30 days of ex vitro cultivation.

Biochar type and concentration	Length (cm)	Surface area (cm ²)	Diameter (mm)	Volume (cm ³)	Number of tips	Number of bifurcations
Control	115.9±20.3 ab	16.48±2.84 ab	0.456±0.016 ab	0.188±0.034 ab	283.6±43.0 c-e	529.5±104.1 c-e
Root BC 4%	127.9±17.7 a	17.96±2.83 a	0.441±0.011 b	0.202±0.036 ab	275.6±45.1 de	643.8±96.5 a-d
Root BC 8%	147.3±18.1 a	21.00±3.00 a	0.446±0.020 b	0.241±0.041 a	403.5±44.8 a-c	824.3±131.1 a-c
Root BC 12%	161.7±18.9 a	23.56±2.92 a	0.465±0.024 ab	0.277±0.039 a	474.3±43.9 a	911.4±116.0 ab
Shoot BC 4%	159.7±14.8 a	22.85±2.23 a	0.455±0.013 ab	0.262±0.028 a	347.9±40.6 a-d	939.8±128.9 a
Shoot BC 8%	152.7±13.3 a	24.15±2.49 a	0.501±0.024 a	0.309±0.044 a	405.3±60.1 a-d	802.3±76.9 a-c
Shoot BC 12%	146.2±11.5 a	23.20±2.41 a	0.499±0.021 a	0.297±0.042 a	449.8±30.8 ab	713.1±73.9 a-d
Flower BC 4%	140.8±30.8 a	22.80±5.75 a	0.489±0.024 ab	0.298±0.088 a	449.4±85.3 a-c	691.1±186.9 b-d
Flower BC 8%	118.3±16.4 a	17.32±2.52 a	0.469±0.015 ab	0.203±0.032 ab	314.6±41.1 b-d	471.8±72.0 de
Flower BC 12%	82.0±15.6 b	11.44±2.20 b	0.445±0.015 b	0.128±0.026 b	222.0±39.4 e	342.5±65.4 e

Fisher's < 0.05; ± standard error. Bifurcation is the place where the main root splits into two lateral roots.

Table 4 Effect of biochar (BC) source and its concentration in the growing substrate on the relative pigment content in chrysanthemum leaves after 30 days of ex vitro cultivation.

Biochar type and concentration	Flavonols	Anthocyanins	Chlorophyll	SPAD
Control	0.552±0.028 bc	0.009±0.002 e	9.56±0.23 a	32.19±0.39 a
Root BC 4%	0.459±0.026 de	0.022±0.001 a	8.60±0.22 bc	30.47±0.41 bc
Root BC 8%	0.410±0.021 de	0.017±0.002 cd	8.63±0.23 bc	30.54±0.40 bc
Root BC 12%	0.619±0.033 b	0.021±0.002 a-c	9.09±0.23 ab	31.35±0.41 ab
Shoot BC 4%	0.471±0.038 e	0.023±0.002 a	7.71±0.16 de	28.79±0.32 ef
Shoot BC 8%	0.498±0.043 c-e	0.016±0.002 d	8.72±0.29 bc	30.60±0.51 bc
Shoot BC 12%	0.489±0.046 de	0.022±0.002 bc	7.22±0.15 e	27.74±0.36 f
Flower BC 4%	0.766±0.036 a	0.016±0.002 cd	7.98±0.18 d	29.31±0.35 de
Flower BC 8%	0.518±0.022 b-d	0.009±0.001 e	8.30±0.16 cd	29.98±0.31 cd
Flower BC 12%	0.512±0.043 c-e	0.017±0.002 b-d	7.38±0.25 e	27.93±0.54 f

Fisher's < 0.05; ± standard error.
SPAD – leaf greenness index.

BC resulted in an average increase in root system surface area of 46.5% and volume of 64.4% compared to the control.

Effect of biochar on chrysanthemum biochemical and physiological activity

The relative content of flavonols in the leaves of chrysanthemums grown in the presence of 4% flower BC was significantly higher than in the control (0.552) and all other treatments (Table 4). This represents a 38.8% increase compared with the untreated group. In contrast, several BC-treatments resulted in a decline in these pigment concentrations, especially with 4% shoot BC (0.489). As for anthocyanins, 4% root and shoot BC stimulated their most intensive synthesis, although most of the studied plants contained more anthocyanins than the control (0.009), except for those grown with 8% flower BC. The highest chlorophyll concentration and SPAD index were observed in the control and in 12% root BC. All remaining treatments led to a decline in their value, with the lowest means observed in the 12% shoot-and-flower BC experimental combinations (Table 4).

None of the tested experimental combinations differed statistically from the control in minimal chlorophyll fluorescence (F_0) (Table 5). On the other hand, plants treated with 12% shoot BC and 4% flower BC had a significantly lower maximal (F_m) and variable (F_v) chlorophyll fluorescence than the control. The F_v/F_m and F_v/F_0 ratio values were the highest in plants cultivated in the presence of 8% root BC. Conversely, none of the other BC treatments resulted in lower values of these parameters than in the control (Table 5).

None of the BC-treated plants had lower total polyphenol content than the untreated control (Table 6). In fact, most factorial treatments resulted in elevated biosynthesis of these phenolics, except for 12% flower BC, and

Table 5 Effect of biochar (BC) source and its concentration in the growing substrate on chlorophyll fluorescence parameters in chrysanthemum leaves after 30 days of ex vitro cultivation.

Biochar type and concentration	F_0	F_m	F_v	F_v/F_m	F_v/F_0
Control	172.7±3.0 a-c	1000.3±13.5 a-c	827.6±12.6 a-c	0.827±0.003 b-d	4.81±0.10 b-d
Root BC 4%	175.9±4.8 a	1018.4±9.6 a	842.5±11.8 ab	0.826±0.006 b-d	4.84±0.15 b-d
Root BC 8%	166.8±1.6 c	1011.1±7.9 ab	844.3±7.2 a	0.835±0.002 a	5.07±0.05 a
Root BC 12%	165.9±1.6 c	989.8±6.9 a-c	823.8±5.7 a-c	0.832±0.001 ab	4.97±0.03 ab
Shoot BC 4%	174.6±1.6 ab	984.5±9.7 b-d	809.9±9.8 cd	0.822±0.002 cd	4.64±0.08 d
Shoot BC 8%	169.8±2.7 a-c	985.1±8.2 b-d	815.3±8.2 b-d	0.827±0.003 b-d	4.81±0.09 b-d
Shoot BC 12%	167.9±1.7 bc	958.6±8.9 de	790.7±8.0 de	0.824±0.002 cd	4.71±0.05 cd
Flower BC 4%	168.6±1.7 a-c	948.5±11.2 e	779.9±10.1 e	0.822±0.002 d	4.63±0.05 d
Flower BC 8%	166.9±2.1 c	979.1±16.3 cd	812.3±14.7 cd	0.829±0.002 a-c	4.87±0.06 a-c
Flower BC 12%	171.9±2.8 a-c	979.4±12.6 cd	807.5±12.6 c-e	0.824±0.001 cd	4.71±0.05 cd

Fisher's < 0.05; ± standard error. F_0 , minimal chlorophyll fluorescence (all PSII reaction centers open); F_m , maximal chlorophyll fluorescence (all PSII reaction centers closed); F_v , variable fluorescence ($F_v = F_m - F_0$).

Table 6 Effect of biochar (BC) source and its concentration in the growing substrate on the content of total polyphenols and flavonoids, as well as the antioxidant activity determined by the ABTS and FRAP assays in chrysanthemum leaves after 30 days of ex vitro cultivation.

Biochar type and concentration	Polyphenols (mg·g ⁻¹ DW)	Flavonoids (mg·g ⁻¹ DW)	FRAP (TEAC·g ⁻¹ DW)	ABTS (mg·ml ⁻¹)
Control	6.12±0.15 e	0.231±0.002 g	50.66±1.12 g	5.96±0.03 c
Root BC 4%	6.64±0.01 d	0.223±0.001 g	60.71±0.89 f	6.21±0.21 b
Root BC 8%	7.09±0.07 c	0.206±0.004 h	52.72±0.97 g	7.44±0.09 a
Root BC 12%	7.33±0.09 c	0.248±0.002 f	66.29±0.11 e	3.65±0.03 d
Shoot BC 4%	8.87±0.04 b	0.357±0.002 a	110.65±1.26 a	3.59±0.03 d
Shoot BC 8%	9.10±0.07 b	0.294±0.004 d	76.39±0.27 d	3.04±0.07 e
Shoot BC 12%	8.89±0.17 b	0.282±0.002 e	80.20±0.13 c	3.00±0.01 e
Flower BC 4%	9.46±0.13 a	0.313±0.002 c	98.73±1.53 b	2.23±0.03 g
Flower BC 8%	9.08±0.10 b	0.345±0.005 b	78.66±2.32 cd	2.50±0.03 f
Flower BC 12%	6.13±0.06 e	0.188±0.002 i	50.16±0.48 g	2.86±0.02 e

Fisher's < 0.05; ± standard error. ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay, DW, dry weight, FRAP, Ferric Reducing Antioxidant Power assay, TEAC, Trolox equivalent antioxidant capacity.

flavonoids, except for 4% and 8% root BC and 12% flower BC. The highest concentration of total polyphenols and flavonoids in the leaves was found after applying 4% flower BC and 4% shoot BC, respectively (Table 6).

The highest FRAP activity (110.65 TEAC g⁻¹ DW) was found after the application of 4% shoot BC (Table 6). In contrast, the lowest FRAP activities were reported in the control and after using 4% and 8% root BC, and 12% flower BC (50.16–60.71 TEAC g⁻¹ DW). As for ABTS, 4% and 8% root BC increased its value (6.21 and 7.44 mg ml⁻¹, respectively) compared to the control (5.96 mg ml⁻¹), whereas all other treatments led to a significant decline, with the lowest ABTS values obtained with flower BC (2.23–2.86 mg ml⁻¹) (Table 6).

The highest SOD activity in plants was observed after adding 8% flower BC to the growing substrate (35.66 U), while the lowest was observed after adding shoot BC at the same concentration (15.90 U) (Table 7). Most factorial treatments increased the GPOX activity compared to the control, with the highest value obtained for flower BC at 4% (11.63 U) and the lowest in the control (2.58 U).

Effect of biochar on plant root microbiome

The control community (no BC supplementation) exhibited the highest overall metabolic versatility across most substrate categories (Table 8). In contrast, bacterial isolates from BC-amended treatments showed a general decline in substrate utilization.

Table 7 Effect of biochar (BC) source and its concentration in the growing substrate on the activity of antioxidant enzymes in chrysanthemum leaves after 30 days of ex vitro cultivation.

Fisher's < 0.05; $\pm$ standard error.
SOD, superoxide dismutase,
GPOX, glutathione peroxidase.

Biochar type and concentration	SOD ($\mu\text{M min}^{-1} \text{mg}^{-1} \text{protein}$)	GPOX ($\mu\text{M min}^{-1} \text{mg}^{-1} \text{protein}$)
Control	26.93 $\pm$ 2.57 b-d	2.58 $\pm$ 0.26 g
Root BC 4%	38.97 $\pm$ 2.45 ab	5.39 $\pm$ 0.60 ef
Root BC 8%	33.32 $\pm$ 5.48 ab	4.20 $\pm$ 0.47 fg
Root BC 12%	26.53 $\pm$ 1.18 b-d	2.15 $\pm$ 0.24 b
Shoot BC 4%	30.80 $\pm$ 0.70 a-c	6.87 $\pm$ 0.66 c-e
Shoot BC 8%	15.90 $\pm$ 0.83 e	8.58 $\pm$ 1.25 bc
Shoot BC 12%	21.44 $\pm$ 0.49 c-e	9.69 $\pm$ 0.48 ab
Flower BC 4%	20.33 $\pm$ 0.47 de	11.63 $\pm$ 1.66 a
Flower BC 8%	35.66 $\pm$ 8.28 a	5.72 $\pm$ 0.62 d-f
Flower BC 12%	22.60 $\pm$ 0.78 c-e	7.81 $\pm$ 1.00 b-d

Average polymer utilization decreased from 38% in the control to 30% (4% root BC), 18% (8% root BC), and 20% (12% root BC). While Tween 40 remained relatively well utilized across treatments, Tween 80 metabolism declined sharply with increasing biochar dose, especially at 8% root BC (10%). Complex polymers, such as α -cyclodextrin and glycogen, were weakly metabolized, with consistently low utilization (10%) observed across all treatments. (Table 9).

Carbohydrate utilization decreased with increasing biochar supplementation: 31% (control), 23% (4% root BC), 19% (8% root BC), and 12% (12% root BC). D-xylose was consistently well utilized in all treatments. However, several carbohydrates (e.g., β -methyl-D-glucoside, i-erythritol, glucose-1-phosphate) were metabolized mainly or exclusively by control isolates, indicating reduced functional diversity under BC amendment (Table 8).

Carboxylic acid utilization also declined with increasing BC dose (41% in control vs. 31%, 19%, and 12% in 4%, 8%, and 12% root BC, respectively). Notably, D-galacturonic utilization decreased with increasing root biochar dose, from 70% in the control to 60% at 4% Root BC, 20% at 8% root BC, and no utilization at 12% root biochar. This suggests a dose-dependent reduction in the ability to metabolize selected organic acids (Table 8).

Amino acids were the most intensively utilized substrates overall, yet their metabolism decreased with biochar application (47% in control, 38% in 4% root BC, 18% in 8% root BC, and 17% in 12% root BC). Although some recovery was observed at the highest BC dose, utilization remained below control levels (Table 8).

Amine utilization declined with increasing biochar dose (50% in the control vs. 30%, 20%, and 10% at 4, 8, and 12% root BC, respectively). The strongest reduction was observed at the intermediate dose (8% root BC). Phenolic compounds were not utilized in any treatment, regardless of BC dose (Table 8).

Effect of biochar on substrate microorganisms

The highest total bacterial counts ($21.6\text{--}22.1 \text{ cfu} \times 10^6 \text{ g}^{-1}$ of horticultural substrate) were observed in the experimental combination with 12% shoot or flower BC, which was almost twofold higher than in the control ($13.2 \text{ cfu} \times 10^6$) (Table 9). Likewise, the maximum number of colony-forming units of copiotrophs was observed with the highest (12%) BC concentration, regardless of its type (Fig. 1). On the other hand, there was no effect of the studied factor on the filamentous fungi multiplication. As for Actinobacteria, flower BC at 8% and 12%, as well as shoot and root BC at 12%, significantly increased their numbers compared to the control (Table 9).

Effect of biochar on substrate enzymatic activity

Application of 8 and 12% shoot BC, as well as 4–12% flower BC, elevated the activity of DEH, BG, and PR, with the highest values obtained with 12% shoot and flower BC (Table 10). In contrast, 8% root BC caused a decline in the activities of DEH and BG. All factorial treatments increased the activity of AIP and CAT compared with the control (except for 4 and 8% root BC in CAT). On the other hand, the highest AcP activity was observed in the control, whereas all other treatments inhibited this enzyme, especially with flower BC at 12% (Table 10).

Table 8 List of compounds in the Biolog Ecoplate grouped into substrate categories.

Compound	Category	Treatment (bacteria in percentage)			
		Control	Root BC 4%	Root BC 8%	Root BC 12%
Tween 40	Polymer	60	50	40	40
Tween 80		70	50	10	20
α -Cyclodextrin		10	10	10	10
Glycogen		10	10	10	10
D-Cellobiose	Carbohydrate	20	20	20	10
α -D-Lactose		20	20	10	10
β -Methyl-D-glucoside		10	10	10	0
D-Xylose		70	60	60	50
i-Erythritol	Carboxylic acid	10	0	0	0
D-Mannitol		50	40	30	30
N-Acetyl-D-glucosamine		40	30	20	10
Glucose-1-phosphate		30	10	10	0
D,L- α -Glycerol phosphate		0	0	0	0
D-Galactonic acid-lactone		60	30	30	10
D-Glucosaminic acid		30	20	20	20
D-Galacturonic acid		70	60	20	0
Pyruvic acid methyl ester		70	50	30	30
γ -Amino butyric acid		70	40	30	20
Itaconic acid		0	0	0	0
α -KetoButyric acid		20	10	10	10
D-Malic acid	Phenolic compound	30	40	20	10
2-Hydroxy benzoic acid		0	0	0	0
4-Hydroxy benzoic acid		0	0	0	0
L-Arginine	Amino acid	70	50	20	10
L-Asparagine		70	60	30	30
L-Phenylalanine		10	0	0	0
L-Serine		70	60	20	20
L-Threonine	Amine	20	20	20	20
β -Hydroxy-Glycyl-L-Glutamic Acid		60	40	20	20
Phenylethylamine		60	20	20	10
Putrescine		40	40	20	10
Polymer	Average	38	30	18	20
Carbohydrate		31	23	19	12
Carboxylic acid		41	31	19	12
Phenolic compound		0	0	0	0
Amino acid		47	38	18	17
Amine		50	30	20	10

Principal component analysis

The first two principal components explained 58.57% of the total variance, with PC1 accounting for 35.22% and PC2 for 23.35% (Fig. 2). The figures showed clear differentiation between the treatment groups and measured variables within the PCA space. Morphological traits, particularly those related to shoot and root growth and biomass, were mainly oriented toward the positive PC1 and/or PC2 directions, whereas several antioxidant, microbiological, and growing-substrate enzymatic variables were oriented toward the negative side of PC1. This opposite orientation indicates contrasting patterns of association within the multivariate dataset. Among the treatments, 4% flower BC and 12% flower BC were located predominantly on the negative side of PC1 and were therefore more closely associated with variables oriented in this direction. In contrast, 4% shoot BC, 8% shoot BC, and 8% root BC were positioned mainly on the positive side of PC1 and showed closer spatial relationships

Table 9 Effect of biochar (BC) source and concentration on the composition of microorganisms in the growing substrate after 30 days of chrysanthemum ex vitro cultivation.

Biochar type and concentration	Total bacteria count (cfu $\times 10^6 \cdot g^{-1}$ of substrate)	Copiotrophs (cfu $\times 10^6 \cdot g^{-1}$ of substrate)	Filamentous fungi (cfu $\times 10^6 \cdot g^{-1}$ of substrate)	Actinobacteria (cfu $\times 10^6 \cdot g^{-1}$ of substrate)
Control	13.2 $\pm$ 1.8 a–c	12.8 $\pm$ 6.2 ab	0.73 $\pm$ 0.22 a	0.15 $\pm$ 0.05 d
Root BC 4%	15.3 $\pm$ 2.7 ab	8.1 $\pm$ 4.0 ab	0.60 $\pm$ 0.20 a	0.25 $\pm$ 0.05 cd
Root BC 8%	7.1 $\pm$ 2.9 bc	7.2 $\pm$ 1.8 ab	1.25 $\pm$ 0.75 a	1.15 $\pm$ 0.35 cd
Root BC 12%	13.2 $\pm$ 0.8 a–c	18.2 $\pm$ 7.8 a	1.15 $\pm$ 0.05 a	3.50 $\pm$ 0.50 bc
Shoot BC 4%	5.5 $\pm$ 0.4 c	3.8 $\pm$ 0.2 b	0.80 $\pm$ 0.20 a	0.35 $\pm$ 0.05 cd
Shoot BC 8%	14.1 $\pm$ 0.9 a–c	15.1 $\pm$ 5.9 ab	0.80 $\pm$ 0.20 a	1.00 $\pm$ 0.00 cd
Shoot BC 12%	21.6 $\pm$ 5.4 a	22.2 $\pm$ 12.8 a	0.75 $\pm$ 0.25 a	3.50 $\pm$ 1.50 bc
Flower BC 4%	19.2 $\pm$ 8.8 ab	15.7 $\pm$ 8.3 ab	0.85 $\pm$ 0.08 a	0.80 $\pm$ 0.20 cd
Flower BC 8%	6.7 $\pm$ 1.7 bc	10.6 $\pm$ 1.4 ab	0.95 $\pm$ 0.02 a	6.50 $\pm$ 1.50 b
Flower BC 12%	22.1 $\pm$ 11.6 a	21.9 $\pm$ 9.0 a	1.05 $\pm$ 0.02 a	12.50 $\pm$ 2.50 a

Fisher's < 0.05 ; $\pm$ standard error. cfu, colony-forming unit.

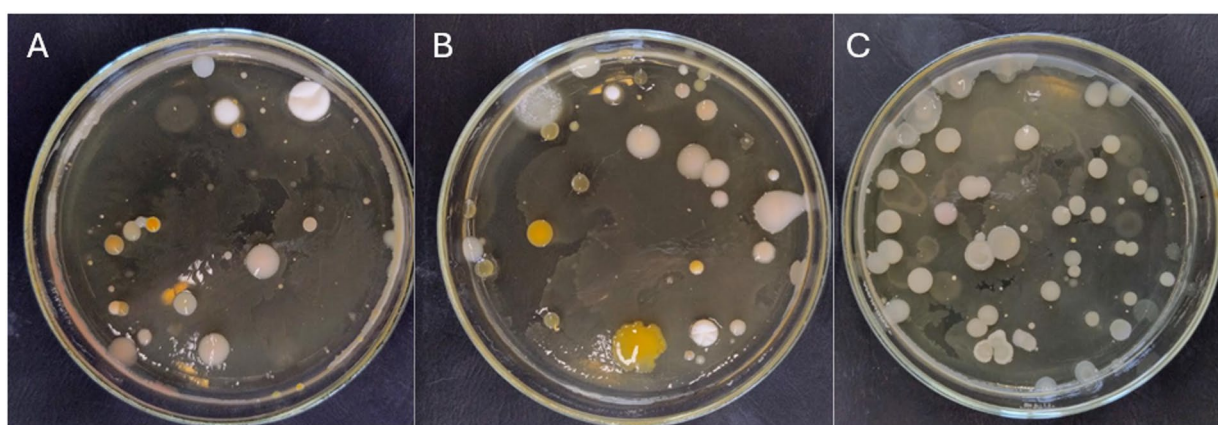


Fig. 1 Bacterial growth on a selective medium for copiotrophic microorganisms from horticultural substrate samples enriched with: A—4%, B—8%, and C—12% flower biochar.

Table 10 Effect of biochar (BC) source and its concentration on the enzymatic activity of growing substrate after 30 days of chrysanthemum ex vitro cultivation.

Biochar type and concentration	DEH	CAT	AIP	AcP	BG	PR
Control	4.02 $\pm$ 0.052 c	1.27 $\pm$ 0.059 e	3.29 $\pm$ 0.088 e	6.06 $\pm$ 0.098 a	1.92 $\pm$ 0.022 cd	29.23 $\pm$ 1.750 c
Root BC 4%	3.92 $\pm$ 0.048 cd	1.18 $\pm$ 0.061 f	3.44 $\pm$ 0.119 d	4.93 $\pm$ 0.754 b	1.63 $\pm$ 0.035 de	28.51 $\pm$ 1.251 c
Root BC 8%	3.53 $\pm$ 0.074 d	1.25 $\pm$ 0.074 e	3.66 $\pm$ 0.050 c	4.39 $\pm$ 0.239 bc	1.26 $\pm$ 0.041 e	29.71 $\pm$ 0.985 c
Root BC 12%	4.10 $\pm$ 0.009 c	1.37 $\pm$ 0.036 d	3.78 $\pm$ 0.072 bc	4.02 $\pm$ 0.216 c	2.11 $\pm$ 0.026 c	31.56 $\pm$ 0.922 c
Shoot BC 4%	4.18 $\pm$ 0.062 c	1.37 $\pm$ 0.088 d	3.71 $\pm$ 0.061 bc	4.18 $\pm$ 0.543 c	2.14 $\pm$ 0.031 c	33.26 $\pm$ 0.894 c
Shoot BC 8%	5.84 $\pm$ 0.058 ab	1.44 $\pm$ 0.059 d	3.87 $\pm$ 0.040 bc	4.32 $\pm$ 0.264 bc	2.75 $\pm$ 0.028 b	41.15 $\pm$ 1.058 b
Shoot BC 12%	6.69 $\pm$ 0.071 a	1.99 $\pm$ 0.044 a	3.99 $\pm$ 0.065 bc	4.42 $\pm$ 0.048 b	3.72 $\pm$ 0.041 a	52.62 $\pm$ 1.114 a
Flower BC 4%	5.74 $\pm$ 0.076 b	1.66 $\pm$ 0.028 c	4.05 $\pm$ 0.037 b	4.59 $\pm$ 0.892 b	2.71 $\pm$ 0.037 b	42.12 $\pm$ 0.917 b
Flower BC 8%	5.57 $\pm$ 0.057 b	1.69 $\pm$ 0.062 c	4.28 $\pm$ 0.065 a	4.13 $\pm$ 0.082 cd	2.63 $\pm$ 0.024 b	43.11 $\pm$ 1.108 b
Flower BC 12%	6.23 $\pm$ 0.044 a	1.77 $\pm$ 0.047 b	4.51 $\pm$ 0.039 a	3.92 $\pm$ 0.117 d	3.26 $\pm$ 0.033 a	53.09 $\pm$ 1.204 a

Fisher's < 0.05 ; $\pm$ standard error. DEH, dehydrogenases (mg TPF $kg^{-1} \cdot 24 h^{-1}$); CAT, catalase (mg H_2O_2 $kg^{-1} h^{-1}$); AIP, alkaline phosphatase (mM pNP $kg^{-1} h^{-1}$); AcP, acid phosphatase (mM pNP $kg^{-1} h^{-1}$); BG, β -glucosidase (mM pNP $kg^{-1} h^{-1}$); PR, protease (mg TYR $kg^{-1} h^{-1}$).

with morphological traits contributing to this region of the biplot. Flower BC (12%) and root BC (4%) also differed along PC2, indicating that these treatments were characterized by distinct combinations of measured variables. The PCA suggests that biochar dose and plant organ contributed jointly to the multivariate differentiation of the treatments. The spatial relationships between treatment points and variable vectors further indicate that

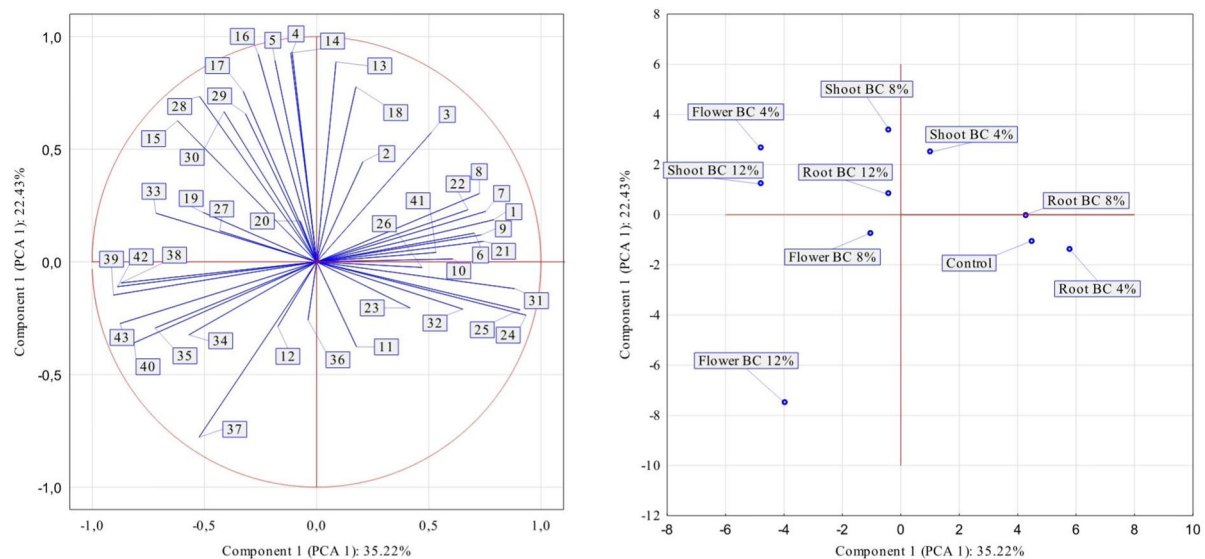


Fig. 2 Principal Component Analysis (PCA) for the measured properties—plot of the first two principal components (PCA 1 and PCA 2). 1-shoot length; 2-number of leaves; 3-shoot FW; 4-root FW; 5-root DW; 6-surface area; 7-perimeter; 8-length; 9-max. width; 10-mean width; 11-aspect ratio; 12-form coefficient; 13-length; 14-surface area; 15-diameter; 16-volume; 17-number of tips; 18-number of bifurcations; 19-flavonols; 20-anthocyanins; 21-chlorophyll; 22-SPAD; 23- F_0 (minimal chlorophyll fluorescence); 24- F_m (maximal chlorophyll fluorescence); 25- F_v (variable fluorescen); 26- F_v/F_m ; 27- F_v/F_0 ; 28-polyphenols; 29-flavonoids; 30-FRAP; 31-ABTS; 32-SOD; 33-GPOX; 34-total bacteria count; 35-Copiotrophs; 36-Filamentous fungi; 37-Actinobacteria; 38-dehydrogenases; 39-catalase; 40-alkaline phosphatase 41-acid phosphatase; 42- β -glucosidase; 43-protease.

the response to biochar was not uniform across plant organs, but was associated with different combinations of morphological, biochemical, microbiological, and substrate enzymatic characteristics.

Discussion

Effect of biochar on the biometric parameters of plants

Overall, the results indicate that biochar is an effective additive that supports ex vitro acclimatization of chrysanthemum ‘Richmond’, confirming our scientific hypothesis. Nevertheless, its final effect depends on both the dose and the plant organ used as feedstock. The longest shoots and the highest number of leaves were observed in the control, whereas the highest BC dose (12%, regardless of type) led to significant shoot shortening and arrested leaf development. On the other hand, biochar derived from shoots improved fresh plant mass and selected root system parameters; BC from roots improved leaf growth and selected root architecture traits; whereas BC from inflorescences was associated with the highest proportion of adverse outcomes. This finding aligns with Ippolito et al. [2020], who identified feedstock choice as a key factor determining biochar properties. Literature indicates that differences in ash content, mineral composition, pH, specific surface area, and sorption properties can lead to widely varying plant responses to BC produced from different feedstocks^{27,28}. However, among the analyzed parameters, it is not only the biomass type that matters, but also its interaction with the applied dosage.

The reduction in leaf number and plant fresh weight at 12% BC, derived from flowers and roots, may indicate physiological stress due to ionic imbalances or limited nitrogen availability. Similar results were previously observed in studies of BC applied at high rates, particularly in systems with limited substrate volume, such as container-based plant production^{29–31}. According to the literature, excessive BC amounts can deteriorate the physicochemical properties of the substrate, e.g., by increasing pH, thereby inhibiting plant growth, as observed

in viola (*Viola cornuta* L.)³¹. Bu et al. showed that, at high application rates of certain BCs, adverse physico-chemical properties of the growing medium limited *Rhododendron delavayi* Franch. Growth³². Regmi et al. reported that the combined use of fertilizer with BC (even at 50% concentration) prevented these negative effects on plant growth³¹, which could also be considered in future chrysanthemum studies. As for maize (*Zea mays* L.), long-term experiments have shown that BC at high concentrations may initially reduce plant growth, though this effect may diminish over time and even become promoting³³. Our experiment lasted for only 30 days, so additional observations are required to learn the final BC effect on chrysanthemum. Nevertheless, shorter plants can be better for production as they are easier to transport and handle and less prone to lodging. We wish to highlight that the highest dosage of 12% was not always detrimental. The greatest root length and the highest number of root tips were observed after incorporating 12% root BC. Thus, plant response depended not only on biochar type and dosage but also on the parameter analyzed.

At the same time, moderate concentrations of BC (4 and 8%), especially produced from shoots, significantly stimulated biomass accumulation in aboveground and belowground plant parts. The highest fresh weight of shoots and roots was obtained with 8% shoot BC, suggesting a beneficial effect of this biochar type on the substrate's air and water conditions and on nutrient uptake efficiency. Likewise, the development of leaves of the greatest size was stimulated by 4% root BC. Similar effects were observed in other horticultural plant species, in which BC at low and medium doses improved substrate structure, increased water retention, and promoted root development^{34,35}. This implies that a moderate application rate could provide favorable conditions for growth. For example, increased water retention could explain why the highest root FW was reported after BC application, whereas the control showed the highest DW. Singh et al. indicate that BC effects can be dose-dependent, with higher rates exerting a stronger influence on growth substrate properties, potentially pushing conditions beyond the optimal range³⁶.

Effect of biochar on the physiology and metabolism of plants

Our results indicate that both the dose and the source of BC affect the physiological responses of the plants studied. No decline in total polyphenol content was detected in any of the BC treatments compared to the control. In fact, in most treatments, increased biosynthesis of polyphenols and flavonoids was observed, particularly with the addition of 4% flower BC (TPC) and 4% shoot BC (flavonoids). Likewise, the highest relative flavonol content was observed in leaves of plants grown in the presence of 4% flower BC, but other combinations led to a decrease in their content. Flavonols play an important role in protecting plants against oxidative stress and UV radiation, and their biosynthesis is strongly influenced by environmental conditions and nutrient availability^{37,38}. Changes in flavanol content resulting from BC application may be driven by alterations in the physicochemical properties of the substrate, mineral availability, and microbiological response³⁹. The increased anthocyanin content in most experimental treatments with BC suggests that this material may act as a mild stressor, stimulating secondary metabolism. Anthocyanins are synthesized in response to environmental stress and function as antioxidants⁴⁰. Our results confirm previous reports indicating that BC can mediate plant secondary metabolism in response to altered environmental conditions induced by its addition^{41,42}. Similar effects were observed in other horticultural plant species, in which the use of BC increased the accumulation of carotenoids, polyphenolic compounds, and anthocyanins^{32,43}.

The highest chlorophyll content and SPAD index were observed in the control plants and in the variant with 12% root BC. A decrease in these values was observed in the remaining combinations, especially at high doses of BC from shoots and flowers. According to He et al.⁴⁴ BC enhances photosynthesis and biomass production in C3 but not C4 plants by increasing photosynthetic rate, improving stomatal conductance, transpiration rate, water use efficiency, and chlorophyll concentration. Our results, however, are consistent with the studies reporting that the effect of BC on photosynthesis is strongly dependent on its dose and chemical properties^{45,46}. Higher concentrations may result in nutrient immobilization or increased substrate salinity, which limits photosynthetic efficiency.

Moreover, C assimilation is vastly dependent on leaf-N concentration, and BC generally contains trace amounts of available N; thus, the induced N-limitation inhibits photosynthesis at high BC dosages⁴⁵.

Chlorophyll fluorescence showed no differences between the treatments in minimum fluorescence (F_0), suggesting that the structure of the photosystem II (PSII) was not damaged by BC application. However, reductions in maximum fluorescence (F_m) and variable fluorescence (F_v) in some variants may indicate limitations in PSII electron transport efficiency. The F_v/F_m and F_v/F_0 parameters reached the highest values in the presence of 8% BC from roots, suggesting enhanced photosynthetic efficiency^{47,48}. Nevertheless, the obtained values of F_v/F_m (0.822–0.835) and F_v/F_0 (4.63–5.07) are within the typical range for plants in good physiological condition^{49,50}.

The studied factor also affected enzymatic activity in the plants. SOD activity was the highest in plants grown in the substrate supplemented with 8% flower BC, while it was the lowest at the same dose of BC from shoots. This enzyme converts superoxide anion radicals, i.e., superoxide anion ($O_2^{\cdot-}$), singlet oxygen (1O_2), and other reactive oxygen species (ROS), into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), and activates the first line of the plant's defense mechanisms⁵¹. A similar trend was observed for GPOX, whose activity was higher in most variants than in the control. GPOX participates in hydrogen peroxide detoxification and in lignification and cell wall strengthening^{52,53}. The increased activity of antioxidant enzymes may be related to the observed total antioxidant activity. The highest FRAP values were observed in plants grown in a substrate supplemented with 4% shoot BC. This may be the result of increased phenolic compound synthesis and activation of enzymatic defense mechanisms. On the other hand, increased ABTS values in the 4% and 8% root BC treatments suggest enhanced free radical scavenging capacity in these combinations. Differences in results between FRAP and ABTS methods are a common finding in plant studies, since these methods rely on different antioxidant reaction mechanisms⁵⁴. These results suggest that biochar addition may induce moderate oxidative stress, leading to activation of both enzymatic (SOD, GPOX) and non-enzymatic antioxidant mechanisms, as reported by^{55,56}.

It is well known that the biological structure of the feedstock affects the properties of the resulting biochar, including morphology, elemental composition, and stability¹³. In the cultivation of pot marigold (*Calendula officinalis* L.) and petunia (*Petunia × hybrida* /Hook./ Regel), BC from forest waste could be safely used as a substrate constituent to improve plant performance (due to its low salinity and CO_2 emissions), but BCs from olive mill waste and hydrochar from forest waste required further improvement before they could be used in plant production⁵⁷. Our study showed that even different organs within the same plant species can affect biochar characteristics. The varied response of chrysanthemums to BC produced from roots, shoots, and flowers suggests a different chemical composition of the starting material. Biochar from shoots may have had a more favorable macro- to micronutrient ratio and greater porosity than biochar from flowers or roots, which resulted in better plant growth conditions. The literature emphasizes that BC derived from different biomass fractions exhibits different sorption and biological properties, and therefore differs in its suitability for plant cultivation^{27,58}.

Effect of biochar on the soil selected microorganisms and enzymatic activity

One factor that affects the quality and properties of BC is the pyrolysis conditions. According to Panditha et al.²⁶, BC pyrolyzed at 500 °C exhibited better characteristics for environmental applications than that produced at 350 °C. However, BC produced at 350 °C was more efficient for the germination of Indian mustard. Moreover, a meta-analysis of over 125 studies revealed that pyrolysis temperature strongly affects enzymatic soil responses⁵⁹. Low-temperature BCs (produced at < 500 °C) tend to trigger the activities of enzymes involved in carbon, nitrogen, and phosphorus acquisition, whereas those produced at ≥ 500 °C have a small impact on soil enzyme response⁵⁹. Interestingly, according to Pokharel et al.²⁴, BC produced at pyrolysis temperatures of 350–550 °C with a high pH (> 10) and low C/N ratio (< 50) increased microbial biomass carbon (MBC), as well as urease and dehydrogenase activities. However, BC increased MBC and N-acquisition enzyme activities only in the field but not in laboratory incubation experiments. Therefore, in the present greenhouse study, we used milder pyrolysis conditions (400 °C), which mostly confirmed the finding of Liao et al.⁵⁹. BC produced from shoots (8–12%) and flowers (4–12%) significantly increased the activity of enzymes responsible for carbon (DEH, BG) and nitrogen

(PR) transformation. Furthermore, the often observed stimulation of AIP with simultaneous strong inhibition of AcP, especially evident in treatment with 12% flower BC, is most likely a physiological response of the microbiome to the alkalizing properties of the added BC, which naturally favors enzymes active at higher pH. It should be emphasized, however, that general trends regarding the effect of pyrolysis temperature can be vastly modified by the type of biomass used. A decrease in DEH and BG enzyme activity following the application of 8% root BC indicates that, in addition to thermal parameters, the specific chemical properties of the feedstock play a key role in determining the enzymatic response of the growth substrate, possibly causing local stress or inhibition. Those changes in enzymatic activity are also related to the activity and diversity of the substrate microbiota.

Dai et al.⁶⁰ reported that improved soil aeration and enhanced microbial abundance are the main factors responsible for elevated DEH activity after BC amendment. CAT reflects the capacity of soil microorganisms to cope with oxidative stress. BC may alleviate environmental stress by improving soil water-air relations and through the adsorption of toxic compounds^{61,62}, which could explain the increased CAT activity in our study. AIP is among the enzymes most strongly stimulated by BC application⁶³. Due to its alkalizing properties, BC often enhances AIP activity at the expense of AcP⁶⁴. BG, which catalyzes the final stage of cellulose degradation, is crucial for the soil carbon cycle. Its increased activity following BC application suggests intensified decomposition of organic matter¹⁴. The increase in PR activity in the BC-amended substrate compared with the control indicates intensified nitrogen cycling, as this enzyme is involved in the mineralization of organic nitrogen by hydrolyzing proteins into amino acids⁶⁵.

Our results showed that the addition of biochar produced from various plant organs affected the abundance of selected microorganism groups in the growing substrate. The highest overall bacterial counts were recorded in the treatments with 12% BC from shoots or flowers ($21.6\text{--}22.1 \times 10^6 \text{ cfu g}^{-1}$), which were nearly two-fold higher compared to the control. This result is consistent with other reports indicating that BC application can increase the abundance and diversity of soil microorganisms by improving soil physicochemical properties⁶⁶. This may be due to the improved porosity of the growing substrate. BC provides a site for microorganisms to colonize and protects them from unfavorable environmental changes, e.g., fluctuations in humidity or temperature, as well as from soil predators, including mites, collembola, protozoans, and nematodes⁶⁷. Moreover, the high specific surface area of BC promotes the adsorption of nutrients and organic compounds, thereby increasing their availability to heterotrophic microorganisms⁶⁸. The observed increase in copiotroph number in the treatments with the highest BC dose (12%) also indicates increased availability of easily digestible organic compounds in the growing substrate. Copiotrophic microorganisms live primarily in environments rich in available carbon substrates⁶⁹. Research indicates that BC can increase the organic carbon and nitrogen content of soil, consequently promoting the development of fast-growing bacteria, such as *Proteobacteria* and *Bacteroidetes*⁷⁰.

Unlike bacteria, our research did not show a significant effect of the tested factor on the number of filamentous fungi. Similar observations have been reported in other studies⁷¹. The lack of a clear response from this group of microorganisms may be due to their greater tolerance to environmental fluctuations and their ability to utilize more complex and difficult-to-decompose fractions of organic matter. Fungi produce an array of enzymes that degrade lignin, cellulose, and other recalcitrant carbon compounds, so their development does not necessarily have to be directly dependent on the availability of easily digestible substrates^{72,73}.

Significant changes were reported with Actinobacteria. Application of BC derived from flowers at concentrations of 8% and 12%, and BC from shoots and roots at 12%, resulted in an increased number of these microorganisms compared to the control. Actinobacteria are microorganisms of great importance in the decomposition of organic matter, especially complex and stable compounds such as lignocellulose and aromatic carbon structures^{74,75}. It has been shown that BC can promote their development by improving soil aeration, increasing pH, and creating numerous colonization microhabitats⁷⁶. Meta-analyses also indicate that physical properties of BC, such as specific surface area and porosity, can increase the activity of Actinobacteria and the total biomass of soil microorganisms⁷⁷.

The Biolog EcoPlate technique is widely used as a quantitative approach to assess microbial functional diversity and carbon-source utilization in biochar studies^{78,79}. Although the effects of biochar on soil microorganisms

have been widely studied, reported responses remain inconsistent. Some studies have shown that biochar enhances microbial abundance, activity, and functional diversity, whereas others have reported declines in carbon utilization and metabolic activity^{79,80}. In our study, although the abundance of selected microbial groups increased, biochar addition reduced the metabolic versatility of bacterial isolates, as reflected by lower carbon-source utilization in the Biolog EcoPlate assay. Our results suggest that root biochar addition reduced the functional diversity and carbon-use capacity of culturable bacterial isolates in a dose-dependent manner. This observation is consistent with studies reporting that biochar may decrease microbial activity, carbon utilization rates, and functional diversity, depending on biochar properties, application rate, and experimental conditions⁸¹. These contrasting results likely reflect differences in biochar properties, application rates, pyrolysis conditions, soil characteristics, and experimental design. Our results indicate that the effect of biochar on microorganisms is complex and multifactorial. Although its application may increase the abundance of selected microbial groups, this does not necessarily correspond to an enhancement of their metabolic activity. On the contrary, biochar has been shown to limit the capacity of bacteria to utilize a broad spectrum of carbon substrates, indicating a reduction in their metabolic versatility. This phenomenon leads to a decline in microbial functional diversity, which may have significant implications for key soil processes, including the turnover and cycling of organic matter.

Conclusions

The obtained results confirm that biochar can be a valuable supplement to horticultural substrates in chrysanthemum production, although its effect depends on the appropriate dose and feedstock selection (even at the level of plant organs). Using 8% shoot BC was particularly beneficial, promoting biomass accumulation and leaf development during acclimatization to *ex vitro* conditions, whereas high BC doses had an inhibitory effect. On the other hand, BC had little effect on root system development, except for stimulating root bifurcation. Biochar stimulated the synthesis of secondary metabolites and the activity of antioxidant enzymes, but negatively impacted chlorophyll concentration. It also influenced the functional structure of the root-associated bacterial community, potentially selecting for a narrower range of metabolic capabilities. In turn, the highest BC doses increased the numbers of bacteria and Actinobacteria in the growth substrate. Consequently, the use of shoot- and flower-derived BC increased soil enzyme activity. Incorporating BC into plant production can minimize peat use, aligning with European Union regulations and the zero-waste policy. In the present study, we did not include any BC mixtures with compost or fertilizer; therefore, further studies are needed to fully understand the potential of BC in horticulture.

Materials and methods

Time and place of the experiments

The experiments were conducted from February to July 2025. Biochar was produced and characterized at the Department of Biogeochemistry, Soil Science, and Water Irrigation of the Faculty of Agriculture and Biotechnology at Bydgoszcz University of Science and Technology. Plants were propagated *in vitro* in the Laboratory of Horticulture and Landscape Architecture and cultivated *ex vitro* at the glasshouses, where they were subjected to biometric and physiological analyses. Additional biochemical scrutiny of plants was conducted at the Department of Agronomy and Food Processing of the same university. Microbiological analyses of the growth substrate and plant roots were performed at the Department of Microbiology and Plant Ecology, Bydgoszcz University of Science and Technology, as well as at the Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University in Toruń.

Biochar preparation and characterization

The feedstock material for BC production was selected from organs of chrysanthemum plants: flowers, shoots, and roots with attached remnants of growing substrate. The production of BC was carried out in the pyrolysis process of air-dried feedstock biomass under atmospheric pressure, in the muffle furnace FCF 22 M (Czylok, Poland). The process parameters were as follows: time to reach operating temperature (400 °C) – 25 min (heating rate: 16 °C min⁻¹ ≈ 0.27 °C·s⁻¹), process execution time – 60 min, material was removed from the furnace—after the furnace chamber had cooled down, pressure—atmospheric. The moisture of air-dried feedstock material: flowers – 6.93%, shoots – 5.06%, roots – 9.55%.

The pH of BC was measured in accordance with the standard PN-ISO 10,390. For this purpose, 0.5 g of BC was mixed with 25 mL of deionized water (pH 5.6, EC 142.6 µS cm⁻¹) and shaken for 5 min; the flask was then set aside for 1 h. The pH was determined using a pH meter (540 GLP, WTW, Germany) with a glass electrode.

Electrical conductivity (EC) was measured according to the standard PN-ISO 11265—soil quality, determination of the specific electrical conductivity. For this analysis, the water extract and BC described above were used. EC was determined using a conductometer (CC-501, Elmetron, Poland).

The ash content in BC was analyzed according to the standard PN-80/G-04512 using the Czylok FCF 22 M furnace at 815 °C for 60 min. The weigh method was used – weigh AS 220/X (Radwag, Poland).

Data on the BC characteristics are presented in Table 11.

The content of macro-elements (K, Ca, Na) was analyzed by flame photometer BWB XP (BWB Flame Technologies, UK). The mineralization of BC samples was performed in sulphuric acid (96%). For this purpose, 1 g of sample was mixed with 10 mL H₂SO₄. Mineralization was performed in a DK20 digester (VELP Scientifica, Italy) at 265 °C for 6 h. After cooling down, the mineralization was continued at 375 °C until the solutions turned dark brown. Then, the solutions were decolorized by adding 30% hydrogen peroxide (5 mL), and the mixtures were digested for an additional 30 min (375 °C). After cooling, the obtained solutions were poured into 100 mL volumetric flasks and made up to volume with deionized water.

The content of microelements in BC was assessed by atomic absorption spectrometry using a AA240FS spectrometer (Varian, Australia). Prior to assay, BC samples were mineralized in a muffle furnace (750 °C, 150 min.); the obtained ash was further mineralized in a mixture of nitric acid (30 mL, 65%) and hydrochloric acid (10 mL, 38%), in an open system, Tecator, Sweden digester, temperature 160 °C, time 120 min. After cooling down, the solutions were poured into 100 mL volumetric flasks and brought to volume with deionized water. After filtration, the resulting mineralization solutions were analyzed using an AAS apparatus. The contents of Cu, Zn, Mn, Mg, and Fe were calculated as the concentrations of BC in ash and, finally, in pure BC.

Data on the content of micro- and macro-elements are presented in Table 12.

In vitro multiplication and ex vitro cultivation of plants

In vitro-derived microshoots of *Chrysanthemum × morifolium* (Ramat.) Hemsl. ‘Richmond’, obtained from our gene bank collection, were used in the experiment. To produce the required number of plants, chrysanthemums were cut into single-node segments and cultured for 3–4 weeks on Murashige and Skoog medium (1962) with 3% sucrose and 0.8% agar, and the pH was adjusted to 5.8 prior to autoclaving⁸². Next, rhizogenesis was initiated by culturing the microshoots for two weeks on MS medium supplemented with 2 mg L⁻¹ indole-3-acetic acid (IAA; Sigma-Aldrich, USA) (Fig. 3A,B).

Table 11 Chrysanthemum biochar characteristics.

Biochar type	Yield (%)	Bulk density (g mL ⁻¹)	Carbon content (%)	Nitrogen content (%)	pH	EC (mS cm ⁻¹)
Flower BC	39.67±1.20	0.038±0.001	59.32±1.48	2.75±0.18	10.01±0.01	3.425±0.231
Shoot BC	33.68±0.57	0.056±0.003	64.49±0.38	2.89±0.01	9.85±0.01	6.470±1.162
Root BC	49.52±1.13	0.177±0.010	64.74±0.40	1.46±0.25	8.11±0.07	0.603±0.123

Table 12 Chrysanthemum biochar content of micro- and macro-elements.

Biochar type	Macro- and micro-elements content ($\text{mg} \cdot \text{g}^{-1}$)							
	K	Ca	Na	Cu	Zn	Mn	Mg	Fe
Flower BC	123.8	8.2	1.5	0.065	0.288	1.239	13.645	0.909
Shoot BC	118.3	9.1	2.2	0.257	0.500	1.489	21.305	1.263
Root BC	5.8	59.2	11.7	0.623	0.719	1.643	47.107	17.057

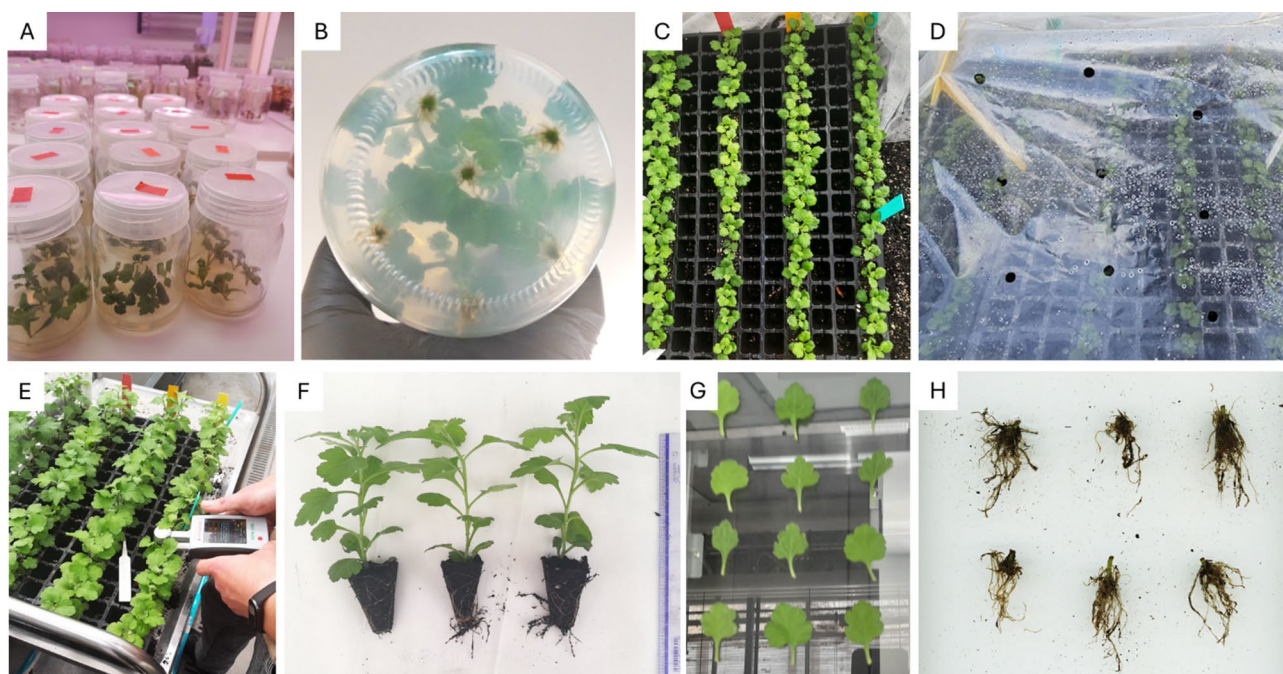


Fig. 3 The course of the experiment and analysis of plant material. (A) chrysanthemum microshoots multiplied in vitro, (B) microshoots with initiated rhizogenesis on the medium with IAA auxin, (C) chrysanthemum plants in a multi-pot tray filled with horticultural substrate and biochar, (D) plants covered with perforated foil during acclimatization, (E) measurements of relative pigment content, (F) month-old plants with fully developed leaves and roots, (G) planimetric measurements of leaves on a scanner, (H) root architecture measurements.

During the in vitro phase of the experiment, a constant temperature of 22 ± 2 °C and 16-h photoperiod were maintained in the growth room. Glass jars (350 mL) sealed with plastic lids were used as culture vessels.

For acclimatization, the plants were taken from the culture medium, and their roots were gently washed with tap water. Then, chrysanthemums were planted in plastic multi-pot trays (one plant per 3×3 cm pot) filled with a mixture of peat and perlite (2:1) and supplemented with various types and concentrations of BC (Fig. 3C; Table 13).

During the first 2 weeks, the plants were covered with perforated foil and agrotexile to protect them from water loss and sunburns, and sprayed with water at least twice a day (Fig. 3D). Next, the covers were gradually removed, and spraying was replaced with watering.

After 1 month of cultivation, detailed biometric, physiological, biochemical, and microbiological analyses were performed on the plants and growth substrate.

Table 13 Composition of the growth substrates used for chrysanthemum acclimatization, detailing the biochar (BC) source organs and applied concentrations based on fresh and dry weight.

Treatment	Base substrate (v/v)	BC source (chrysanthemum organ)	BC concentration (% FW)	BC concentration (% DW)
Control	Peat : Perlite (2:1)	None	0	0
Root BC 4%		Roots	4	~7
Root BC 8%			8	~15
Root BC 12%			12	~24
Shoot BC 4%		Shoots	4	~7
Shoot BC 8%			8	~15
Shoot BC 12%			12	~24
Flower BC 4%		Flowers	4	~7
Flower BC 8%			8	~15
Flower BC 12%			12	~24

FW, fresh weight; DW, dry weight. Dry weight values are approximate.

Biochar type and concentration

Biochar obtained from roots, shoots, or flowers of in vivo-grown chrysanthemum ‘Richmond’ plants was added to the growth substrate at the levels of 4, 8, and 12% fresh weight (FW), i.e., approximately 7%, 15%, and 24% dry weight (DW), and mixed thoroughly with peat and perlite (Table 13).

Analyses of plant material

Biometric traits

Biometric traits of the plants were analyzed, including shoot length, leaf number, shoot fresh weight, as well as root fresh and dry weight (Fig. 3F). Leaf planimetry analysis was performed using a Perfection V800 scanner (Epson, Japan) and WinFOLIA 2016b imaging software (Reagen Instruments, Canada) (Fig. 3G). These analyses included leaf area (A), perimeter (P), horizontal length (L), vertical width (W), and average width, as well as the aspect ratio (W/L) and the form coefficient (calculated as $4\pi A/P^2$)⁸³. Root system architecture was measured using WinRhizo 2016a software (Reagen Instruments, Canada), including the total root length and area, root volume, mean diameter, length per volume, number of tips and forks (branching points, i.e., places where one root segment divides into two or more daughter segments) (Fig. 3H).

Pigment content and chlorophyll fluorescence

The relative contents of flavonols, anthocyanins, chlorophyll, and Soil Plant Analysis Development (SPAD) in the fresh leaves were measured using a portable MPM-100 multi-pigment meter (Opti-Sciences Inc., USA) (Fig. 3E). The relative values of F_0 (minimal chlorophyll fluorescence), F_V (variable fluorescence), F_M (maximal fluorescence), F_V/F_M , and F_V/F_0 ratios in the leaves were analyzed using the OS-30p+ chlorophyll fluorimeter (Opti-Sciences Inc., USA).

Enzymatic activity

To determine the activity of antioxidant enzymes, the Bagherzadeh Homaei and Ehsanpour procedure was applied⁸⁴. Fresh leaves were homogenized in phosphate buffer (pH 7.4) with 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 2% polyvinylpyrrolidone (PVP). The homogenates were centrifuged (MPW-260R, MPW MED INSTRUMENTS, Poland), and the resulting supernatants were used to determine the total protein content, as described by⁸⁵. The activities of guaiacol peroxidase (GPOX) and superoxide dismutase (SOD) were performed using the NP80 NanoPhotometer® (Implen GmbH, Germany) at specific

wavelengths: for GPOX at 430 nm^{86,87}, for SOD at 560 nm⁸⁸, and for proteins at 595 nm⁸⁵. Enzymatic activity was expressed as $\mu\text{mol} \cdot \text{min}^{-1}$ per mg of total protein.

Total polyphenol and flavonoid content, antioxidant capacity: FRAP and ABTS assays

Fresh plant leaves were stored at $-20\text{ }^{\circ}\text{C}$ (AFG 6402 E-B freezer, Whirlpool, Italy). The material was lyophilized (Alpha 1–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to a moisture content below 2% and ground in a mill to a fine powder (0.3–0.5 mm). For extraction, the samples were mixed with a methanol-water mixture (70:30% v/v) and shaken for 1 h (KS 130 basic shaker, IKA, Germany). Next, the samples were centrifuged for 15 min at 3000 rpm, and $4\text{ }^{\circ}\text{C}$ (ROTINA 420R centrifuge, Hettich, Germany). The resulting solution was decanted into a flask, which was then filled with the methanol-water mixture and mixed to obtain the final extract for the analyses.

The total polyphenol content (TPC) was determined according to Keutgen and Pawelzik⁸⁹. Absorbance was measured at 735.5 nm with a UV-1800 spectrophotometer (Shimadzu, Japan). Results were calculated based on the gallic acid calibration curve and expressed as $\text{mg} \cdot \text{g}^{-1}$ dry weight.

Total flavonoids were determined according to Papoutsis et al.⁹⁰ using a colorimetric reaction with sodium nitrite, aluminum chloride, and sodium hydroxide. The extract was sequentially mixed with distilled water, 5% NaNO_2 , 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, and 1 M NaOH, with appropriate incubation times between each step. A blank sample was prepared using methanol instead of the plant extract. Absorbance was measured at 510 nm using a SHIMADZU UV-1800 spectrophotometer (UV-Vis Spectral Photometer System, Japan). Total flavonoid content was calculated from a quercetin calibration curve, prepared under the same conditions as the analyzed samples, and expressed as mg quercetin equivalents (QE) per g of dry weight.

The ferric reducing antioxidant power (FRAP) was measured as described in the Benzie and Strain⁹¹ protocol. A working FRAP reagent was prepared by mixing acetate buffer (pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ) solution, iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution, and distilled water (10:1:1:1.2 ratio) at $37\text{ }^{\circ}\text{C}$. The extract sample was then mixed with the FRAP reagent (1:10 ratio) and incubated for 4 min at $37\text{ }^{\circ}\text{C}$. Next, the mixture was cooled to room temperature, and absorbance was measured at 593 nm using a UV-1800 spectrophotometer. Results were expressed in Trolox equivalent antioxidant capacity (TEAC).

As for the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay⁹², the ABTS radical cation was generated by incubating 7 mM ABTS with 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ (1:0.5) in the dark for 12 h. Before measurement, the solution was diluted with phosphate buffer (pH 7.4) to an absorbance of 0.700 (± 0.02) at 734 nm. Then, the diluted extract or standard was mixed with the ABTS working solution (1:39) and incubated in the dark at room temperature for 6 min. Absorbance was measured at 734 nm (Shimadzu UV-1800), and the inhibition rate was calculated as:

$$\text{Inhibition rate} = (A_{\text{ref}} - A_{\text{sample}}) / A_{\text{ref}} \times 100\% \quad (1)$$

where A_{ref} and A_{sample} are the absorbance values for the standard sample and extract, respectively.

To evaluate the antioxidant activity of each sample, the EC_{50} , indicating the concentration required to inhibit 50% of the ABTS radical, was calculated.

Microbiological analyses

Root-associated bacteria were isolated from plant root systems (treated with root-derived BC) collected for analysis. The analysis was limited to root BC based on the hypothesis of the unique influence of exudates and phytochemical compounds specific for this plant organ on the metabolic activity of the soil.

Briefly, 5 g of fresh root material previously rinsed to remove adhering soil particles were weighed aseptically. The material was subjected to a series of tenfold serial dilutions in sterile physiological saline (0.85% NaCl).

Aliquots of appropriate dilutions were surface-spread onto Plate Count Agar (PCA, Biomaxima, Poland) and incubated at 22 °C for 48 h.

After incubation, ten morphologically distinct colonies were selected from each sample based on differences in size, shape, color, margin, and surface characteristics. The selected colonies were purified by repeated streaking on PCA plates using the quadrant streak method until pure cultures were obtained. Purified isolates were maintained on agar slants and stored at 4 °C for further analysis.

For physiological profiling, bacterial strains were revived from agar slants and cultured in Tryptic Soy Broth (TSB Biomaxima, Poland) under appropriate growth conditions. Cell suspensions were prepared and adjusted to an optical density corresponding to 0.2 on the McFarland scale. Subsequently, 100 µL of each standardized suspension was inoculated into individual wells of EcoPlates™ (Biolog®, USA). The plates were incubated at 22 °C for 4 days. Following incubation, metabolic activity was assessed by measuring absorbance at 590 nm using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific, USA).

The results were expressed as the percentage of microorganisms capable of utilizing a given carbon source within each functional category. The percentage was calculated as the number of isolates showing a positive response (color or turbidity development) to substrates belonging to a specific carbon group, relative to the total number of tested isolates. Carbon sources were grouped into functional categories according to the Biolog Eco-Plate classification, and the proportion of positive responses within each category was used to characterize substrate utilization patterns.

Analyses of growth substrate

Microbiological analyses

Microbiological array of the growth substrate samples included quantitative determinations of the following microorganism groups: Actinobacteria⁹³, total bacterial counts, total filamentous fungi⁹⁴, and copiotrophic microorganisms⁹⁵.

Ringer's solution (90 mL; Merck, Germany) was added to 10 g of the growth substrate samples, and the mixture was shaken at room temperature. A series of tenfold dilutions was then prepared. The sample solution was transferred to selective media. Incubation of the microorganisms took place at 25–28 °C: 4 days for bacteria, fungi, and copiotrophs (BD 400/BD 720 Incubator, Thermo Scientific, USA) and 10 days for Actinobacteria (INC 125 F digital Incubator, IKA, Poland). The resulting number of colony-forming units (cfu) was determined per 1 g of growth substrate DW⁹⁶.

Enzymatic activity analyses

The activities of selected enzymes belonging to the oxidoreductase class (dehydrogenases and catalase) and hydrolases (alkaline and acid phosphatases, β -glucosidase, and proteases) were determined in fresh growth substrate samples sieved to <2 mm. Prior to analyses, the samples were stored at 4 °C for a maximum of two weeks.

- Dehydrogenase activity (DEH) was determined according to the method proposed by Thalmann⁹⁷, based on the reduction of 2,3,5-triphenyltetrazolium chloride to triphenylformazan (TPF), which was quantified spectrophotometrically at 546 nm and expressed as mg TPF kg⁻¹ substrate per 24 h.
- Catalase activity (CAT) was measured following the procedure of Johnson and Temple⁹⁸ using hydrogen peroxide (0.3%) as a substrate, with the residual H₂O₂ determined by titration with 0.02 M KMnO₄ under acidic conditions.

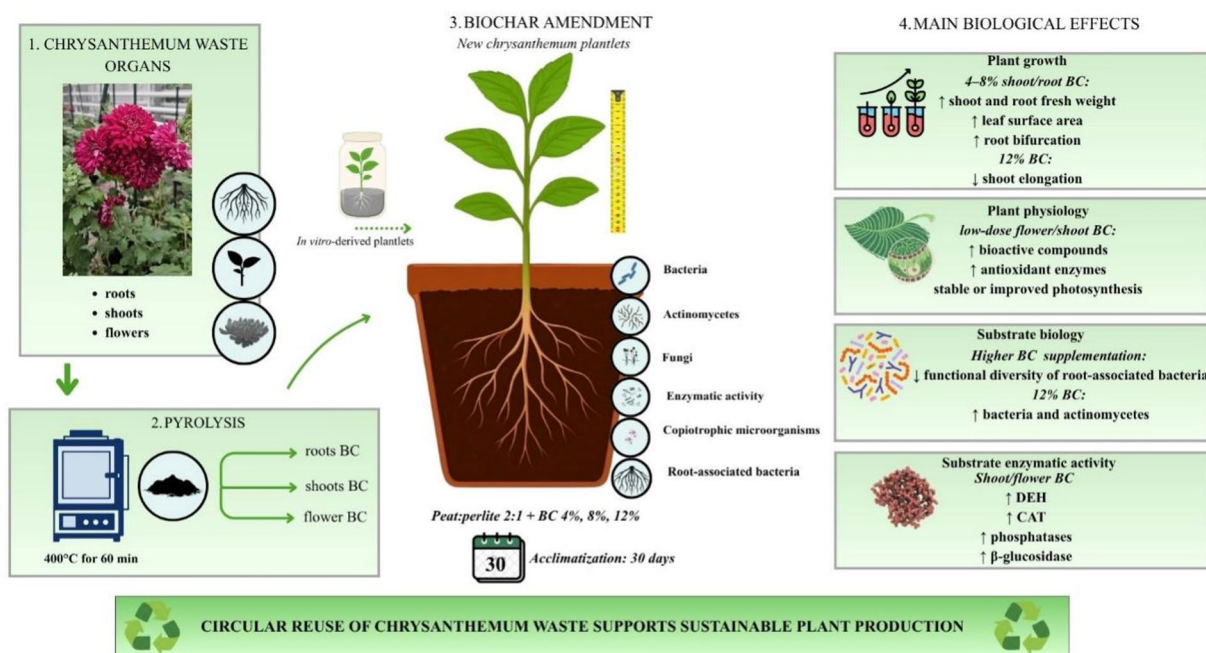


Fig. 4 Schematic demonstration of the experiment and main results.

- Activities of alkaline (AIP) and acid phosphatase (AcP) were assessed by quantifying p-nitrophenol (pNP) released after incubation at 37 °C for 1 h, at pH approximately 11.0 for alkaline phosphatase and approximately 6.5 for acid phosphatase Tabatabai and Bremner⁹⁹, .
 - β-glucosidase activity (BG) was determined using the method of Eivazi and Tabatabai¹⁰⁰ with p-nitrophenyl-β-D-glucopyranoside as the substrate. The released p-nitrophenol was measured at 400 nm immediately after alkalization with Tris/NaOH buffer (pH 10.0) and CaCl₂.
 - Protease activity (PR) was evaluated according to the method described by Ladd and Butler¹⁰¹ based on the determination of tyrosine (TYR) released from sodium caseinate after sample incubation. The concentration of tyrosine was quantified spectrophotometrically at 680 nm using a UV–Vis Thermo Scientific Evolution 201.
- The summary of the experiment and its main results are shown in Fig. 4.

Statistical analysis

The experiment followed a factorial design with 10 treatments: three types of BC (root-, shoot-, and flower-derived) applied at three concentrations (4, 8, and 12%) as fixed factors and a BC-free control. A total of 150 plants were studied, with 15 per experimental treatment (one plant per replication). Data were log₁₀ or logit-transformed prior to statistical analysis to meet assumptions of normality and homogeneity of variance. The data were analyzed via analysis of variance (ANOVA) and a post-hoc Fisher's test at $p \leq 0.05$ using Statistica 13.3 software (TIBCO Software Inc., USA). Values are expressed as mean ± standard error (SE). Letter designations accompanying the means in Tables indicate statistical significance: values sharing at least one common letter do not differ significantly from one another, whereas values with different letters show a significant difference. Given the large number of variables assessed, Principal Component Analysis (PCA) was used as a complementary multivariate approach to visualize relationships among the experimental treatments and the measured plant morphological, physiological and biochemical traits, microbiological parameters, and growing substrate enzyme activities (Statistica 13.3).

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Data availability All data supporting the findings of this study are included in this published article and its figures and tables.

Declarations

Competing interests The authors declare no competing interests.

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