



Biochars as culture medium additives influence organogenic potential of plant explants through changes in endogenous phytohormone and carbohydrate contents in *Daphne* species

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Abstract

Biochar applicability as culture medium amendment is rarely investigated due to its uncovered impact on in vitro cultured plants. This study assessed the feasibility of several biochars as alternatives to activated charcoal, for micropropagation of two ornamental *Daphne* species (Thymelaeaceae). We distinguished metabolic responses that were specific for: a) medium supplementation with any type of charcoal; b) biochar addition; c) biochar activation; d) the process of charcoal activation itself. We compared impacts of industrially produced biochar and three different biochars made in small-scale stoves (from residues of *Gliricidia sepium*) on organogenic capacity and physiological status in *Daphne* sp. shoots. Metabolite profiling revealed that biochars differently modulated accumulation of endogenous phytohormones and osmolytes in the shoots. Biochars reduced accumulation of proline, betaines and stress-related phytohormones (ABA, jasmonates), and impacted carbohydrate profile. For *D. jasminea* the most impactful was biochar activation, which increased micropropagation efficiency by elevating cytokinin and soluble sugars content. For *D. tangutica* charcoal/biochar activation, and application of biochar reduced accumulation of ABA and jasmonic acid, increased level of gibberellins, salicylic and benzoic acid, and altered carbohydrate composition. The study revealed that tested biochars can be used as a sustainable medium supplements without negative impact on production of new microshoots. Based on studied species we showed that biochar supplements could stimulate plantlet formation (*D. jasminea*), ameliorate stress response (*D. tangutica*), and constitute a way of undesired biomass utilization (invasive species *G. sepium*).

Keywords Activation · Biochar · Micropropagation · Phytohormones · Carbohydrate profile

Introduction

Biochar is a carbon-rich product, obtained by biomass pyrolysis under low oxygen conditions (Woelf et al. 2010). It has been originally used as a soil amendment to increase productivity of agricultural land areas (Glaser 2007; Rodrigues

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et al. 2019). Its application stimulates enzymatic activity in a soil (Oleszczuk et al. 2014), and improves root system architecture and growth parameters in poor soil conditions (Abiven et al. 2015). Biochar addition was also reported to increase productivity of aboveground plant organs, promote nutrient elements cycling and accretion of soil microbial biomass (Biederman and Harpole 2013; Latawiec et al., 2019). The scale of biochar production ranges from industrial facilities to individual farms (Woelf et al. 2010; Cornelissen et al. 2016), thus its yield and physicochemical properties may vary depending on the composition and type of biomass, as well as on production conditions. Such diversity contributes to complexity of biochar-soil interactions (Olmo et al. 2016). Nowadays new directions of biochar application are emerging, involving other fields of agriculture and environmental practice. Biochar can be transformed into potent, nutrient-enriched fertilizers to achieve further improvement of crop productivity (Borges et al. 2020). It is also an efficient biosorbent for detoxification of soil and water contaminants, including toxic trace metals, radionuclides, and pesticides (Oleszczuk et al. 2014; Wiszniewska et al. 2016; Jin et al. 2018; Bartoli et al. 2020). Biochar is also exploited as a means of energy storage and conversion (Liu et al. 2019), organic waste management (Jindo et al. 2020) and mitigation of climate change (Jeffery et al. 2011).

Despite that the long-term environmental effects of biochar exploitation are yet to be verified (Vithanage et al. 2015; Gelardi et al. 2019), perspectives of its use has already increased its global production and distribution. This material is considered low-cost and renewable source of activated carbon (AC) (Tan et al. 2017). Use of biochar as AC alternative is substantiated by biochar's low energy demand and reduced impact on global warming (Alhashimi and Aktas 2017). Properties of biochars can be further improved by chemical activation, which increases a number of functional groupings on the surface (Kalinke et al. 2017). Such prepared biochar-derived materials can be suitable for horticultural uses, as a substrate for growing plants (Blok et al. 2017; Jindo et al. 2020). High purity AC has been used as a component of growing media in plant production, particularly in micropropagation and in studies on plant regeneration in various in vitro culture systems (Thomas 2008; Wiszniewska and Pindel 2009; Oláh 2017; Chin et al. 2019). Growth-promoting features of AC are attributed to its surface porosity. This feature facilitates adsorption of inhibitors and toxic metabolites secreted by cells during culture (Xu et al. 2014), and gradual release of beneficial compounds bound to its particles (Thomas 2008). A replacement of AC by biochar may be "low-cost tissue culture technology" approach, aimed at reducing the unit cost of plant production, without compromising the quality of plants (Savangikar 2004). In fact, Di Lonardo et al. (2013) reported that micropropagation parameters of white poplar were the same in media

containing biochar and AC. However, it is still unclear how various biochars affect physiological status and organogenic abilities of cultured plant organs.

This study investigated the feasibility of several biochar types as alternative medium supplements for micropropagation of two ornamental *Daphne* species. Studied plants differ in growth habit and have contrasting in vitro regeneration potential (Wiszniewska et al. 2013). Suitability of tested biochars for replacement of commercially available AC was evaluated on a basis of growth parameters and multiplication efficiency, as well as on physiological status of propagated shoots. We hypothesized that distinct charcoal/biochar types may differentially affect the vigor and propagation of shoots, as well as accumulation of phytohormones, carbohydrates, osmolytes, phenolic compounds and chloroplast pigments. We compared effects exerted by industrially produced biochar and three different biochars made in small-scale stoves from *Gliricidia sepium* biomass. This species, native from Mexico to Colombia and well established in other parts of the tropical region, provides a high biomass production capacity (Latawiec et al. 2019). Because it is a perennial plant and easily cultivated for green manure (N fixation) and mulch, the thicker stems can be used for the production of biochar, as, in some circumstances, they have no alternative use and can cause unwanted shade. In addition, in the area where the biochar was produced (Seropédica, Rio de Janeiro state, Brazil) *Gliricidia* spreads into native forests competing with local vegetation (Castro et al. 2018). Biochar production from *G. sepium* may therefore be helpful in preventing unintended impacts caused by uncontrolled growth of this species.

Materials and methods

Plant material

Stock shoot cultures of *Daphne jasminee* (Sibth. & Sm.) and *Daphne tangutica* (Maxim.) (Thymeleaceae) were maintained on modified basal woody plant medium (WPM) (Lloyd and McCown 1980), supplemented with 12.3 μM N6-[2-isopentyl]adenine (2iP), 5.37 μM 1-naphthaleneacetic acid (NAA), 20.0 $\text{g}\cdot\text{L}^{-1}$ sucrose and solidified with 0.8% Difco agar. Medium pH was adjusted to 5.6 prior autoclaving.

Biochar production and characteristics.

Industrially produced biochar (Biochar I) was produced in the low-temperature flash pyrolysis (300 °C). Feedstock was pine and spruce wood chips. Biochar was alkaline (pH 8) and contained 52% of carbon. At the level of detection, it was free from polycyclic aromatic hydrocarbons (according to manufacturer's information – Fluid S.A., Poland).

Self-produced biochar (Biochar II) was made of the residue biomass of *Gliricidia sepium* (Jacq.) Kunth ex Walp. of Fabacea family. A 1.5 m diameter floorkiln, with a 45° sloping edge and around 60 cm deep hole was used to produce the biochar (Suppl. Material 1a–c). The pyrolysis process in this kiln lasted for about 1 h. To finish the process by pyrolysis quenching, 6.5 L of water (water-activated biochar II) or cow urine (urine-activated biochar II), respectively, were used per 19.5 kg of *Gliricidia* biomass. This resulted in approximately 6 kg of activated biochar.

Culture conditions and growth response evaluation.

Shoot cultures were established by explanting apical parts of the shoots from stock cultures onto culture medium containing $0.6 \text{ g} \cdot \text{L}^{-1}$ of respective charcoal additive. The experimental treatments were as follows: AC—medium supplemented with commercially available “plant cell, tissue culture tested” activated charcoal (Sigma Aldrich); BCF – medium supplemented with industrially produced pine and spruce biochar (Fluid S.A.) (referred also to as biochar I); BCB – medium supplemented with self-produced *Gliricidia sepium* biochar (referred also to as biochar II); ABC-W – medium supplemented with water-activated biochar II; ABC-U – medium supplemented with cow urine-activated biochar II. Control medium (referred to as C-0) did not contain charcoal in any form.

Explants were cultured in 250 ml Erlenmayer flasks containing 50 ml of medium. For *D. jasminea* ten 5 mm-long, while for *D. tanguitica* five 15 mm-long explants were cultured per flask. Minimum 30 and 20 explants, for *D. jasminea* and *D. tanguitica* respectively, were used per treatment within experiment replicate. The experiment was replicated three times. Shoots were grown for 16 weeks in a growth chamber at 22 °C, under 16 h photoperiod (irradiance $80 \mu\text{mol m}^{-2} \text{ s}^{-1}$), with one subculture after 8 weeks.

After 16 weeks, the shoots were counted and micropropagation coefficient was calculated using the following formula:

$\text{MC} = (\text{number of developed adventitious shoots} / \text{total number of explants})$.

Shoots were measured and weighted. For dry matter determination the plant material was dried at 105 °C in an oven for 24 h and weighted afterwards.

Extraction and quantification of phytohormones

Extraction and quantification of phytohormones was performed according to Dziurka et al. (2016) and Dziurka et al. (2022). Plant material was lyophilized and pulverized. To each sample internal isotopic standard (ISTD) mixture was added. The samples were extracted with 1 ml of methanol/water/formic acid mixture (MeOH/H₂O/HCOOH 15/4/1 v/v). Details on procedure are included in Suppl. Material 2. The following hormones were monitored: ABA,

auxins, cytokinins, gibberellins, jasmonic acid, salicylic acid and benzoic acid. All phytohormone standards were purchased from Olchemim (Olomouc, Czech Republic) at the highest available purity, whereas all solvents were of HPLC-grade and purchased from Sigma-Aldrich (Poznań, Poland). The content of ethylene precursor, 1-aminocyclopropanecarboxylic acid (ACC) was estimated by means of stable isotope dilution method with use of [²H₄]1-aminocyclopropanecarboxylic acid (D-ACC, Olomouc, Czech Republic). Lyophilized samples spiked with D-ACC were extracted and cleaned up like described above for other phytohormones. UHPLC separation of 100% CAN dissolved samples was performed by hydrophilic interaction liquid chromatography (HILIC) on an Agilent 1260 Infinity system coupled to MS/MS (Agilent 6410). Separation was achieved on a AsecentisExpress HILIC (3.0 × 100 mm, 2,7 μm, Supelco, USA) at 35 °C. Details on procedure are included in Suppl. Material 2. Detected phytohormones were classified into several groups and presented accordingly as described previously (Wiszniewska et al. 2019).

Analyses of betaines and free proline

Betaine (glycine betaine, Gly-bet), stachydrine (proline betaine Pro-bet) and free proline (Pro) were estimated in the same samples simultaneously with ACC, and D-ACC was used as internal standard. Details on procedure are included in Suppl. Material 2.

HPLC analysis of carbohydrate profile

Lyophilized and homogenized plant samples were extracted with 1 ml of ultra-pure water, and after dilution with acetonitrile 1:1 (v/v) and filtration, were analyzed on HPLC for soluble sugar content using Agilent 1200 chromatograph (Agilent, Germany) equipped with pulsed amperometric detector (ESA, USA) The following compounds were determined: inositol, glycerol, arabinol, mannitol, kestose, nystose, kestopentatose, raffinose, stachyose, maltotriose, glucose, fructose, sucrose, maltose, glucuronic acid, galacturonic acid. Details on procedure are included in Suppl. Material 2. Detected compounds were classified into groups: sugar alcohols, oligosaccharides (including fructans), mono- and disaccharides, and uronic acids, and presented accordingly (Wiszniewska et al. 2019).

The content of chlorophyll, carotenoids and phenolic compounds

Lyophilized leaf tissue was homogenized using a ball mill (MM200, Retsch, Germany) for 2 min at maximum frequency (30 Hz). Five mg DW were then extracted in 1.5 ml of 95% ethanol for 15 min and centrifuged at 2,000 g

(Universal 32R, Hettich, Germany) for 10 min. An aliquot of the ethanol extract (100 µl) was added to a 96-well microplate, and absorbances at 470, 648 and 664 nm were read on Synergy II (Bio-Tek, USA) spectrophotometer with a microplate reader. The concentrations of total chlorophyll a + b (Chl_{a+b}) and carotenoids (Car) were calculated according to (Lichtenthaler and Buschmann 2001). Concentrations reported here are averages of three biological replications, each consisting of two analytical replications, and are expressed as pigment content per gram DW (mg g^{-1} DW).

Total phenolic compounds (PhC) were measured according to the modified method of Singleton et al. (1999). An aliquot of the extract (50 µl) was diluted in 0.5 ml of deionized water and 0.2 ml of Folin-Ciocalteu reagent, and after 10 min 0.7 ml saturated Na_2CO_3 was added. After 2 h incubation the samples were mixed and transferred to 96-well plates. Absorbance at 765 nm was read on a microplate reader (Synergy II, Biotek). Results were presented as chlorogenic acid equivalent.

Statistical analysis

Data were statistically analyzed using STATISTICA 13.0 software (StatSoft, Tulsa, OK, USA). One way ANOVA and post hoc Tukey's test were used to assess differences between responses to applied biochar treatments in each *Daphne* species.

Results

Growth parameters and organogenesis of *D. jasminea* and *D. tangutica* microplantlets.

D. jasminea. Shoots were healthy and multiplied vigorously in all experimental conditions (see representative cultures on Fig. 1a–f). Micropropagation coefficient (MC) was the highest on media containing activated biochars (7.4 and 7.8 in ABC-W and ABC-U, respectively). Activated charcoal (AC) and both non-activated biochars (BCF, BCB) did not alter the number of newly developed shoots in comparison with the control ($\text{MC}=4.8\text{--}5.9$, $P>0.05$) (Table 1). Shoot height increased significantly only on ABC-U medium (Table 1). Each tested biochar stimulated fresh biomass accumulation (FW), but did not affect dry biomass content. In turn, AC declined fresh weight by 64%, and increased dry biomass by 35% in comparison with the control (Table 1). Shoot explants formed callus and adventitious roots (or root primordia) at the shoot base. Frequency of adventitious rooting was the lowest on media containing activated carbon forms (both activated charcoal and biochars) (Fig. 1g–l).

D. tangutica. Shoots multiplied with moderate efficiency (Fig. 2m–s). Micropropagation coefficient (MC) amounted to

3.2 in control conditions (Table 1). Only BCF biochar stimulated development of new shoots ($\text{MC}=6.2$) and enhanced fresh biomass accumulation. Other amendments did not influence MC and fresh biomass. Dry matter content ranged from 16.1–18.5% ($P>0.05$) and was comparable among the treatments. Shoots grew significantly shorter in a presence of BCF and BCB (Table 1). The longest shoots developed on ABC-U medium. Adventitious buds emerged on the shoots from the control, BCF, ABC-W and ABC-U media (data not shown). *D. tangutica* shoots did not form adventitious roots on studied media.

Phytohormonal profiling in *Daphne jasminea* and *D. tangutica* shoots

Auxins

D. jasminea. Total content of endogenous auxins (Aux) amounted to $33.93 \text{ nmol} \cdot \text{g}^{-1}$ DW in the control shoots (Table 2). All tested charcoal amendments reduced total Aux content. The lowest content was determined in AC treatment ($14.65 \text{ nmol} \cdot \text{g}^{-1}$ DW), while in the case of biochars it ranged from 21.99 to $27.74 \text{ nmol} \cdot \text{g}^{-1}$ DW ($P>0.05$). Considering a profile of endogenous auxins, conjugated Aux prevailed in each treatment, and constituted 89.5–95% of the total Aux pool ($P>0.05$) (Table 2). Indole-3-carboxylic acid (I3CA) was the most abundant Aux conjugate. (Table 2). The highest proportion of active Aux was noted in the presence of AC, while in the case of control and ABC-W -the lowest. 4-chloroindole-3-acetic acid was a predominant active Aux in all media.

D. tangutica. Total Aux content amounted to $19.62 \text{ nmol g}^{-1}$ DW (Table 3) in control shoots. There was no effect of any amendment type on Aux concentration. Amino acid-conjugates constituted the highest proportion of Aux (approx. 83–88%, depending on the treatment), with predominant indole-3-carboxylic acid (Table 3). ABC-U elevated a proportion of active Aux by approx. 40%, in comparison with the control (Table 3). 4-chloroindole-3-acetic and 5-chloroindole-3-acetic acids were the most abundant active Aux. Each biochar enhanced auxin oxidization in *D. tangutica*.

Cytokinins

D. jasminea. Total content of cytokinins (Cyt) did not exceed 0.9 nmol g^{-1} DW (Table 2) ($P>0.05$) in most treatments. Only activated biochars (ABC-W and ABC-U) enhanced Cyt accumulation. Cyt concentration doubled (to 2.1 nmol g^{-1} DW) in the case of ABC-U, whereas it raised almost 19-times to $15.1 \text{ nmol} \cdot \text{g}^{-1}$ DW in the case of ABC-W (Table 2). Active Cyt constituted over 98% and 77% of total Cyt pool in the shoots from ABC-W and ABC-U media, respectively. It was attributed to an accumulation of kinetin (Table 2). In contrast, active Cyt constituted about half of

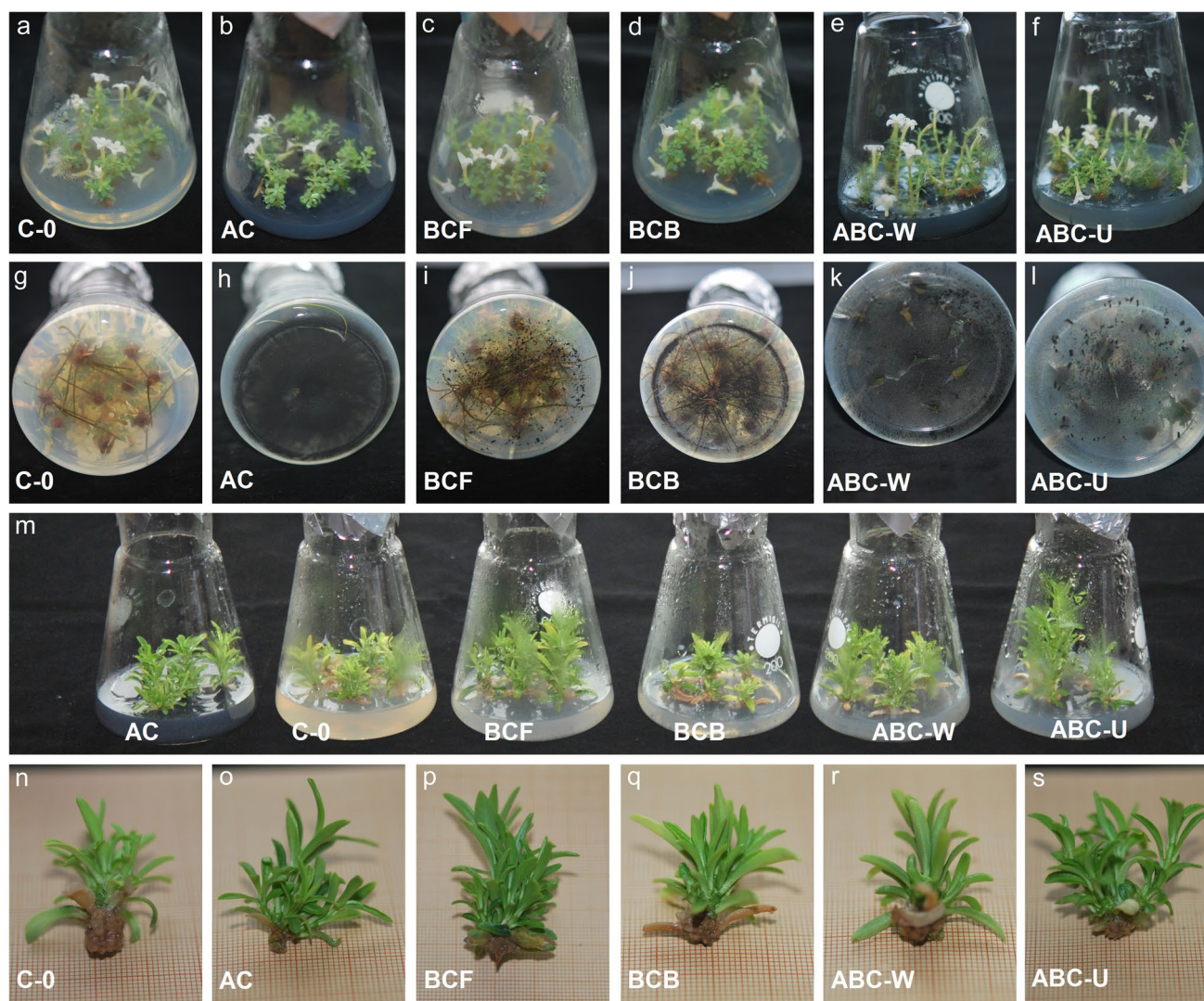


Fig. 1 In vitro cultures of two *Daphne* species on media with charcoal and biochar amendments. a–f –shoots of *D. jasminee* developed on tested media after 16 wk of culture; g–l – adventitious rooting of *D. jasminee* on tested media, m– cultures of *D. tangutica*; n–s –shoots

of *D. tangutica* developed on tested media tested media after 16 wk of culture. C-0 control medium (no charcoal), AC- activated charcoal, BCF – biochar I, BCB – biochar II, ABC-W–water-activated biochar II, ABC-U–urine-activated biochar II

total Cyt content in control medium, and in the presence of AC and non-activated biochars (BCF and BCB). N6-isopentenyladenine prevailed in these treatments. Levels of Cyt intermediates (ribosides) and glucoside conjugates were comparable among the media (Table 2).

D. tangutica. Total Cyt content amounted to $0.81 \text{ nmol g}^{-1} \text{ DW}$ in the control shoots (Table 3). ABC-W treatment increased total Cyt by 20% (Table 3), owing to elevated accumulation of N6-isopentenyladenine (Table 3). The profile of active Cyt was altered also in AC treatment, where enhanced accumulation of kinetin and reduced content of zeatin occurred (without changes in the total Cyt concentration) (Table 3). Cytokinin intermediates (ribosides)

constituted about half of the total Cyt pool, irrespectively of the treatment ($P > 0.05$).

Gibberellins

D. jasminee. Total pool of gibberellins (GAs) amounted to $140.6 \text{ nmol g}^{-1} \text{ DW}$ in control shoots. It increased to 174.7 and $173.6 \text{ nmol g}^{-1} \text{ DW}$ as a result of medium supplementation with AC and ABC-W, respectively (Table 2). Application of BCB and ABC-U did not change total GAs content, while BCF decreased it (Table 2). Deactivated GAs (GA8) predominated in each treatment and constituted 55–72% of total GAs, depending on the medium. Intermediates (GA5, GA6, GA7, GA9) constituted 23–41% of total gibberellin

Table 1 Growth and micropropagation efficiency parameters

Biochar Code	Treatment – charcoal type	MC ¹	Shoot height (mm)	Shoot FW ² (g)	Shoot biomass (%; DW ³)
<i>D. jasminea</i>					
C-0	Control (no charcoal)	5.1 ± 1.3b	13.2 ± 3.4b	0.42 ± 0.4b	14.6 ± 0.7b
AC	Activated charcoal	4.8 ± 0.5b	14.7 ± 4.9b	0.15 ± 0.2c	19.8 ± 2.0a
BCF	Biochar I	5.6 ± 0.4b	12.5 ± 2.3b	0.52 ± 0.3a	14.4 ± 1.4b
BCB	Biochar II	5.9 ± 0.3b	13.5 ± 2.5b	0.49 ± 0.3a	14.7 ± 1.4b
ABC-W	Water-activated biochar II	7.4 ± 1.3a	14.4 ± 1.5b	0.56 ± 0.0a	14.6 ± 1.5b
ABC-U	Urine-activated biochar II	7.8 ± 0.9a	21.7 ± 1.9a	0.53 ± 0.4a	16.5 ± 0.9b
<i>D. tangutica</i>					
C-0	Control (no charcoal)	3.2 ± 1.2b	21.5 ± 2.5b	0.68 ± 0.07b	16.1 ± 2.0a
AC	Activated charcoal	2.4 ± 1.8b	22.5 ± 4.0b	0.27 ± 0.05d	17.2 ± 2.6a
BCF	Biochar I	6.2 ± 1.7a	14.4 ± 1.5c	0.78 ± 0.05a	17.3 ± 2.5a
BCB	Biochar II	1.6 ± 1.3b	11.2 ± 4.8c	0.33 ± 0.13d	16.9 ± 1.7a
ABC-W	Water-activated biochar II	1.8 ± 1.3b	22.7 ± 4.5b	0.54 ± 0.03c	18.5 ± 1.0a
ABC-U	Urine-activated biochar II	1.4 ± 0.6b	29.7 ± 3.7a	0.38 ± 0.04d	17.8 ± 2.0a

¹Micropropagation coefficient²fresh weight³dry weight

Values are means ± SD, (n = 3). For each species different lower case letters indicate significant differences among treatments according to ANOVA and post hoc Tukey's test ($P \leq 0.05$)

concentration. The content of active GAs decreased by half in the presence of activated biochars (Table 2).

D. tangutica. Total GAs concentration reached 126.3 nmol g⁻¹ DW in control shoots. It increased by 33% in a presence of AC, and by approx. 10 and 20% in a presence of both activated biochars (ABC-W and ABC-U treatments, respectively) (Table 3). GAs content declined in the case of BCF medium. Gibberellin intermediates were the most abundant group in each treatment, owing to high content of GA6 (Table 4). The contribution of active GAs (particularly GA3) was the highest in AC and ABC-U-supplemented media (Table 3). Level of deactivated GAs was low and did not exceed 2.5% of the total pool (Table 3).

Stress-related PGRs: abscisic acid, jasmonates and ethylene precursor

D. jasminea. The content of ABA was the highest on the control medium (111.98 nmol g⁻¹ DW). It dropped significantly in a presence of studied charcoals (Table 2). Total ABA level declined by 72–75% in the case of activated biochars, by 37% in the presence of AC, and by 13% in the presence of non-activated biochars.

Total content of jasmonates (JA) was similar in most of the treatments (4.93–6.51 nmol g⁻¹ DW, $P > 0.05$). An exception was ABC-W-medium, where JA content dropped by half in comparison with the control (Table 2). The level of OPDA, the jasmonate precursor, declined in a presence of biochars (Table 2). The content of ethylene precursor (ACC)

went up in the presence of AC and ABW-U, while remained unchanged in other charcoal treatments (Table 2).

D. tangutica. Total ABA content was elevated by 45% (to 84.74 nmol g⁻¹ DW) in the presence of AC, owing to enhanced accumulation of ABA active forms (Table 3). In turn, BCB and ABC-U reduced ABA content by 20 and 32%, respectively. The content of both active and deactivated ABA forms declined (Table 3). In each treatment, active ABA predominated over its deactivated glucosylated form.

None of the charcoal treatments induced changes in total JA content, in comparison with the control (Table 3). Concentration of ethylene precursor increased significantly by 438% and 372% in media enriched with BCB and ABC-W, respectively (Table 3).

Amino acids and organic acids

D. jasminea. Proline (Pro) content increased 2.5-fold in AC treatment, in comparison with the control (Table 4). In contrast, both activated biochars reduced Pro content (247 and 316 µg·g⁻¹ DW in a presence of ABC-W and ABC-U, respectively). The charcoals influenced also the level of glycine betaine (GlyB). Its concentration increased by 101% and 61% in AC and ABC-W, respectively. GlyB content declined in the case of non-activated biochars (BCF and BCB, by approx. 34%), and urine activated one (ABC-U, by half) (Table 4). Proline betaine (ProB) was not as massively accumulated as Pro, but both compounds were influenced similarly by tested charcoal amendments (Table 4).

Fig. 2 Carbohydrate profile: mono and disaccharides **a**, sugar alcohols **b**, oligosaccharides (total and only fructans) **c** and uronic acids **d** in the shoots of *Daphne jasminea* multiplied on media supplemented with charcoal and biochars. C-0 control medium (no charcoal), AC- activated charcoal, BCF – biochar I, BCB – biochar II, ABC-W–water-activated biochar II, ABC-U–urine-activated biochar II. Values are means $\pm$ SD, $n=3$. Different letters indicate significant differences between means (acc. to ANOVA and post-hoc Tukey test at $P \leq 0.05$)

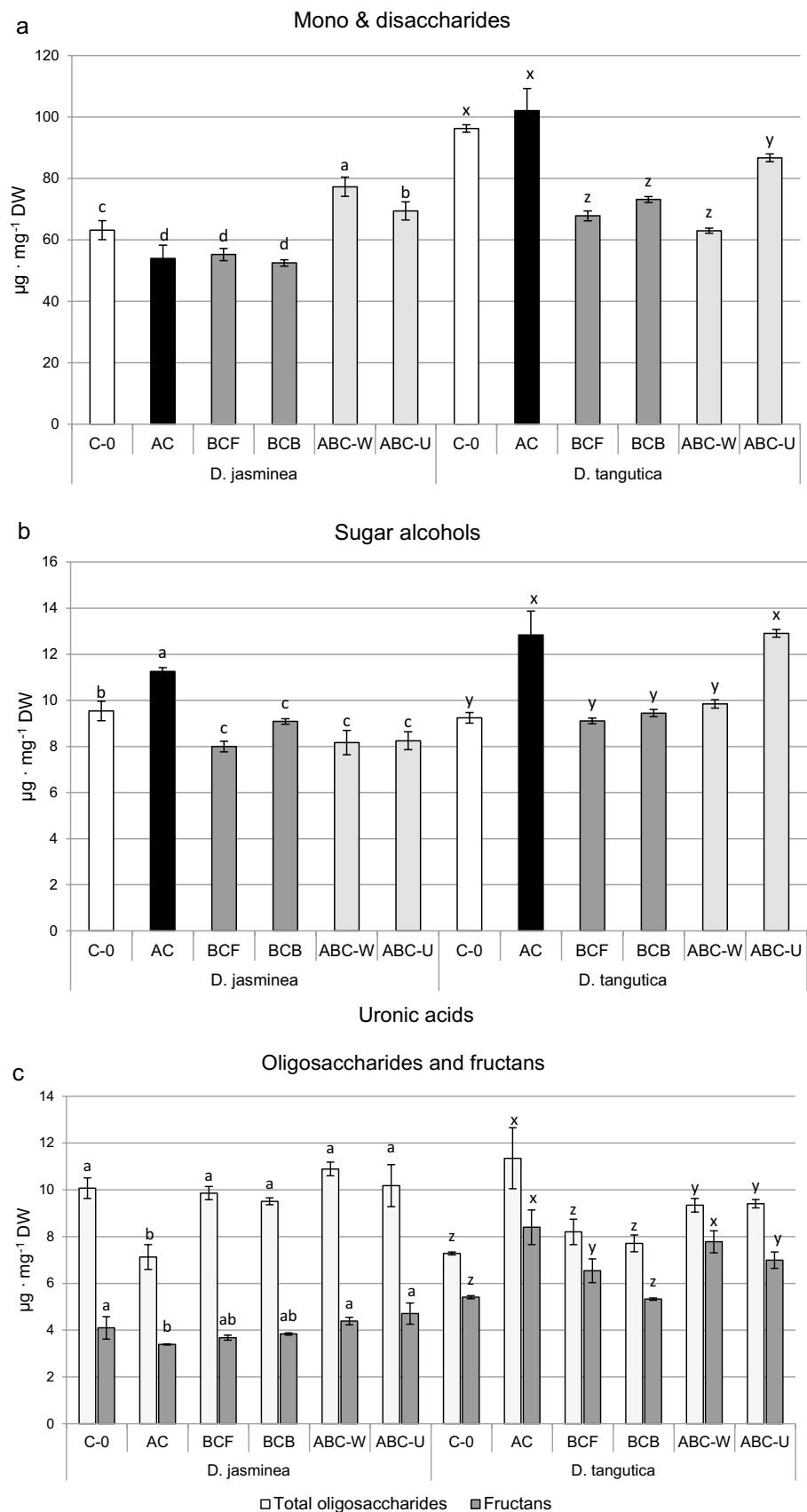
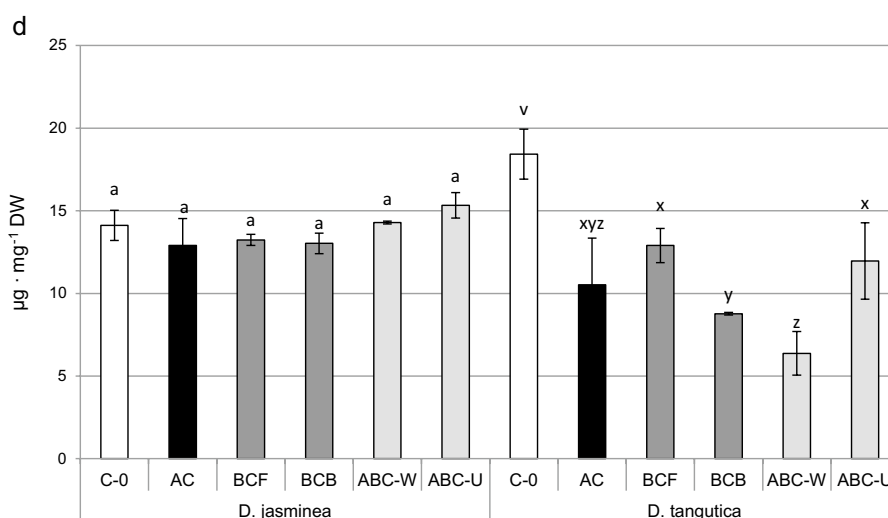


Fig. 2 (continued)



Considering organic acids of regulatory function, the content of salicylic acid (SA) increased significantly only in ABC-U-medium (by 18%, in comparison with the control). SA level remained the same in AC, BCB and ABC-W treatments, and decreased in BCF (Table 4). The content of benzoic acid (BA) did not vary among tested media (Table 4).

D. tangutica. Pro content declined in the presence of tested charcoals. It decreased by approx. 70% in BCB and ABC-U, by 77% in ABC-W, and by over 80% in BCF and AC treatments, in comparison with the control (Table 4). GlyB content decreased by over 90% in each test treatment (Table 4). Accumulation of ProB was enhanced by non-activated BCB biochar and its activated variants (ABC-W and ABC-U) (Table 4).

Concentration of SA increased in a presence of all tested biochars (Table 4). The highest SA contents were determined in the case of non-activated biochars (5.19 and 4.81 nmol g⁻¹ DW in BCF and BCB, respectively ($P > 0.05$)), in comparison with 3.40 nmol g⁻¹ DW in the control. Less pronounced increments occurred in the presence of both activated biochars. All activated charcoals: AC, ABC-W and ABC-U, elevated BA accumulation by approx. 25–30% (Table 4).

Carbohydrate profiling

D. jasminea. The content of mono- and disaccharides amounted to 63 µg · mg⁻¹ DW in the control shoots (Fig. 2a). The content of these carbohydrates went up to 77.29 and 69.42 µg · mg⁻¹ DW after treatment with ABC-W and ABC-U, respectively. In contrast, the level of mono- and disaccharides decreased to approx. 53–55 µg · mg⁻¹ DW in the presence of AC, BCF and BCB (Fig. 2a). Glucose and sucrose were predominant in all treatments (Suppl Material 3). Concentrations of sugar alcohols and oligosaccharides increased, while uronic acids declined in a presence of AC, in comparison with other treatments (Fig. 2b,c,d). Raffinose

always predominated in the pool of oligosaccharides, and kestose—among fructans. Inositol and glucuronic acid predominated among sugar alcohols and uronic acids, respectively (Suppl Material 3).

D. tangutica. Mono- and disaccharide content reached 96.27 µg · mg⁻¹ DW in control shoots (Fig. 2a). AC did not alter their concentration, whereas each biochar decreased it (by 10–35%, depending on the treatment) (Fig. 2a). The main compound was sucrose (Suppl Material 3). Application of AC and ABC-U enhanced accumulation of sugar alcohols by 39% in comparison with the control (predominantly inositol, Suppl Material 3) (Fig. 2b). Oligosaccharide content increased by 56, 28 and 29% in a presence of AC, ABC-W and ABC-U, respectively (Fig. 2c). Kestose predominated in most of the treatments (Suppl Material 3). The content of uronic acids decreased significantly by 29–66% (depending on the treatment) after addition of any charcoal type (Fig. 2d). Glucuronic acid was the main compound in this group (Suppl Material 3).

Pigments and phenolic compounds

D. jasminea. Total chlorophyll content increased by 15% only in a presence of ABC-U, in comparison with the control and other treatments (Fig. 3a). ABC-U also stimulated carotenoid accumulation (an increase by 18% in relation to the control) (Fig. 3a).

D. tangutica. Total chlorophyll content increased by 54, 12 and 29% in a presence of AC, ABC-W and ABC-U, respectively (Fig. 3b). The increments were attributed to enhanced accumulation of Chl a and Chl b in AC and ABC-U, and only Chl a in ABC-W. Non-activated biochars (BCF and BCB) did not influence chlorophyll, but reduced carotenoid content (Fig. 3b). Significant increase of carotenoid concentration occurred only in the presence of AC (Fig. 3b).

Table 2 Phytohormonal profile of *D. jasminea* shoots multiplied on media supplemented with charcoal and biochar

PGRs	Group	Compound name	Concentration in respective medium (nmol·g ⁻¹ DW)					
			C-0	AC	BCF	BCB	ABC-W	ABC-U
Auxins	Oxidized (Ox Aux)	Oxindole-3-acetic acid (OxIAA) [2-oxo-3-indolineacetic acid]	0.36 ± 0.06a	0.25 ± 0.07b	0.45 ± 0.02a	0.47 ± 0.05a	0.30 ± 0.02ab	0.42 ± 0.06a
	Conjugated	Indole-3-acetyl-L-asparatic acid (IAAsp)	0.30 ± 0.06	0.27 ± 0.08	0.42 ± 0.23	0.50 ± 0.00	0.17 ± 0.08	0.20 ± 0.07
		Indole-3-acetyl-L-glutamic acid (IAGlu)	0.53 ± 0.07	0.36 ± 0.01	0.45 ± 0.02	0.51 ± 0.00	0.64 ± 0.08	0.52 ± 0.08
		Indole-3-carboxylic acid (I3CA)	28.54 ± 3.77	10.12 ± 2.91	18.98 ± 3.18	18.12 ± 1.84	21.94 ± 1.73	17.28 ± 2.98
		Indole-3-acetic acid methyl ester (IAAME)	2.83 ± 0.78	2.34 ± 2.06	1.83 ± 1.67	1.10 ± 1.46	3.47 ± 1.95	3.50 ± 0.72
	Sum of Conj Aux		32.20 ± 4.68a	13.10 ± 5.06c	21.68 ± 5.10b	20.24 ± 3.30b	26.22 ± 3.84ab	21.49 ± 3.85b
	Active	Indole-3-acetic acid (IAA)	0.31 ± 0.11	0.33 ± 0.04	0.32 ± 0.04	0.30 ± 0.02	0.26 ± 0.10	0.29 ± 0.03
		4-chloroindole-3-acetic acid (4ClIAA)	0.68 ± 0.02	0.67 ± 0.07	0.82 ± 0.07	0.71 ± 0.07	0.73 ± 0.12	0.69 ± 0.08
		5-chloroindole-3-acetic acid (5ClIAA)	0.35 ± 0.05	0.25 ± 0.02	0.24 ± 0.11	0.26 ± 0.03	0.22 ± 0.07	0.20 ± 0.07
		Indole-3-butyric acid (IBA)	0.03 ± 0.03	0.05 ± 0.02	0.04 ± 0.00	0.02 ± 0.02	0.02 ± 0.03	0.02 ± 0.00
	Sum of Act Aux		1.37 ± 0.21a	1.30 ± 0.15a	1.42 ± 0.20a	1.29 ± 0.14a	1.23 ± 0.32a	1.20 ± 0.18a
	Total Auxins		33.93 ± 4.95a	14.65 ± 5.28c	23.55 ± 5.32b	21.99 ± 3.49b	27.74 ± 4.18b	23.11 ± 4.09b
Cytokinins	Conjugated/Deactivated	Trans-zeatin-7-glucoside (tZ7G)	0.00 ± 0.0	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.0	0.01 ± 0.01	0.01 ± 0.01
		Trans-zeatin-O-glucoside (tZOG)	0.04 ± 0.003	0.04 ± 0.003	0.04 ± 0.002	0.04 ± 0.003	0.03 ± 0.002	0.03 ± 0.003
	Sum of Conj Cyt		0.04 ± 0.003a	0.05 ± 0.013a	0.05 ± 0.012a	0.04 ± 0.003a	0.04 ± 0.012a	0.04 ± 0.013a
	Intermediates	Trans-zeatin riboside (tZR)	0.05 ± 0.005	0.05 ± 0.005	0.05 ± 0.004	0.05 ± 0.002	0.05 ± 0.002	0.05 ± 0.000
		Dihydrozeatin riboside (DZR)	0.07 ± 0.003	0.06 ± 0.002	0.08 ± 0.006	0.06 ± 0.004	0.09 ± 0.008	0.09 ± 0.008
		Cis-zeatin riboside (cZR)	0.04 ± 0.000	0.04 ± 0.002	0.03 ± 0.002	0.04 ± 0.000	0.03 ± 0.002	0.04 ± 0.002
		Kinetin riboside (KR)	0.09 ± 0.008	0.08 ± 0.005	0.08 ± 0.005	0.09 ± 0.007	0.08 ± 0.002	0.07 ± 0.01
		Meta-topolin riboside (mTR)	0.08 ± 0.006	0.08 ± 0.004	0.08 ± 0.003	0.08 ± 0.002	0.08 ± 0.003	0.08 ± 0.000
		N6-isopentenyladenosine (iPR)	0.09 ± 0.003	0.1 ± 0.002	0.08 ± 0.003	0.1 ± 0.000	0.09 ± 0.009	0.1 ± 0.002

Table 2 (continued)

PGRs	Group	Compound name	Concentration in respective medium (nmol·g ⁻¹ DW)					
			C-0	AC	BCF	BCB	ABC-W	ABC-U
		Sum of Int Cyt	0.42 ± 0.025a	0.41 ± 0.020a	0.40 ± 0.023a	0.42 ± 0.015a	0.42 ± 0.026a	0.43 ± 0.022a
	Free active	Trans-zeatin (tZ)	0.04 ± 0.005	0.02 ± 0.002	0.05 ± 0.017	0.04 ± 0.000	0.02 ± 0.005	0.03 ± 0.005
		Dihydrozeatin (DZ)	0.1 ± 0.005	0.08 ± 0.014	0.10 ± 0.016	0.09 ± 0.013	0.08 ± 0.013	0.07 ± 0.007
		cis-zeatin (cZ)	0.02 ± 0.001	0.02 ± 0.003	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.003	0.02 ± 0.002
		Kinetin (K)	0.06 ± 0.009	0.06 ± 0.009	0.07 ± 0.019	0.07 ± 0.015	14.33 ± 2.569	1.34 ± 0.039
		Meta-topolin (mT)	0.04 ± 0.006	0.04 ± 0.006	0.04 ± 0.005	0.04 ± 0.002	0.04 ± 0.006	0.04 ± 0.004
		N6-isopentenyladenine (iP)	0.12 ± 0.016	0.14 ± 0.006	0.12 ± 0.019	0.13 ± 0.003	0.11 ± 0.009	0.11 ± 0.015
		Sum of Act Cyt	0.37 ± 0.042c	0.38 ± 0.040c	0.41 ± 0.078c	0.38 ± 0.035c	14.60 ± 2.61a	1.61 ± 0.072b
		Total Cyto-kinins	0.80 ± 0.07c	0.83 ± 0.07c	0.85 ± 0.11c	0.85 ± 0.05c	15.07 ± 2.76a	2.07 ± 0.11b
Gibberellins	Deactivated (Deact GA)	Gibberellin A8	92.79 ± 5.67 b	125.69 ± 13.95a	63.42 ± 7.14d	73.40 ± 0.07c	121.29 ± 7.16a	78.22 ± 4.21c
	Active	Gibberellic acid (GA3)	5.97 ± 0.98	7.45 ± 2.32	4.31 ± 0.55	4.74 ± 0.96	2.99 ± 0.47	3.45 ± 1.05
		Gibberellin A1	0.01 ± 0.02	0.04 ± 0.05	0.06 ± 0.06	0.04 ± 0.03	0.05 ± 0.06	0.04 ± 0.04
		Gibberellin A4	0.09 ± 0.06	0.19 ± 0.03	0.18 ± 0.08	0.22 ± 0.07	0.13 ± 0.05	0.20 ± 0.03
		Sum of Act GA	6.08 ± 1.06a	7.68 ± 2.40a	4.55 ± 0.69a	5.01 ± 0.76a	3.17 ± 0.58b	3.68 ± 1.12b
	Intermediates	Gibberellin A7	0.74 ± 0.45	1.61 ± 0.06	1.24 ± 0.21	1.50 ± 0.32	1.41 ± 0.28	1.31 ± 0.13
		Gibberellin A9	0.22 ± 0.06	0.19 ± 0.04	0.24 ± 0.13	0.21 ± 0.06	0.13 ± 0.02	0.18 ± 0.07
		Gibberellin A5	7.01 ± 0.59	6.27 ± 1.72	6.02 ± 0.65	6.88 ± 0.28	5.79 ± 0.36	4.63 ± 0.70
		Gibberellin A6	33.78 ± 4.91	33.22 ± 0.51	31.67 ± 3.26	46.09 ± 4.91	41.79 ± 5.71	45.15 ± 5.93
		Sum of Int GA	41.75 ± 5.48b	41.29 ± 2.33b	39.17 ± 4.25b	54.68 ± 5.57a	49.12 ± 6.37ab	51.26 ± 6.98a
		Total Gib-berellins	140.61 ± 12.21b	174.66 ± 18.68a	107.14 ± 12.08c	133.08 ± 6.40b	173.57 ± 14.11a	133.16 ± 12.31b
Abscisic acid	Inactive (Deact ABA)	(±)-cis,trans-abscisic acid glucosylester (ABAGlc)	7.84 ± 3.58a	6.03 ± 1.67a	7.14 ± 1.07a	6.01 ± 0.37a	2.59 ± 0.75b	2.88 ± 0.80b
	Active	(+)-trans,trans-abscisic acid (S-ttABA)	103.53 ± 4.33	64.19 ± 6.06	91.75 ± 5.14	91.32 ± 2.57	28.55 ± 6.35	24.82 ± 4.98
		(±)-cis,trans-abscisic acid	0.61 ± 0.04	0.48 ± 0.04	0.57 ± 0.17	0.46 ± 0.07	0.40 ± 0.04	0.38 ± 0.21
		Sum of Act ABA	104.14 ± 4.37a	64.67 ± 6.10b	92.32 ± 5.31ab	91.76 ± 2.64b	28.95 ± 6.39d	25.21 ± 5.19d
		Total ABA	111.98 ± 7.95a	70.70 ± 7.77c	99.46 ± 6.38b	97.79 ± 3.01b	31.53 ± 7.14d	28.09 ± 5.99d
Jasmonates	Precursor	12-oxo-phyto-dienoic acid (OPDA)	0.48 ± 0.07a	0.38 ± 0.13ab	0.27 ± 0.04c	0.37 ± 0.05b	0.25 ± 0.03c	0.28 ± 0.02c
		(±)-jasmonic acid (JA)	5.89 ± 0.92	4.52 ± 0.64	4.58 ± 0.25	6.05 ± 0.25	2.38 ± 0.36	4.57 ± 2.36
	Other JA	(±)-jasmonic acid methyl-ester (MeJA)	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.09 ± 0.00	0.04 ± 0.01	0.08 ± 0.03

Table 2 (continued)

PGRs	Group	Compound name	Concentration in respective medium (nmol·g ⁻¹ DW)					
			C-0	AC	BCF	BCB	ABC-W	ABC-U
		Sum of other JA	5.98 ± 0.93a	4.60 ± 0.66b	4.66 ± 0.26b	6.14 ± 0.25a	2.42 ± 0.37c	4.65 ± 2.39ab
		Total jasmonates	6.46 ± 1.00a	4.98 ± 0.79b	4.93 ± 0.30b	6.51 ± 0.30a	2.68 ± 0.40c	4.93 ± 2.41ab
Ethylene precursor		1-aminocyclopropane-1-carboxylic acid (ACC)	1.94 ± 0.50b	3.94 ± 0.38a	3.54 ± 0.49ab	2.29 ± 0.91b	3.42 ± 0.47ab	4.02 ± 0.26a

C-0 control medium (no charcoal), AC activated charcoal, BCF biochar I, BCB biochar II, ABC-W – water-activated biochar II, ABC-U – urine-activated biochar II

Values are means ± SD, n=3. For each group of compounds (bolded), different lower case letters indicate significant differences among treatments, according to one way ANOVA and post hoc Tukey's test ($P \leq 0.05$). For table clarity, statistical comparisons within each single compound were omitted

In bold are indicated only those parameters that were evaluated statistically

D. jasminea. The content of phenolic compounds (PhC) amounted to 48.66 mg g⁻¹ DW in control shoots (Fig. 4a). Application of BCB biochar and its activated derivatives (ABC-W and ABC-U) reduced PhC content by 16–21% ($P > 0.05$) (Fig. 4a).

D. tangutica. PhC content reached 10.54 mg·g⁻¹ DW in control shoots (Fig. 4b). This parameter increased in a presence of AC and ABC-W (by approx. 17% in both the cases), whereas declined by 14% in BCB medium (Fig. 4b).

Discussion

Conducted study revealed that supplementation of in vitro culture medium with various types of charcoals influences micropropagation efficiency, biomass accretion, and biochemical composition of cultured organs. Charcoal-induced improvement of micropropagation was achieved in *D. jasminea*, whereas alterations in culture development and in accumulation of endogenous compounds were observed in both *Daphne* species. Studied plants differ in their organogenic capability in in vitro culture (Wiszniewska et al. 2013). *D. jasminea* propagates quickly and efficiently, thus the main obstacle in its propagation is fast ageing of explants. In contrast, culture conditions for *D. tangutica* need to be improved in order to obtain higher number of new plants. Stimulation of organogenic and embryogenic ability of cultured explants in a presence of activated charcoal is often reported in in plant vitro cultures, including orchids (Prizao et al. 2012; Chin et al. 2019; Manokari et al. 2021), grapevine (López-Pérez et al. 2005; Oláh 2017), and woody species (von Aderkas et al. 2002; Möller et al. 2006; Tretyakova et al. 2019). Metabolic responses of cultured organs are modified by enhanced adsorption of toxic metabolites and gradual release of growth-promoting compounds (Thomas 2008). Changes in transcription of development-related genes (Racioppi et al. 2019) and rearrangements of endogenous

phytohormone profile (von Aderkas 2002; Farhangi-Abriz and Torabian 2018) affect organogenic potential of explants. Our study shows that not only commercially available charcoal, but also self-produced biochars and their activated variants were capable of inducing changes in growth and proliferation capacity. Biochemical status of explants was also significantly altered. Rearrangements occurred in phytohormonal, carbohydrate and osmolyte profiles, as well as in accumulation of regulatory organic acids, chloroplast pigments and phenolic compounds. Similarly, Di Lonardo et al. (2013) reported that biochar stimulated ethylene release in the culture of poplar clones. This supports our finding that biochar as medium supplement affected physiological condition of cultured explants. It has not been fully elucidated in previous works whether the changes were beneficial or detrimental to overall plant performance. To provide an insight into physiology of micropropagated *Daphne* shoots, we focused on distinguishing growth and metabolic responses that were specific for: a) medium supplementation with any type of charcoal – commercially available charcoal and biochars; b) biochar addition – irrespectively whether activated or not; c) biochar activation – only activated biochars; d) the process of charcoal activation itself – for both activated biochars and activated charcoal. The analysis revealed that for *D. jasminea* the most beneficial was the use of activated biochar (biochar activation). Activated biochars (water- and cow urine-activated) significantly increased micropropagation efficiency, a crucial parameter of in vitro culture protocol. For *D. tangutica* application of biochar and the process of charcoal activation contributed to significant metabolic alterations, although without direct influence on growth parameters. Determined responses were summarized on Fig. 5 (only statistically significant differences were included). Their impact and implications for in vitro propagation are discussed below in detail.

Table 3 Phytohormonal profile of *D. tangutica* shoots multiplied on media supplemented with charcoal and biochar

PGRs	Group	Compound name	Concentration in respective medium (nmol·g ⁻¹ DW)					
			C-0	AC	BCF	BCB	ABC-W	ABC-U
Auxins	Oxidized (Ox Aux)	Oxindole-3-acetic acid (OxIAA) [2-oxo-3-indoline-acetic acid]	0.99 ± 0.06c	1.08 ± 0.14b	1.32 ± 0.06b	1.23 ± 0.09b	1.84 ± 0.08a	1.19 ± 0.23b
	Conjugated	Indole-3-acetyl-L-aspartic acid (IAAsp)	0.83 ± 0.26	0.22 ± 0.03	0.25 ± 0.06	0.29 ± 0.02	0.55 ± 0.23	0.16 ± 0.02
		Indole-3-acetyl-L-glutamic acid (IAGlu)	0.32 ± 0.08	0.16 ± 0.05	0.24 ± 0.07	0.18 ± 0.01	0.19 ± 0.02	0.12 ± 0.00
		Indole-3-carboxylic acid (I3CA)	16.17 ± 0.57	16.73 ± 2.87	13.15 ± 2.32	19.65 ± 1.43	19.76 ± 3.49	13.07 ± 4.53
		Indole-3-acetic acid methyl ester (IAAME)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Sum of Conj Aux		17.32 ± 1.82ab	17.11 ± 2.95ab	13.64 ± 2.45b	20.12 ± 1.46a	20.51 ± 3.74a	13.35 ± 4.55b
	Active	Indole-3-acetic acid (IAA)	0.22 ± 0.04	0.30 ± 0.03	0.22 ± 0.04	0.29 ± 0.08	0.38 ± 0.03	0.30 ± 0.05
		4-chloroindole-3-acetic acid (4ClIAA)	0.58 ± 0.16	0.85 ± 0.35	0.43 ± 0.04	0.46 ± 0.04	0.48 ± 0.03	0.56 ± 0.04
		5-chloroindole-3-acetic acid (5ClIAA)	0.46 ± 0.00	0.40 ± 0.03	0.50 ± 0.03	0.54 ± 0.04	0.63 ± 0.21	0.61 ± 0.08
		Indole-3-butyric acid (IBA)	0.04 ± 0.03	0.04 ± 0.02	0.04 ± 0.00	0.12 ± 0.03	0.05 ± 0.04	0.01 ± 0.00
		Sum of Act Aux	1.30 ± 0.23a	1.59 ± 0.43a	1.18 ± 0.11a	1.41 ± 0.19a	1.53 ± 0.31a	1.47 ± 0.34a
	Total Auxins		19.62 ± 2.11a	19.78 ± 3.52a	16.15 ± 2.62a	22.76 ± 1.74a	23.88 ± 4.13a	16.02 ± 5.12a
Cytokinins	Conjugated/Deactivated	Trans-zeatin-7-glucoside (tZ7G)	0.00 ± 0.000	0.01 ± 0.011	0.00 ± 0.000	0.01 ± 0.012	0.02 ± 0.000	0.01 ± 0.011
		Trans-zeatin-O-glucoside (tZOG)						
			0.05 ± 0.005	0.04 ± 0.003	0.04 ± 0.002	0.05 ± 0.003	0.05 ± 0.005	0.05 ± 0.005
	Sum of Conj Cyt		0.05 ± 0.005a	0.04 ± 0.014a	0.04 ± 0.002a	0.06 ± 0.015a	0.07 ± 0.005a	0.06 ± 0.015a
	Intermediates	Trans-zeatin riboside (tZR)	0.05 ± 0.002	0.05 ± 0.002	0.08 ± 0.004	0.05 ± 0.003	0.05 ± 0.003	0.05 ± 0.002
		Dihydrozeatin riboside (DZR)	0.12 ± 0.002	0.15 ± 0.004	0.14 ± 0.038	0.12 ± 0.015	0.19 ± 0.017	0.14 ± 0.000
		Cis-zeatin riboside (cZR)	0.03 ± 0.002	0.03 ± 0.000	0.03 ± 0.002	0.03 ± 0.000	0.03 ± 0.000	0.03 ± 0.000
		Kinetin riboside (KR)	0.08 ± 0.009	0.08 ± 0.009	0.07 ± 0.006	0.07 ± 0.006	0.07 ± 0.005	0.09 ± 0.016
		Meta-topolin riboside (mTR)	0.08 ± 0.000	0.08 ± 0.002	0.08 ± 0.000	0.08 ± 0.000	0.08 ± 0.002	0.04 ± 0.035
		N6-isopentenyladenosine (iPR)	0.05 ± 0.002	0.05 ± 0.000	0.05 ± 0.002	0.06 ± 0.002	0.05 ± 0.000	0.05 ± 0.002
	Sum of Int Cyt		0.41 ± 0.017b	0.43 ± 0.017b	0.41 ± 0.052b	0.42 ± 0.026b	0.47 ± 0.027a	0.41 ± 0.055b
	Free active	Trans-zeatin (tZ)	0.04 ± 0.005	0.02 ± 0.000	0.05 ± 0.007	0.06 ± 0.007	0.05 ± 0.003	0.05 ± 0.002
		Dihydrozeatin (DZ)	0.04 ± 0.003	0.04 ± 0.000	0.04 ± 0.002	0.05 ± 0.002	0.05 ± 0.002	0.05 ± 0.003
		cis-zeatin (cZ)	0.01 ± 0.002	0.01 ± 0.000	0.01 ± 0.000	0.01 ± 0.000	0.01 ± 0.002	0.01 ± 0.000
		Kinetin (K)	0.03 ± 0.024	0.09 ± 0.013	0.04 ± 0.006	0.06 ± 0.017	0.08 ± 0.029	0.03 ± 0.005
		Meta-topolin (mT)	0.04 ± 0.003	0.04 ± 0.006	0.04 ± 0.004	0.04 ± 0.003	0.05 ± 0.005	0.05 ± 0.005
		N6-isopentenyladenine (iP)	0.18 ± 0.018	0.15 ± 0.000	0.11 ± 0.006	0.12 ± 0.011	0.21 ± 0.049	0.13 ± 0.011
	Sum of Act Cyt		0.35 ± 0.055b	0.35 ± 0.019b	0.29 ± 0.025c	0.38 ± 0.040b	0.44 ± 0.090a	0.31 ± 0.026c
	Total Cytokinins		0.81 ± 0.077b	0.83 ± 0.050b	0.74 ± 0.079c	0.85 ± 0.081b	0.98 ± 0.122a	0.77 ± 0.092c
Gibberellins	Deactivated (Deact GA)	Gibberellin A8	1.83 ± 0.67b	3.85 ± 0.88a	1.32 ± 0.11bc	0.64 ± 0.43c	1.54 ± 0.28b	1.34 ± 0.86b
	Active	Gibberellic acid (GA3)	12.19 ± 0.09	19.85 ± 4.32	8.42 ± 0.51	10.49 ± 2.86	11.44 ± 1.84	18.24 ± 3.19
		Gibberellin A1	0.08 ± 0.06	0.19 ± 0.08	0.11 ± 0.03	0.08 ± 0.05	0.07 ± 0.07	0.28 ± 0.15
		Gibberellin A4	0.19 ± 0.05	0.21 ± 0.04	0.24 ± 0.04	0.28 ± 0.05	0.40 ± 0.04	0.36 ± 0.02
	Sum of Act GA		12.45 ± 0.20b	20.25 ± 4.44a	8.78 ± 0.58b	10.84 ± 2.96b	11.91 ± 1.95b	18.88 ± 3.19a
	Intermediates	Gibberellin A7	3.87 ± 0.57	1.01 ± 0.39	5.57 ± 1.54	0.73 ± 0.29	7.29 ± 2.74	3.36 ± 2.88
		Gibberellin A9	0.23 ± 0.05	0.14 ± 0.02	0.20 ± 0.09	0.15 ± 0.05	0.24 ± 0.16	0.19 ± 0.06
		Gibberellin A5	0.84 ± 0.27	0.75 ± 0.29	0.87 ± 0.32	0.53 ± 0.24	0.64 ± 0.27	0.71 ± 0.07
		Gibberellin A6	107.03 ± 2.97	142.69 ± 1.46	92.95 ± 3.89	103.53 ± 7.43	116.87 ± 10.20	127.36 ± 2.76
	Sum of Int GA		111.98 ± 3.86c	144.60 ± 2.16a	99.59 ± 5.84c	104.95 ± 8.01c	125.03 ± 8.53b	131.62 ± 5.77b
	Total Gibberellins		126.26 ± 4.73c	168.69 ± 7.48a	109.69 ± 6.53d	116.43 ± 11.40c	138.49 ± 10.76b	151.84 ± 9.82b

Table 3 (continued)

PGRs	Group	Compound name	Concentration in respective medium (nmol·g ⁻¹ DW)					
			C-0	AC	BCF	BCB	ABC-W	ABC-U
Absciscic acid	Inactive (Deact ABA)	(±)-cis,trans-abscisic acid glucosylester (ABAGlc)	10.97 ± 0.55a	10.01 ± 0.55a	5.53 ± 0.19b	5.73 ± 3.91b	5.19 ± 0.02b	3.99 ± 3.45b
	Active	(+)-trans,trans-abscisic acid (S-ttABA)	47.08 ± 3.85	74.33 ± 0.51	50.41 ± 3.66	41.22 ± 2.74	59.33 ± 3.97	35.99 ± 3.59
		(±)-cis,trans-abscisic acid	0.32 ± 0.03	0.40 ± 0.03	0.21 ± 0.02	0.24 ± 0.02	0.30 ± 0.02	0.22 ± 0.00
	Sum of Act ABA		47.40 ± 3.88c	74.73 ± 0.54a	50.62 ± 3.68c	41.46 ± 2.76d	59.63 ± 3.99b	36.21 ± 3.59d
	Total ABA		58.37 ± 4.43b	84.74 ± 1.09a	56.15 ± 3.87b	47.19 ± 6.67c	64.83 ± 4.01b	40.19 ± 7.04c
Jasmonates	Precursor	12-oxo-phytodienoic acid (OPDA)	0.31 ± 0.07b	0.23 ± 0.03c	0.21 ± 0.02c	0.49 ± 0.05a	0.50 ± 0.02a	0.48 ± 0.06a
	Other JA	(±)-jasmonic acid (JA)	1.14 ± 0.03	0.97 ± 0.17	1.12 ± 0.29	1.16 ± 0.17	0.88 ± 0.11	0.79 ± 0.03
		(±)-jasmonic acid methylester (MeJA)	0.12 ± 0.05	0.07 ± 0.00	0.09 ± 0.03	0.08 ± 0.04	0.07 ± 0.02	0.07 ± 0.00
	Sum of other JA		1.26 ± 0.08a	1.04 ± 0.17b	1.21 ± 0.32a	1.24 ± 0.21a	0.95 ± 0.13b	0.86 ± 0.03b
	Total Jasmonates		1.57 ± 0.15a	1.27 ± 0.20a	1.42 ± 0.34a	1.73 ± 0.27a	1.44 ± 0.15a	1.33 ± 0.09a
Ethylene precursor		1-aminocyclopropane-1-carboxylic acid (ACC)	0.36 ± 0.04b	0.42 ± 0.17b	0.39 ± 0.17b	1.94 ± 0.94a	1.7 ± 0.34a	0.6 ± 0.23b

C-0 control medium (no charcoal), AC activated charcoal, BCF biochar I, BCB biochar II, ABC-W – water-activated biochar II, ABC-U – urine-activated biochar II

Values are means ± SD, n=3. For each group of compounds (bolded), different lower case letters indicate significant differences among treatments, according to one way ANOVA and post hoc Tukey's test ($P \leq 0.05$). For table clarity, statistical comparisons within each single compound were omitted

In bold are indicated only those parameters that were evaluated statistically

Effects of medium supplementation with any charcoal type

Daphne jasmineea. Each type of charcoal decreased total auxins, auxin conjugates, and ABA content (Fig. 5). Auxins, together with brassinosteroids, were crucial for growth performance of charcoal-treated *Arabidopsis* and lettuce (Viger et al. 2015). Decline in total auxin concentration observed in *D. jasmineea* was due to reduced content of reversibly conjugated auxins (Table 3). The pool of active compounds remained unchanged, unlike in *Arabidopsis* and lettuce (Viger et al. (2015), where the content of active auxins increased in parallel with decline in auxin conjugates. In our study, auxin availability could drop as a result of their binding to charcoal particles. This could influence the overall auxin homeostasis in the shoots. Lower content of auxins may have positive impact on shoot branching, affecting apical dominance.

Each type of charcoal reduced ABA content in *D. jasmineea* shoots (Fig. 5). Previous studies showed that in a presence of activated charcoal, ABA content in culture medium rapidly declined, preventing from explant aging and senescence, and stimulating embryogenic development (Thomas 2008). Increased accumulation of ABA was detected during whole period of callus induction, whereas the decline was associated with organ regeneration (Huang et al. 2012). This is partially in line with our results, since only if ABA content dropped below 30 nmol g⁻¹ DW, shoot formation increased. This was observed here in the case of activated

biochar. ABA content also declined in salinity-treated bean plants in a presence of biochar (Farhangi-Abriz and Torabian 2018). ABA improves plant survival under stress of suboptimal temperatures and salinity (He et al. 2014), and is involved in water deficit resistance (Souza et al. 2017). Its content usually increases under stress, interacting with other hormonal pathways (Sharma et al. 2019). Organic compounds present in biochar leachates affect ABA signaling pathway, and are capable of inducing responses similar to those exerted by exogenously applied ABA, acting as its analog (Yuan et al. 2017). The presence of such compounds in charcoal-supplemented media could influence phytohormonal balance of cultured *D. jasmineea* shoots and diminish endogenous ABA content. The reduction was particularly pronounced in a presence of activated charcoals, indicating that activation process had an impact on the composition of charcoal leachates and their biological activity. The role of charcoal activation in modulation of shoot physiological performance is discussed in separate subsection below.

Daphne tangutica. Each charcoal type reduced concentration of proline, glycine betaine and uronic acids (Fig. 5). Proline content declined by 3–5 times, whereas glycine betaine by 8–12 times. These osmoprotective compounds are involved in stabilization of cell structures during osmotic impairments and radical scavenging, thus are usually synthesized and accumulated in stressed plants (Kanechi et al. 2013; Xu et al. 2018). Decline in their contents in *D. tangutica* may suggest that charcoal optimized osmotic conditions in culture environment and ameliorated stress. Similar

Table 4 Amino acids (proline (Pro), glycine betaine (GlyB), proline betaine (ProB)) and organic acids (salicylic acid (SA), benzoic acid (BA)) concentrations in the shoots of two *Daphne* species multiplied on media supplemented with charcoal and biochars

Medium	Amino acids ($\mu\text{g}\cdot\text{g}^{-1}$ DW)			Organic acids ($\text{nmol}\cdot\text{g}^{-1}$ DW)	
	Pro	GlyB	ProB	SA	BA
<i>D. jasminea</i>					
C-0	393.07 $\pm$ 23.35 b	146.72 $\pm$ 19.50c	1.82 $\pm$ 0.02b	28.20 $\pm$ 2.12b	110.04 $\pm$ 15.73a
AC	1028.51 $\pm$ 50.93a	296.11 $\pm$ 30.84a	2.95 $\pm$ 0.45a	20.26 $\pm$ 1.91bc	109.74 $\pm$ 18.97a
BCF	432.24 $\pm$ 28.67 b	101.04 $\pm$ 34.60c	1.46 $\pm$ 0.27c	18.93 $\pm$ 2.36c	101.28 $\pm$ 7.42a
BCB	420.61 $\pm$ 51.42 b	98.35 $\pm$ 8.14 d	2.76 $\pm$ 0.39a	25.57 $\pm$ 2.21b	108.39 $\pm$ 5.94a
ABC-W	246.94 $\pm$ 37.00 d	237.25 $\pm$ 36.08b	1.58 $\pm$ 0.16c	23.22 $\pm$ 4.86b	99.00 $\pm$ 9.85a
ABC-U	316.39 $\pm$ 34.16 c	72.69 $\pm$ 18.22d	1.57 $\pm$ 0.02c	33.38 $\pm$ 1.27a	95.30 $\pm$ 5.57a
<i>D. tangutica</i>					
C-0	811.29 $\pm$ 38.54a	125.60 $\pm$ 4.65a	0.32 $\pm$ 0.15c	3.40 $\pm$ 0.01c	132.86 $\pm$ 12.43b
AC	165.72 $\pm$ 10.71d	16.01 $\pm$ 0.61b	0.21 $\pm$ 0.04c	3.50 $\pm$ 0.21c	165.11 $\pm$ 9.88a
BCF	153.22 $\pm$ 8.73d	10.05 $\pm$ 0.42c	0.32 $\pm$ 0.08c	5.19 $\pm$ 0.58a	137.67 $\pm$ 6.67b
BCB	261.97 $\pm$ 10.16b	11.69 $\pm$ 0.42c	1.07 $\pm$ 0.37a	4.81 $\pm$ 0.31a	143.17 $\pm$ 11.31b
ABC-W	189.42 $\pm$ 9.82c	10.42 $\pm$ 0.39c	0.69 $\pm$ 0.17b	4.26 $\pm$ 1.27b	171.92 $\pm$ 1.20a
ABC-U	258.03 $\pm$ 11.57b	16.55 $\pm$ 0.71b	0.60 $\pm$ 0.24b	4.32 $\pm$ 0.01b	168.37 $\pm$ 9.19a

C-0 control medium (no charcoal), AC activated charcoal, BCF biochar I, BCB biochar II, ABC-W – water-activated biochar II, ABC-U – urine-activated biochar II

Values are means $\pm$ SD, (n = 3). For each species different lower case letters indicate significant differences among treatments according to ANOVA and post hoc Tukey's test ($P \leq 0.05$)

finding was reported in biochar-treated mung bean exposed to arsenic (Alam et al. 2019). Reduction of glycine betaine pool was associated with an inhibition of jasmonic acid synthesis in watermelon cell culture (Xu et al. 2018). This could explain some of our results, since among tested charcoals only activated ones reduced jasmonate content (Fig. 5, Table 4). Notwithstanding, the presence of any charcoal type in the soil or other growing substrates improves their water capacity properties, what can be manifested by reduced accumulation of osmoprotectant amino acid derivatives in plants (Alam et al. 2019; Bitarafan et al. 2019).

Effects of medium supplementation with biochar

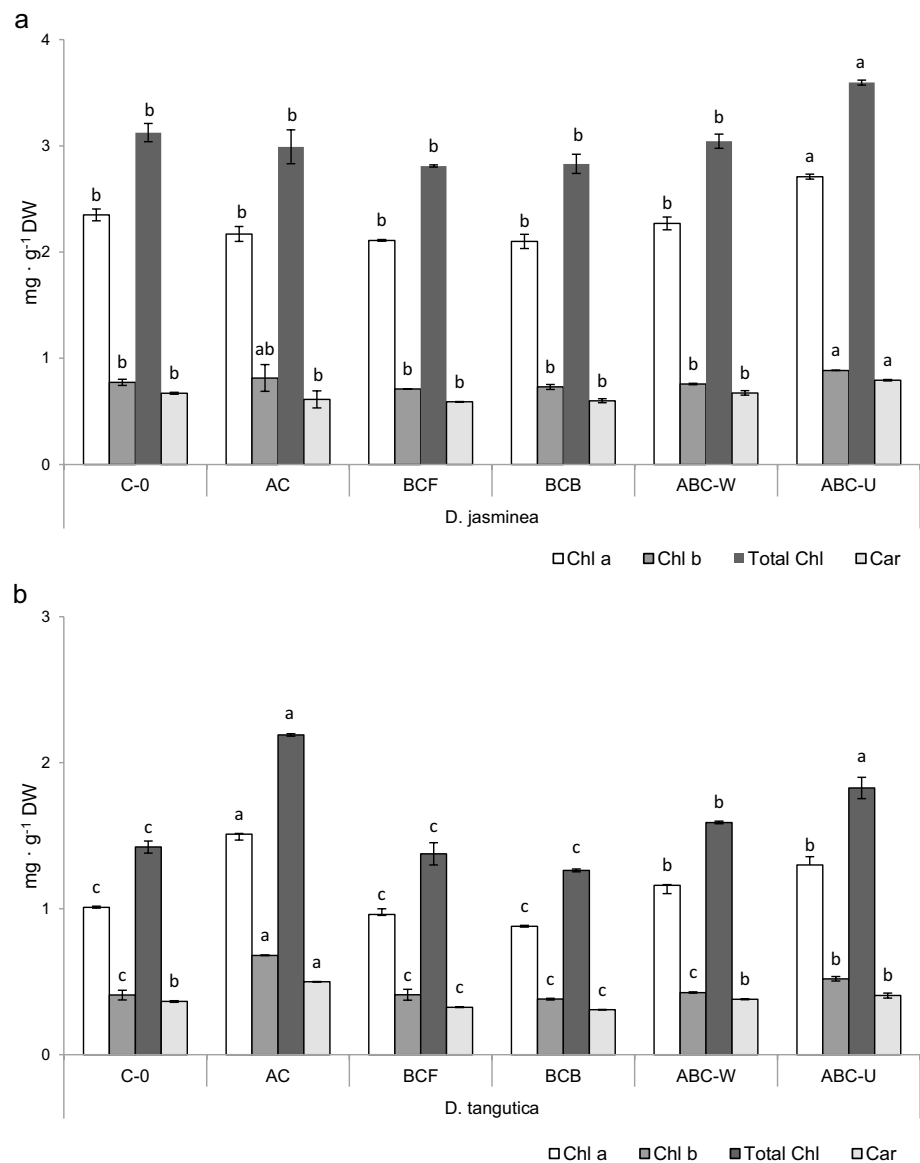
Daphne jasminea. Fresh biomass increased in a presence of each tested biochar (Fig. 5). Observed elevation in fresh biomass content was probably related to promoted hydration of developed shoots, as it was not accompanied by dry weight increase and any specific metabolic changes. Excessive water storage in in vitro cultured shoots may lead to hyperhydricity. Shoots with such disorder accumulate water in intercellular spaces, what stimulates synthesis of gaseous stress phytohormones, mainly ethylene and methyl jasmonate (van den Dries et al. 2013). However, despite high water content, *D. jasminea* shoots had no hyperhydricity symptoms, and enhanced accumulation of ethylene precursor was not specific to each biochar treatment. Several previous reports showed that the growth-promoting effect of biochar is negligible in non-stressful conditions (Zhang et al.

2013; Polzella et al. 2019; Martos et al. 2020). This seems to be in line with our results on *D. jasminea*. The major risk in biochar exploitation is its potential biotoxicity deriving from undefined concentrations of inherent pollutants formed during biochar production (Wang et al. 2017; Zheng et al. 2018). Biochars did not exert stressful or phytotoxic effects on this species. This finding may facilitate biochar utilization as a medium component in commercial plant propagation.

Daphne tangutica. Biochar application increased the content of oxidized auxins (oxoIAA) (Fig. 5). Oxidation is a mode of irreversible auxin inactivation and it serves as a major pathway of auxin pool regulation (Casanova-Sáez et al. 2021). Inactive oxoIAA itself do not exert any effects in planta (Zhang and Peer 2017). The level of auxin oxidation increases with increasing auxin supply (Kubeš et al. 2012). This mode of active auxin termination prevails during plant development, as a constitutive mechanism to gradually decrease auxin activity (Zhang et al. 2016). Elevated accumulation of oxidized auxins could be also associated with a response to abiotic stimuli (Korver et al. 2018). Biochar-induced auxin oxidation was in parallel with elevated level of endogenous salicylic acid (Fig. 5). Such interplay was previously reported to promote adventitious rooting in apple microcuttings (De Klerk et al. 1997). Since salicylic acid inhibits auxin conjugation (Dong et al. 2020), enhanced oxidation could be a way of regulating active auxin pool in *D. tangutica* under biochar treatment.

A specific response of *D. tangutica* to biochar was reduced concentration of ABA-glucosyl ester (ABA-GE), deactivated form of ABA (Fig. 5). This compound has

Fig. 3 Pigment accumulation in the shoots of two *Daphne* species multiplied on media supplemented with charcoal and biochars. a) *Daphne jasminea*, b) *Daphne tangutica* C-0 control medium (no charcoal), AC- activated charcoal, BCF – biochar I, BCB – biochar II, ABC-W – water-activated biochar II, ABC-U – urine-activated biochar II Values are means $\pm$ SD, different letters indicate significant differences between means (acc. to ANOVA and post-hoc Tukey test at $P \leq 0.05$)



limited biological activity and is considered a storage or transport form of active ABA. ABA-GE concentration usually decreases under stress, when the compound is hydrolyzed to release active ABA. As a result, the content of active ABA increases (Piotrowska-Niczyporuk and Bajguz 2011). This was not the case in *D. tangutica*, since the pool of active ABA did not increase in all biochar treatments. This is another premise of significant impact of biochar on plant hormonal homeostasis, and its potential to modulate plant responses to environmental factors. As suggested above, biochar leachates may contain ABA analogs that interfere with the pool of endogenous ABA, regulating the ratio of its active and inactive forms (Yuan et al. 2021).

Biochar induced changes in carbohydrate profile of *D. tangutica*. The alterations involved suppressed accumulation of soluble carbohydrates, particularly mono- and disaccharides, and

uronic acids (Fig. 5). Soluble sugars constitute a pool of compatible solutes, synthesized primarily to counteract osmotic imbalance, but also to protect cells from oxidative damage (Keunen et al. 2013). Uronic acids are constituents of mucilage and their accumulation usually increases under osmotic and ionic stress (Ghanem et al. 2010). Biochar is typically applied to alleviate response to stressors, including those associated with osmotic disorders: drought, salinity or cold (Yuan et al. 2017; Farhangi-Abriz and Torabian 2018; Hafez et al. 2020). Therefore there are scarce and contradictory reports examining carbohydrate profile in non-stressed plants grown in a presence of biochar. Non-stressed tomato did not exhibit any alterations in carbohydrate profile after biochar treatment (Kolton et al. 2017), contrary to pak choi, where carbohydrate content increased (Song et al. 2020). The content of soluble sugars either dropped (Hafez et al. 2020), or increased (Yuan et al. 2017) in stressed

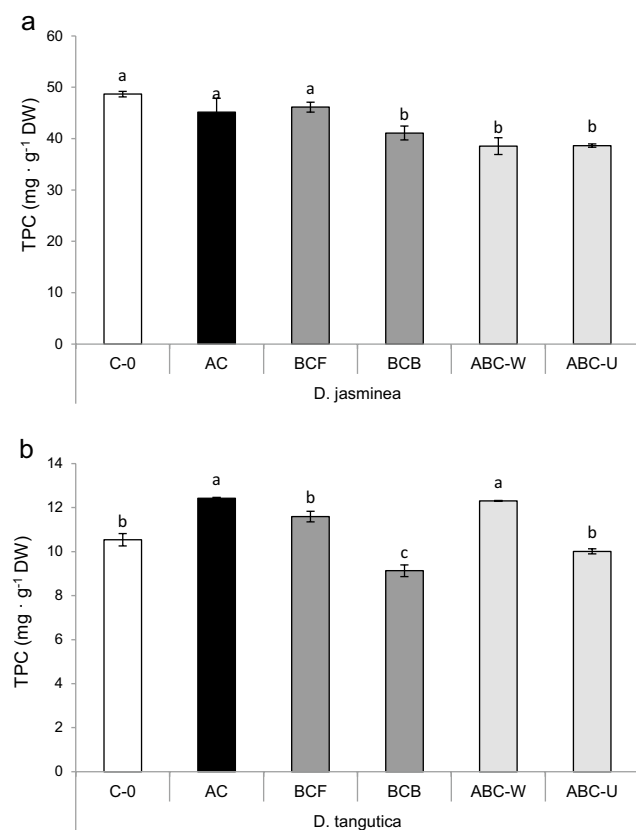


Fig. 4 Total phenolic content (TPC) in the shoots of two *Daphne* species multiplied on media supplemented with charcoal and biochars. a) *D. jasminea*; b) *D. tangutica* C-0 control medium (no charcoal), AC- activated charcoal, BCF – biochar I, BCB – biochar II, ABC-W – water-activated biochar II, ABC-U – urine-activated biochar II. Values are means $\pm$ SD, different letters indicate significant differences between means (acc. to ANOVA and post-hoc Tukey test at $P \leq 0.05$)

plants. Such diverse data suggest that biochar-induced changes in carbohydrate content and profile depend on the species and environmental factors. In *D. tangutica* lower content of soluble sugars and uronic acids may indicate amelioration of osmotic impairments and optimal growth conditions for cultured organs.

Effects of medium supplementation with activated biochar

Daphne jasminea. Growth and metabolic responses of this species were significantly impacted by activated biochars. These amendments stimulated formation of adventitious shoots, expressed as micropagation coefficient (Fig. 5). Such effect was absent in the media containing other tested charcoal types, including non-activated starting product (BCB, biochar II). Therefore growth promotion should be attributed to an activation process. Functional groupings are more abundant on the surface of activated biochars, resulting in their higher adsorptive capacity in comparison

with non-activated materials (Kalinke et al. 2017). Biochar adsorptive properties involve binding of organic compounds, including natural organic matter (Vithanage et al. 2015; Jung et al. 2015) that could exert growth-promoting effect.

Stimulation of shoot formation by activated biochars could be related to changes in the content of growth promoting phytohormones. Activated biochars elevated total cytokinins and reduced a pool of active gibberellins (Fig. 5). Shoot formation rate could be also influenced by reduced total auxin content, specific for the shoots cultured in a presence of all charcoal types. Recently significant alterations in profiles of growth promoters were observed in plants treated with biochar. Jaiswal et al. (2020) reported that promotion of tomato plant growth by biochar was attributed to upregulation of genes involved in auxin, cytokinin and gibberellin biosynthesis pathways.

Activated biochars affected also osmotic conditions of the cells by changes in accumulation of several osmolytes. Proline content decreased, while mono- and disaccharides increased. Signaling pathways of proline and soluble sugars interact with each other, as both contribute to osmotic adjustment and ROS scavenging (Moustakas et al. 2011). Increased accumulation of proline and soluble sugars is usually observed during stress, because these compounds function as osmolytes, membrane stabilizers and antioxidants (Keunen et al. 2013). Proline was a major osmoprotective amino acid in *D. jasminea*. Significant decline in its content may suggest that activated biochars optimized osmotic conditions in the medium, making massive accumulation of proline redundant. Efficient shoot multiplication supports an assumption of non-stressful culture conditions in the presence of activated biochars.

Enhanced accumulation of soluble mono- and disaccharides could be associated with higher carbon supply (Huang et al. 2017). Carbon release from biochar and its bioavailability may increase after dissolving in culture medium. Water soluble carbon nanoparticles were detected in biochar suspension and were found to promote growth of young plants (Saxena et al. 2014). Fluctuations in the content of growth-promoting phytohormones could further contribute to elevated sugar accumulation (Javid et al. 2011; Sharma et al. 2019; Rogach et al. 2020). Similarly, phytohormonal cross-talks could have an impact on proline metabolism (Farhangi-Abriz and Torabian 2018; Sharma et al. 2019).

Daphne tangutica. No response specific for activated biochar was distinguished in *D. tangutica*. However, tested activated biochars distinctly impacted phytohormonal and carbohydrate status. The contents of endogenous cytokinins, ABA and ACC were higher in the presence of water-activated biochar (ABC-W) that in the presence of urine-activated one (ABC-U). In turn, ABC-U stimulated accumulation of mono- and disaccharides, sugar alcohols

		<i>D. jasminea</i>				<i>D. tangutica</i>			
		Charcoal	Biochar	Activated biochar	Charcoal activation	Charcoal	Biochar	Activated biochar	Charcoal activation
Growth parameters	MC			↑					
	SH								
	FW		↑	↑					
	DW								
Pigments and phenolic compounds	Chl								↑
	Car								
	TPC								
Growth promoting PGRs	T Aux	↓	↓	↓					
	Act Aux								
	Conj Aux	↓	↓	↓					
	Ox Aux						↑	↑	
	T Cyt			↑					
	Act Cyt								
	Int Cyt								
	Deact Cyt								
	T GA								↑
	Act GA			↓					
	Int GA								
	Deact GA								
Stress-related PGRs	T ABA	↓	↓	↓					
	Act ABA								
	Deact ABA						↓	↓	
	T JA								
	Other JA								↓
	Precursor								
Organic acids and amino acids	SA						↑	↑	
	BA								↑
	Prol			↓		↓	↓	↓	
	Prol Bet								
	Gly Bet	↓	↓	↓		↓	↓	↓	
Carbohydrate profile	Su Alc		↓	↓					
	Mono&Di			↑			↓	↓	
	Oligo								↑
	Fruct								
	UA	↓	↓	↓		↓	↓	↓	

responses specific to charcoal application

responses specific to biochar application

responses specific to activated biochar application

responses specific to charcoal activation

↑/↓

increase/decrease

Fig. 5 Growth and metabolic responses of *Daphne* sp. shoots related to medium supplementation with all types of charcoals, biochars and their activated variants The abbreviations are explained in the main text. Colored boxes indicate responses that are statistically significant

and uronic acids, in comparison with ABC-W. Shoots were longer and had higher content of chloroplast pigments in the presence of ABC-U than ABC-W. Our observations

indicate that physiological responses and growth performance of plants depend on the mode of biochar activation. This process can be performed physically or chemically

(Maroušek et al. 2017; Mendonça et al. 2017), and is aimed at improving biochar's quality: increased surface porosity and micropore volume, better absorbing properties (Tan et al. 2017). To date benefits of activated biochar utilization for plant production were mainly reported for the soil-based cultivation systems. Our results contribute to deciphering an impact of activated biochar on plant physiology in soilless cultivation systems, including in vitro culture and may facilitate optimization of such growth conditions.

Effects of activation process

Daphne jasminea. In *D. jasminea* we were incapable of distinguishing responses specific for activation process itself, because the effects exerted by activated charcoal and activated biochars were too differential (Fig. 5). AC and ABC influenced physiological status of shoots in an independent way. Benefits obtained with a use of activated biochars, described in a subsection above, were not achieved in a presence of commercially available activated charcoal. AC presence induced several specific metabolic modifications: elevated synthesis of osmolytes: proline, glycine betaine and polyols, higher dry biomass accumulation, and lower fresh weight. AC caused also the most pronounced decline in the content of total auxins, and the highest increase of active gibberellins. Rearrangements in phytohormonal status and synthesis of osmoprotectants may indicate that *D. jasminea* shoots counteracted osmotic imbalance in the presence of this amendment.

Daphne tangutica. The activation process had the most significant impact on physiological responses of cultured *D. tangutica* shoots. All activated charcoal products elevated the content of chlorophylls (Fig. 5). Higher concentration of chlorophylls reflects improved growth response, conditioned by photosynthetic activity and nutritional status (Shadchina and Dmitrieva 1995; Luo et al. 2019), and indicates stress tolerance (Koźmińska et al. 2019). It facilitates acclimatization and increases survival rate in in vitro cultured plants (de Paiva Neto et al. 2013). Stimulation of chlorophyll accumulation in the presence of activated charcoal was observed in in vitro cultures of orchids (Dehestani-Ardakani et al. 2020). Chlorophyll content increased also in mung bean and sweet basil treated with biochar (Torabian et al. 2018; Ding et al. 2020).

Activated amendments influenced phytohormonal profile of *D. tangutica* by increasing contents of active gibberellins and benzoic acid, and reducing level of jasmonates (Fig. 5). Observed alterations could be involved in acquiring higher adjustability to stressful conditions of in vitro culture. An interplay between gibberellins and salicylic acid, which is the main BA derivative, was responsible for tolerance to salt, oxidative and heat stresses in

A. thaliana (Alonso-Ramírez et al. 2009). Benzoic acid itself was capable of inducing tolerance to suboptimal temperatures and drought in garden bean (Senaratna et al. 2003). Exogenous BA is frequently determined in biochar particles and therefore biochar application of may promote accumulation of endogenous BA (Biederman and Harpole 2013; Graber et al. 2015). An elevated level of gibberellins repressed jasmonate accumulation, since a crosstalk between these two classes of phytohormones is regulated by the same transcription factors (Riemann et al. 2015).

Activated biochars and charcoal enhanced also an accumulation of oligosaccharides. These compounds are compatible solutes (dos Santos et al. 2011), act as antioxidants (Pena et al. 2020), and may have signaling properties (Kollárová et al. 2018). Increased oligosaccharide content is also related to higher vigor of germinating seeds (Li et al. 2017). It appears that activated biochars and charcoal induced mild osmotic stress in *D. tangutica* shoots. Plants responded by synthesis of signaling and osmoprotective carbohydrates, gibberellins and benzoic acid, thus moderate stress did not deteriorate culture development.

Concluding remarks and future prospects

Effects of biochar application to the soil on plant growth performance and physiology were evaluated in numerous greenhouse and field studies (Rees et al. 2016, 2017; Hafez et al. 2020; Martos et al. 2020; Jaiswal et al. 2020). In turn, its impact on in vitro cultured plants was rarely investigated. In this study we showed for the first time how the contents and profiles of several endogenous compounds are modulated during in vitro organogenesis in a presence of activated charcoal and biochars. Tested medium amendments altered the concentrations of compounds that are crucial for proper differentiation and development of cultured explants. The most pronounced changes concerned carbohydrate metabolism, synthesis of osmoprotectants, homeostasis between active and inactive forms of growth-promoting phytohormones and ABA, as well as accumulation of signaling organic acids.

Important obstacles limiting using biochar for plant production include an unpredictable variations in its properties (Amin et al. 2016; Jindo et al. 2020) and unknown biotoxicity (Buss and Mašek 2014; Wang et al. 2017). Final product should be optimized prior to application, usually by using mixed biochars of desired characteristics (Alhashimi and Aktas 2017). Our study confirmed that effects exerted by biochars in in vitro culture also depend on the species and biochar type. No phytotoxicity symptoms were observed in studied *Daphne* cultures, most probably due to low concentration of biochar used for medium preparation. We have also observed reduced accumulation of stress-related compounds,

mainly osmolytes and ABA. These findings indicate that biochars could successfully replace activated charcoal in studies on other plant genera. In vitro cultures of numerous orchids require an addition of charcoal for efficient non-symbiotic seed germination (Shin et al. 2011), organ formation (Pacek-Bieniek et al. 2010; Kim et al. 2019) and shoot proliferation (Manokari et al. 2021). Charcoal reduces the content of endogenous auxins in cultured explants, contributing to developmental alterations and higher proliferation capacity. In orchid *Vanda tessellata* efficient micropropagation was assured by reduced content of auxins (Manokari et al. 2021). Our study shows that biochars may exert similar effect. Biochars could be also applied in experiments on somatic embryogenesis. Phytohormonal changes induced by charcoal and biochar in explants, particularly decreased level of auxins and increased concentration of cytokinins, may stimulate induction of somatic embryogenesis as well as accelerate embryo maturation and conversion, as revealed in hybrid larch (Von Aderkas et al. 2002), grapevine (López-Pérez et al. 2005) and purple coneflower (*Echinacea purpurea*) (Dehestani-Ardakani et al. 2020).

To conclude, biochars can be used as a low-cost medium supplements without compromising production of new shoots, and also as a components facilitating plantlet formation and ameliorating stress responses. For *D. jasminea* activated biochar was the most promising alternative to commercially available activated charcoal. Biochar exploitation in in vitro laboratories could also contribute to sustainable management of biomass residues. It could be an efficient way of biomass utilization of high yielding invasive plants, as was *Gliricidia sepium* in our study. Similar approach was found economically viable in conventional soil-based cultivation system (Vithanage et al. 2015), but has not been reported previously as suitable for in vitro culture.

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Author contributions AW- experiment design and conduction, data collection and analysis, manuscript writing, supervision. KD – biochemical analysis, data collection and analysis, manuscript writing, MD – UHPLC and HPLC analysis, data collection and analysis, AFR – biochar production, AEL – biochar production and supply.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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