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Biochar enhances root development and aloin content of mature leaves in containerized *Aloe arborescens* Mill.

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ABSTRACT

The leaves of the medicinal plant *Aloe arborescens* Mill. (*Asphodelaceae*) contain significant amounts of bioactive metabolites, including aloin (a mixture of the two diastereoisomers, aloin A and aloin B), aloesin, isoaloeresin D, and aloenin A. The presence of these metabolites varies considerably depending on the plant's growth conditions, including the used growing substrate. In recent years, there has been growing interest in using biochar for potted plants cultivation. However, there is currently no available information regarding the suitability of biochar for the containerized cultivation of *A. arborescens*. A pot experiment was conducted with the hypothesis that biochar could influence the growth and phytochemistry of *A. arborescens*. The growing medium was supplied with increasing proportions of biochar (1: 100 % commercial substrate; 2: mixed 50 % (v/v) substrate; 3: 100 % conifers wood biochar). Over the course of three years, the plants were closely monitored, and several key growth parameters were measured, including plant height, stem diameter, number and weight of leaves, and the number of suckers. After the first year, the content of selected active metabolites was assessed. This evaluation also involved a comparison of the respective levels in the leaves taken from the apical, median, and basal sections of the stem. The leaves collected from the median section of plants were found to be larger and exhibited the highest percentage of spikes, epidermis, and gel on fresh weight. As a general trend, it was observed that in plants cultivated within the highest amount of biochar, the leaves collected from the intermediate stem portion contained the highest quantity of secondary metabolites.

1. Introduction

Aloe arborescens Mill. is a perennial plant belonging to the *Asphodelaceae* family, likely native to South Africa. The species has established itself in a wide range of environments worldwide, both in wild conditions and as a cultivated plant. This multi-branched shrub can reach heights of up to 3 m, and brings the leaves in dense rosettes at the branch apices. Its leaves are greyish-green and fleshy, with yellow marginal teeth (Smith et al., 2008, 2012).

A. arborescens is grown as an ornamental plant, but its primary significance lies in its medicinal properties. The plant's leaves produce two distinct materials: leaf exudate, primarily employed to prepare laxatives, and inner mesophyll, otherwise termed gel, which, like its more well-known relative *A. vera,* finds use in skin ailments (Grace, 2011).

A shared characteristic among *Aloe* species is the presence of a relatively high concentration of bioactive metabolites in leaf extracts, encompassing anthraquinones, anthrones, chromones, coumarins, saponins, and polysaccharides (Akaberi et al., 2013; Olennikov et al., 2008). The well-known bitter taste of *Aloe* juice results from the presence of aloin, a blend of aloin A (barbaloin) and aloin B (isobarbaloin), two diastereoisomers of the glycoside 10-β-d-glucopiranosil-aloe-emodina (Sharma et al., 2014). Studies have demonstrated that aloin exhibits antioxidative and anti-inflammatory properties in murine macrophages (Park et al., 2009, 2011; Silva et al., 2014). Additionally, aloin displays a marked anti-proliferative effect on various human cancer cell lines (Esmat et al., 2006; Wan et al., 2017). Research by Niciforovic et al. (2007) has revealed the significant antitumor effects of aloin in the HeLaS3 cervical cancer cell line, suggesting it may have fewer undesirable side effects compared to current drugs for uterine cervix carcinoma treatment. A recent study has also shown that aloin can shield macrophages from an inflammatory response induced by lipopolysaccharide (LPS) by inhibiting the NF-κB signaling pathway (Luo et al., 2018).

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Chromones found in *Aloe* species are frequently mentioned for their beneficial effects on the skin. This biological activity is often associated with aloesin (also known as aloeresin B) and isoaloeresin D, which are of particular interest to the cosmetic industry (Añibarro-Ortega et al., 2021). Aloesin, in particular, can impede the initial stage of the conversion of tyrosine into melanin and is, therefore, a valuable ingredient for skin-care cosmetics.

On the other hand, aloenin A is a pyrone monoglycosidic derivative that has recently been identified as a novel natural product with the potential to inhibit pancreatic lipase, hinting at its possible role in treating obesity (Deora and Venkatraman, 2023).

A prior study has demonstrated the impact of the growing substrate on the yield performance and the content of active metabolites of *A. arborescens* (Lazzara et al., 2021). In this research, the addition of 20 % perlite to the growing substrate led to improved leaf and root development, although no significant effect was observed on the content of active metabolites. Thus, there is room for exploring the effects of other substrates on plant growth and phytochemical responses. Among the available growing substrates, biochar appears to hold particular promise. Biochar is the solid, carbonaceous, low-density by-product resulting from the pyrolytic treatment of various organic biomasses, such as municipal and industrial wastes, agricultural and forestry residues, and sewage sludge (Conte et al., 2021; EBC, 2021–2022; Ferlito et al., 2020). The physico-chemical properties of biochar vary depending on the pyrolysis temperature, duration, and the feedstock used (Fascella et al., 2020a). Furthermore, biochar's use in agricultural practices as a soil amendment is gaining traction, as it has been shown to enhance soil porosity, nutrient and water availability, and microbial activities, thereby improving overall plant growth and quality (Brassard et al., 2016; Alvarez-Campos et al., 2018; Kavitha et al., 2018). Recent experiments worldwide have assessed the benefits of incorporating biochar into the growing media of various horticultural and ornamental containerized crops, either partially or entirely replacing peat (Dispenza et al., 2016; Fascella et al., 2018; 2020b; Gasco et al., 2018; Margenot et al., 2018). Recently, we have tried to ascertain the potential and the ideal proportion of conifer wood biochar as a growing substrate for Carrizo citrange rootstock nursery production and potted *Murraya paniculata* plants (Roccuzzo et al., 2018; Fascella et al., 2021). However, to the best of our knowledge, its suitability as a growing medium for *A. arborescens* in soilless cultivation has not been explored. Therefore, with the hypothesis that biochar could influence the growth and phytochemistry of *A. arborescens,* this study was aimed to evaluate the response of potted *A. arborescens,* concerning growth, development, and content of selected secondary metabolites, to varying levels of biochar in the growing medium.

2. Material and methods

2.1. Experimental layout and measurements on substrate characteristics and plants growth

The trial was started in December 2018. For plant establishment, uniform suckers (5–8 cm in length from base to apex) of *A. arborescens* were sourced from the Cooperative Company "Le Shiare" in Marsala (TP, Sicily), and then transferred to the CREA-DC facilities in Bagheria (PA, Sicily) for transplantation. This operation was carried out using small plastic pots (7 × 7 cm), filled with three growing substrates:

1 100 % commercial substrate;
2 A blend of 50 % (v/v) substrate, consisting of 50 % biochar and 50 % commercial substrate;
3 100 % conifer wood biochar.

The biochar employed was produced through pyrolysis (450 °C for 4 h) of trunks and branches from various woody plant species, including black pine, larch, Scots pine, silver fir, and spruce. The obtained material was sieved using a 5 mm mesh and then incorporated in the selected proportions.

The commercial substrate utilized was "Vigorplant Terriccio Cinquestelle®" (Vigorplant Italia srl), which comprises a mixture of sphagnum peat, graded volcanic rock and bentonite clay. This commercial substrate had previously demonstrated success in growing *A. arborescens* in pots (Lazzara et al., 2021).

Regarding the initial physical characteristics of the three growing substrates, at the beginning of the trial, three samples from each substrate were water-saturated into a double ring and equilibrated on a sand box at a water pressure head of −10 cm. Subsequently, the main physical features were determined based on the wet and dry weights of samples in the lower ring. The water content at 5 kPa was measured by drying the samples at 105 °C for 24 h and assessing the pressure from the middle of the lower ring. Total porosity was obtained using the formula: 1.1 - (Bulk density/particle density), with bulk density derived from the dry mass samples/ring volume ratio, and particle density determined from the organic matter and ash density of the samples. As for the initial chemical characteristics of the three substrates, the pH was measured on three samples per substrate by means of a pH-meter (HI 9025C, Hanna Instruments, Padua, Italy) in a settling suspension of 60 g sample and 300 mL of deionized water (1:5; v:v), after shaking at room temperature for 1 h. Electrical conductivity (EC) was measured on the same water extract used for pH measurement using an EC-meter (XS 80 Pro-Stirrer, XS Instruments, Modena, Italy). The total content of nutrient content (phosphate, potassium, calcium, magnesium, and sodium) was determined after acid-digestion of 200 mg of dry sample in a microwave oven (Mars 5, CEM, Matthews, NC, USA), followed by filtration, dilution, and analysis with an ion chromatography system (Dionex ICS-6000, Thermo Fisher Scientific, Waltham, MA, USA).

Thirty plants were transplanted in each substrate, resulting in a total of 90 plants for the entire experiment. These plants were then moved to a greenhouse for full rooting and establishment, where they were regularly monitored for their phytosanitary conditions, and watered as needed. After reaching full establishment, on 15 January 2020, all one-year-old plants underwent a series of initial measurements. Six representative plants were randomly selected for each treatment (6 × 3 = 18 plants) to measure their height (cm), stem diameter (mm), and the total number of leaves (excluding the three not fully developed leaves at the apex of the plant). On that date, 18 plants for each treatment (18 × 3 = 54 plants) were transplanted into larger pots (22 cm in diameter, approximately 4 liters each), filled with the previously described substrates.

Non-destructive measurements, including plant height, stem diameter, number of leaves, and number of suckers, were taken on the following dates: 15/01/2020, 16/06/2020, 21/10/2020, 15/06/2021, and 06/06/2022.

On 21/10/2020 and 06/06/2022, a series of destructive measurements were conducted. Six plants for each treatment were carefully removed from their pots, weighed, and separated into hypogeal (roots) and aerial parts (leaves and stem). These parts were individually weighed, both immediately after cutting (fresh weight) and after drying in oven at 104 °C for 24 h to obtain the dry matter. On the same dates, all leaves on each sampled plant were counted (excluding the three not fully developed leaves at the apex of the plant), and three leaves were randomly chosen from the basal (B), median (M), and apical (A) sections of each plant. These three leaves were individually weighed, and the respective fractions of spikes (S), epidermis (E), and inner gel (G) were separated and evaluated separately, both in terms of fresh weight (FW) and dry matter (DM). In October 2020, 100 g samples of leaves from each substrate (1, 2, and 3), and leaf

Table 1
Physical and chemical characteristics of the studied growing substrates.

	Substrate 1 (100 % commercial substrate)	Substrate 2 (biochar+commercial substrate 50:50)	Substrate 3 (100 % biochar)
Water content (% v:v)	58.7 a	49.6 b	40.2 c
Air content (% v:v)	27.4 c	32.2 b	39.3 a
Total porosity (% v:v)	88.9 a	90.5 a	91.2 a
Particle density (g/L)	1605.0 c	1726.1 b	1860.3 a
Bulk density (g/L)	320.6 c	452.1 b	642.2 a
pH	5.7 c	7.1 b	8.5 a
EC (dS/m)	2.1 c	7.3 b	11.5 a
P (g/kg DW)	29.3 a	15.4 b	4.0 c
K (g/kg DW)	102.5 b	131.2 a	138.7 a
Ca (g/kg DW)	105.0 a	46.6 b	16.1 c
Mg (g/kg DW)	37.5 a	16.5 b	5.8 c
Na (g/kg DW)	14.8 a	16.1 a	7.0 b

Each value is the average of three replicates; in each row, values followed by the same letter are statistically not different at $P \leq 0.05$ (Duncan's test).

position (A, M, and B) were lyophilized and used for chemical determination of the major active metabolites (aloesin, aloenin A, aloin A, aloin B, and isoaloeresin D).

2.2. Chemicals

The solvents employed for sample preparations (methanol) and for chromatographic runs (methanol and water) were HPLC grade and were purchased from VWR International Srl (Milan, Italy). Pure standards of Aloenin A, Aloin A, Aloin B, Aloesin and Isoaloeresin D were obtained from Labochem Science SRL (Catania, Italy). PTFE filters were sourced from Pall Corporation (NY, USA).

2.3. Sample preparation for chemical analysis

500 mg of lyophilized plant material were cut into small pieces, approximately 5 mm^2 in size, and ground in a porcelain mortar until a fine powder was achieved. The powder was then extracted in 8 mL glass vials for 24 h using methanol (MeOH), with continuous agitation, all the while being shielded from light. The resulting suspensions, which exhibited a light green color, were filtered through PTFE filters with a 0.45 μm pore size. Subsequently, the filtered extracts were transferred into 2 mL amber vials and stored at +4/6 °C until analytical determinations.

2.4. HPLC/DAD/MS quantitative analyses

Quantitative analysis of the target metabolites was conducted using a Thermofisher Ultimate3000 instrument equipped with a binary high-pressure pump and a Photodiode Array Detector (Thermo Scientific, Milan, Italy). Each sample was analyzed on a reverse-phase column (Gemini C_{18}, 250 × 4.6 mm, 5 μm particle size, Phenomenex, Milan, Italy). The analysis followed previously published conditions (Lazzara et al., 2021) with slight modifications, as outlined before. A gradient of B (MeOH) in A (Water) was applied as follows: 0 min: 25 % B, 5 min: 30 % B, 15 min: 35 % B, 50 min: 70 % B, 60 min: 70 % B, 65 min: 25 % B, then held at 25 % B for 5 min. The solvent flow rate was set at 0.7 mL/min. Quantifications were performed, using the corresponding reference substances, at 293 nm for aloenin A (R^2 = 0.9996), aloin A (R^2 = 0.9939), aloin B (R^2 = 0.9999), isoaloeresin (R^2 = 0.9893) and aloesin (R^2 = 0.997). Peak assignments were confirmed through a series of HPLC/ESI/MS analyses, performed on a significant number of samples. The same HPLC apparatus, chromatographic column, and elution program were used. ESI mass spectra were acquired by a Thermo Scientific Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Inc., Milan, Italy) with a heated electrospray ionization (HESI II) interface. The mass spectra were recorded in negative ion mode, as previously reported (Napoli et al., 2020). Data acquisition and analyses were conducted using the Xcalibur software (Thermo Fisher Scientific, Inc., Milan, Italy).

2.5. Statistical data management

All data were subjected to analysis of variance (ANOVA) using the statistical package "Minitab" v. 17. The General Linear Model (GLM) procedure was employed, with all measurements as dependent variables (Y), and the experimental factors as independent variables (X). For the destructive and non-destructive measurements on whole plants, the analysis considered the experimental factors "Date" and "Substrate". In the case of destructive measurements on leaves, an additional factor, "Leaf position" (basal, B; median, M; and apical, A), was included in the analysis. All experimental sources of variation were treated as fixed factors, and the factors "Substrate" and, when applicable, "Leaf position" were nested within the factor "Date". In cases where a variable yielded a statistically significant result in the ANOVA, post-hoc testing was carried out using Tukey's post-hoc test at a significance level of $P \leq 0.05$, to identify differences between mean values (Gomez and Gomez, 1984).

3. Results

3.1. Physical–chemical properties of growing substrates

The main physical and chemical characteristics of the three studied growing substrates at the beginning of the experiment are summarized in Table 1. Generally, the physical properties were

Table 2
Results of the ANOVA (*F*-values) for the non-destructive measurements on *A. arborescens* plants, according to date and substrate (within date).

Source of variability	DF	Height of plants (cm)	Stem diameter (mm)	N. of leaves/plant	N. of suckers/plant
Date	4	254.01*	58.97*	110.66*	68.68*
Substrate (Date)	10	<1 n.s.	1.51 n.s.	1.47 n.s.	4.00*
Error	75				
Total	89				

n.s.: not significant.
* Difference significant at $P \leq 0.001$.

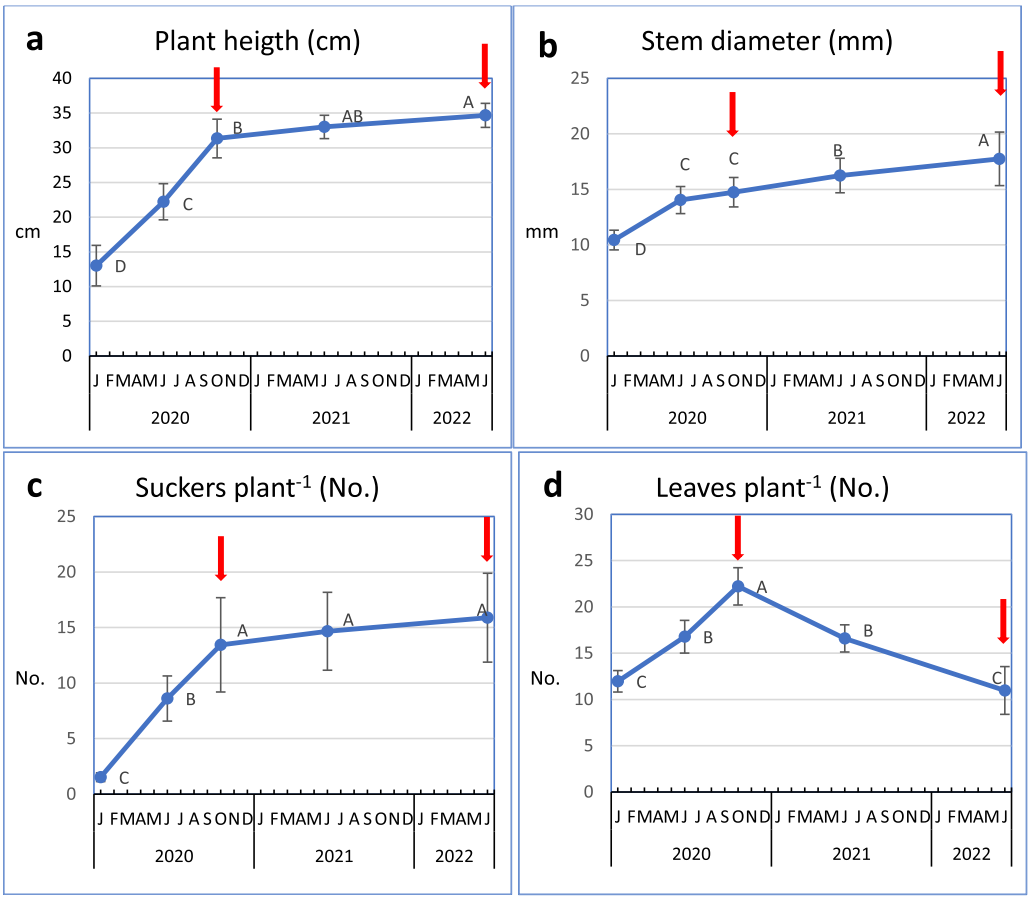

Fig. 1. Trend over time of *A. arborescens* plant height (cm), diameter of stem (mm), number of suckers per plant, and number of leaves per plant. For each date, vertical lines represent the standard deviation of mean. Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test). The red arrows indicate the dates of destructive measurements.

influenced by the proportion of biochar in each substrate. Specifically, as the biochar content in the tested substrates increased, there was a reduction in their water content, decreasing from 58.7 % to 40.2 % for substrates 1 and 3, respectively, along with a moderate increase in air content, ranging from 27.4 % to 39.3 %. These findings align with other studies that have reported a decrease in water content and an increase in air content with higher levels of biochar amendment in substrates (Gasco et al., 2018; Guo et al., 2018). Furthermore, the particle density increased as the biochar percentage increased, from 1605 g/L to 1860 g/L for substrates 1 and 3, respectively, and the bulk density also increased, from 320.6 g/L to 642 g/L (Table 1).

In terms of the chemical characteristics, an increase in pH, rising from 5.7 to 8.5 for substrates 1 and 3, respectively, and electrical conductivity (EC) from 2.1 to 11.5 dS/m, was observed as the biochar content in the growing substrates increased. Conversely, the phosphorus (P) content decreased from 29.3 to 4.0 g/kg DW for substrates 1 and 3, respectively, as the biochar percentage increased (Table 1). The potassium (K) content of the three substrates increased, from 102.5 to 138.7 g/kg DW for substrate 1 and 3, respectively, with the rise in their biochar concentration. A reduction in calcium (Ca) and magnesium (Mg) content was noted as the biochar amendment increased (Table 1). These findings are consistent with those reported by other researchers who have observed an increase in pH, EC, and K content, as well as a decrease in P concentration with increasing biochar amendment in substrates (Vaughn et al., 2015; Prasad et al., 2018; Chrysargyris et al., 2019).

Table 3
Results of the ANOVA (*F*-values) for the destructive measurements on *A. arborescens* plants, according to date and substrate (within date).

Source of variability	DF	Suckers/plant			Stem			Leaves/plant			Aerial part		Roots	
		n°	FW (g)	DW (g)	Diameter (mm)	FW (g)	DW (g)	n°	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)
Date	1	4.36	39.83**	21.79**	21.96**	80.43**	10.11*	213.30**	70.68**	21.57**	111.43**	19.07**	512.30**	158.68**
Substrate (Date)	4	3.95	8.72**	2.76 n.s.	1.20 n.s.	<1 n.s.	1.00 n.s.	1.04 n.s.	4.35*	2.19 n.s.	3.11	1.78 n.s.	3.69	1.44 n.s.
Error	30													
Total	35													

n.s.: not significant; FW: fresh weight (g); DW: dry weight (g).
* Difference significant at $0.001 < P \leq 0.01$.
** Difference significant at $P \leq 0.001$.

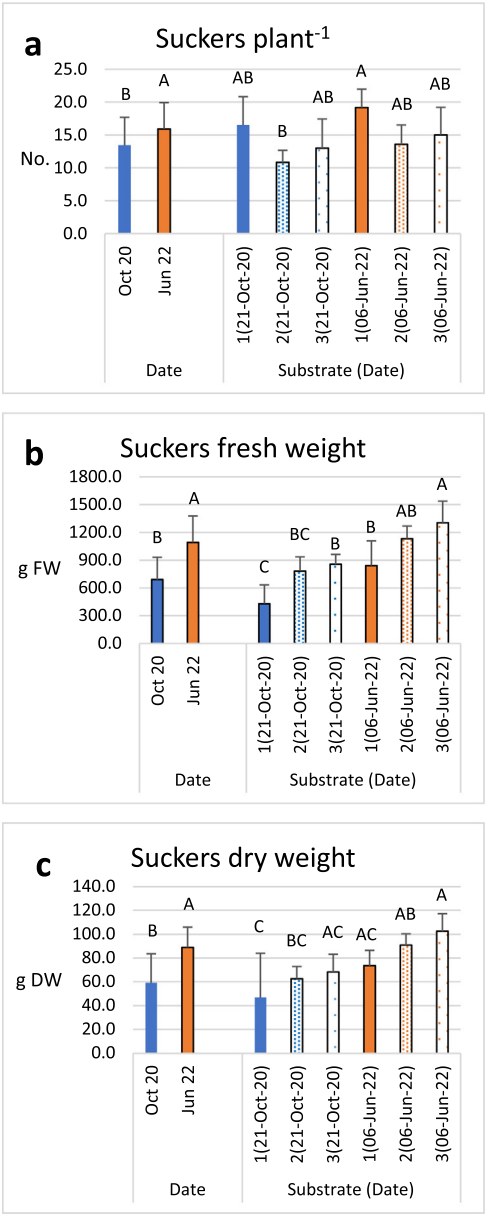

Fig. 2. Number and weight (fresh - FW and dry - DW) of *A. arborescens* suckers per plant according to date of measurement and growing substrate within each date. For each value, vertical lines represent the standard deviation of mean. Values marked by the same letter (including unreported intermediates) indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

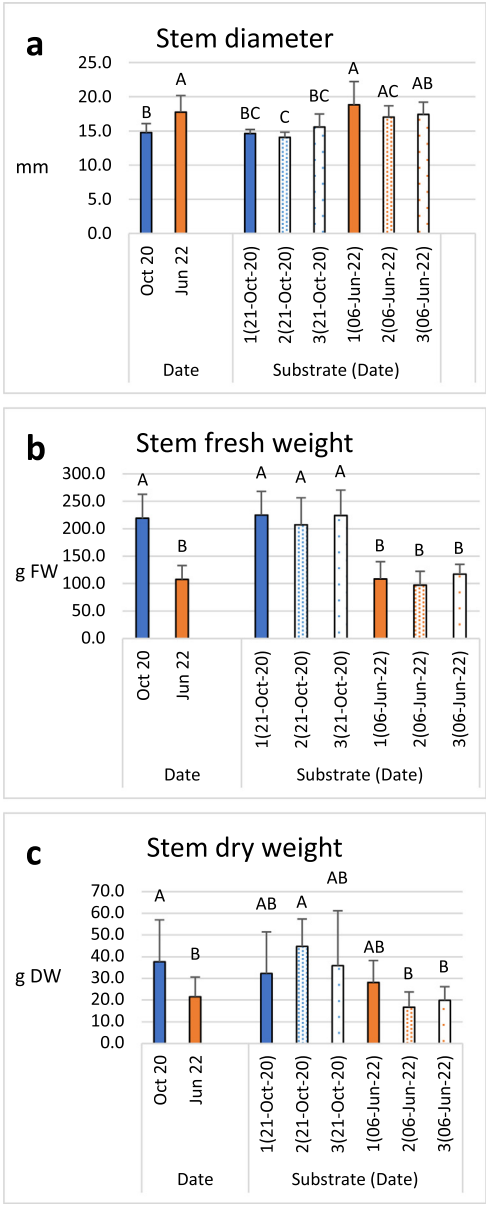

Fig. 3. Diameter (mm) and weight (fresh - FW and dry - DW) of *A. arborescens* stem according to date of measurement and growing substrate within each date. For each value, vertical lines represent the standard deviation of mean. Values marked by the same letter (including unreported intermediates) indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

3.2. Plant growth and development

Table 2 presents the results of the ANOVA for the non-destructive measurement, taken on five different dates throughout the observation period, which extended from the beginning of 2020 to May 2022. The corresponding measurements are graphically illustrated in Fig. 1, showing the growth trend over this observation period. Mean values are reported based on the observation date, which was the most significant factor at the ANOVA (Table 2). As shown, plant height (Fig. 1a), stem diameter (Fig. 1b), and number of suckers plant^{-1} (Fig. 1c) exhibited rapid growth in the first cultivation year, showing increases from 13 to 31 cm, from 10.4 to 14.0 mm, and from 1.5 to 13.4 units, respectively. However, in the following two years (2021 and 2022) their growth, while still evident, slowed down. A similar initial trend was observed in the average number of leaves per plant (Fig. 1d), which increased from around 12 to 22 in the first year but saw a notable reduction in 2021 and 2022, reaching the final value of about 11 leaves plant^{-1}.

The ANOVA results for data from the two destructive measurements (Table 3) also confirmed the highly significant effect of the "Date" factor on all plant components. However, the number and the fresh weight of suckers per plant appeared to be significantly influenced also by the substrate. In both years, the substrate 1 (100 % commercial substrate) consistently resulted in a higher number of suckers per plant (16.5 and 19.2 in both years, respectively), although they were smaller in size (430.7 and 840.7 g of fresh weight per plant in 2020 and 2022, respectively). In contrast, the fresh weight of suckers increased with higher proportion of biochar in the growing substrate (Fig. 2a–c).

Measurements on stems also exhibited a significant effect of the "Date" factor at the ANOVA. Stems were, on average, larger from one survey to the next (14.8 and 17.8 mm in the two surveys,

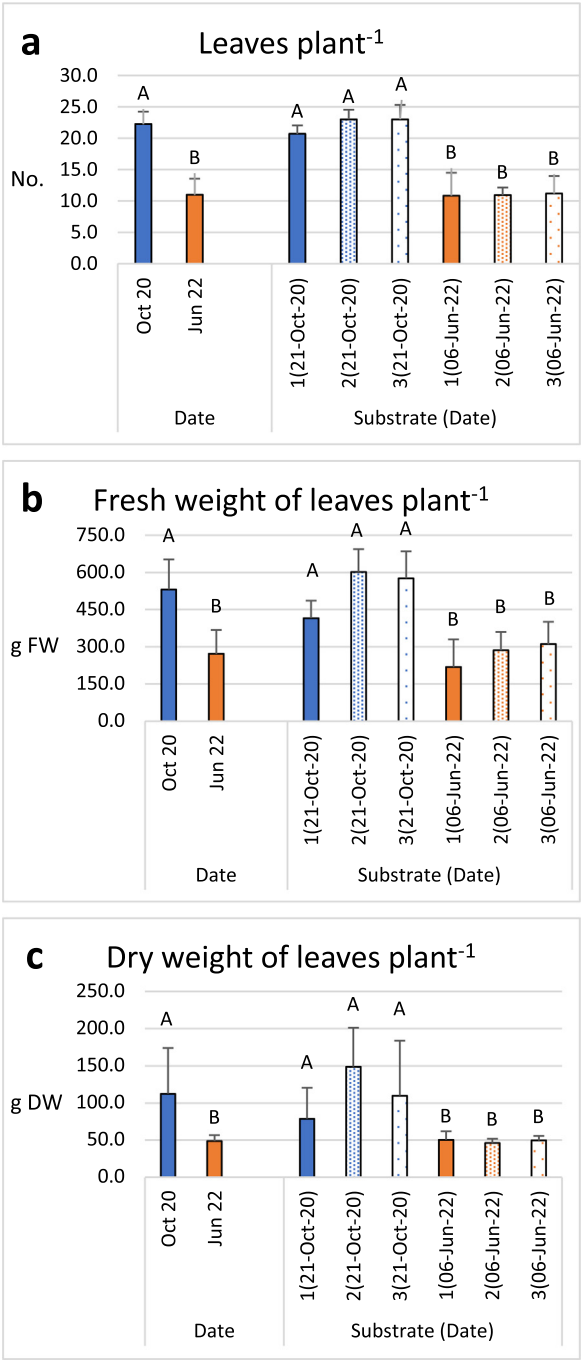

Fig. 4. Number and weight (fresh - FW and dry - DW) of A. arborescens leaves per plant according to date of measurement and growing substrate within each date. For each value, vertical lines represent the standard deviation of mean. Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

respectively), but also lighter, showing a decrease from 218.9 to 107.5 g of fresh weight (Fig. 3a–c), likely due to the occurrence of suberification processes in the inner tissues. The different substrates did not show any significant effect on stems.

Furthermore, the number of leaves per plant decreased, as did their size, from the first to the second measurement date. This was supported by the ANOVA results (Table 3), and it can be observed in Fig. 4a–c, which illustrate the statistically significant reduction in the number of leaves per plant (from 22.2 to 11.0) and their corresponding weight (from 531.1 to 271.2 g of fresh weight). As a result of the concurrent decrease in the weight of both stem and leaves, their combined weight (defined as the average weight of the plant's aerial part - Fig. 5a and b) significantly decreased from the first to the second survey, going from 750.0 to 378.7 g of fresh weight, respectively). In contrast, the weight of the below-ground parts (hypogeal mass) exhibited a substantial increase during the same period, with values of 179.8 and 834.7 g of fresh weight in the first and second measurements, respectively (Table 3; Fig. 6a and b). At the second measurement date, a significant effect of the different substrates on roots weight was observed. Specifically, substrate 3 (100 % biochar) led to a greater root development compared to substrate 1 (100 % commercial substrate), and this difference was statistically significant ($P < 0.05$) for the fresh weight of roots, reaching values of 923.0 g for substrate 3 and 732.3 g for substrate 1 (Fig. 6a).

3.3. Leaf composition

The average weight of one mature leaf, both fresh and dried, exhibited significant variability across all tested experimental factors (date, substrate, and leaf position), as well as, in some cases, according to their interactions (Table 4; Fig. 7a and b). Notably, larger and heavier leaves were observed during the first measurement date, with an average weight of 29.8 g fresh weight (FW), compared to 22.4 g FW during the second measurement date. However, a closer examination of each measurement date reveals substantial variations in these values based on the substrate and leaf position.

On average, substrate 3 consistently yielded bigger leaves compared to substrate 1, with means of 28.3 g FW and 22.7 g FW, respectively. The mixed substrate (substrate 2) occupied an intermediate position between the other two. Furthermore, a highly significant effect ($P < 0.001$) of the "Leaf position" factor was observed (Table 4; Fig. 7a and b). In most cases, median leaves (M), located in the central part of the stem, were consistently larger and heavier, while basal (B) and apical (A) leaves consistently displayed lower weights, often without differentiation between the two groups.

The leaves (Fig. 8a and b) are composed of three distinguishable components: spikes (9.5–11.8 % of leaf FW, and 21.7–22.2 % of DW), the outer green rind (referred to as "epidermis", accounting for 39.8–40.8 % of leaf FW and 65.5–67.7 of DW), and the inner parenchymatic tissue ("gel", comprising 47.4–50.7 of leaf FW and 10.1–12.8 % of DW). A previous study by Lazzara et al. (2021) indicated that the partitioning of leaves into these three components can vary under different growth conditions, such as illumination levels. In this experiment, the fresh weight of all three components was significantly affected by the diverse substrates. However, changes in DW values were only observed in the weight of the epidermis, with no noticeable impact on the DW values of spikes and gel. This suggests that the contrasting substrates primarily influenced water absorption, with minimal effects on building new plant biomass.

The average composition of leaves in spikes, epidermis, and gel, was also influenced by leaf position (Table 4, Fig. 8a and b). Generally, leaves in the intermediate position (M), which were larger than leaves in other positions, also yielded a higher fresh and dry weight of epidermis and gel in both years. However, this difference was less apparent when the relative amounts were expressed as percentages of the total leaf weight: in general, basal leaves produced a higher percentage of gel, even though (being smaller than others) the absolute amount was not always the highest.

3.4. Phytochemical analyses

The ANOVA on pooled data (data not shown) did not reveal any significant effects of the tested experimental factors on the levels of investigated secondary metabolites. Consequently, a more in-depth statistical analysis was performed on data obtained from each substrate (Table 5). This approach helped to uncover statistically significant differences among leaves positioned at various heights on the

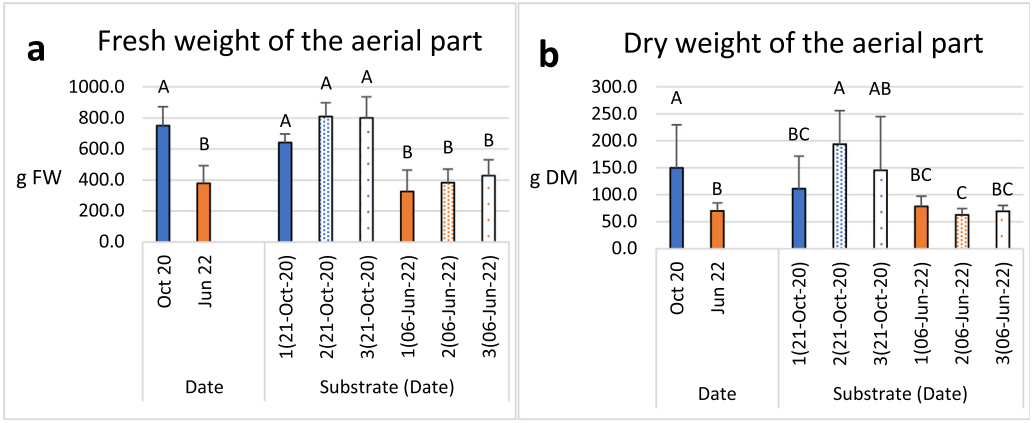

Fig. 5. Mean values of fresh (FM) and dry (DM) weight of the whole aerial part (leaves + stem) of *A. arborescens* plant according to date of measurement (2020 and 2022) and growing substrate within each date. For each bar, vertical lines represent the standard deviation of mean. Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

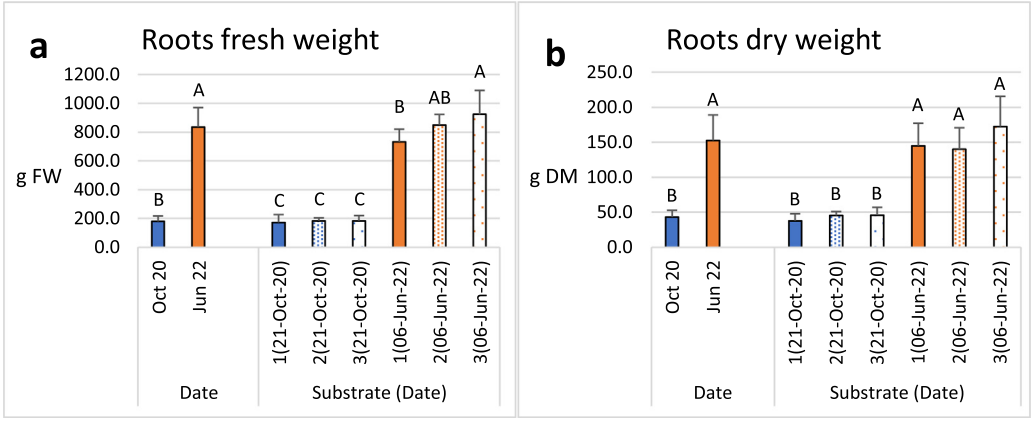

Fig. 6. Mean values of fresh (FW) and dry (DW) weight of *A. arborescens* roots according to date of measurement (2020 and 2022) and growing substrate within each date. For each bar, vertical lines represent the standard deviation of mean. Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

plant height, but only in the third substrate (100 % biochar), specifically in the levels of isoaleresin D, aloin A, and the cumulative amount of aloin A + aloin B (Table 5; Figs. 9 and 10).

The aloesin content in the leaves (Fig. 9) consistently remained low, ranging from a minimum of 0 (not detected) to a maximum of 0.087 mg per 100 mg of lyophilized plant material. The average value across all samples was 0.030, closely mirroring the median of 0.025. Although no systematic variations could be attributed to the tested experimental factors, data sorted by substrate and leaf position showed a different distribution. In substrates 2 and 3, aloesin content exhibited more variability in the median leaves, followed by the basal leaves. In contrast, in the apical leaves the detected values were lower and ranged within a narrower interval. However, in substrate 1 (100 % commercial substrate), the leaves from the three positions displayed similar values and distributions.

Aloenin A in a previous study (Lazzara et al., 2021) ranged from 1.4 % to 2 % depending on different illumination levels, while in the present study, it ranged between 0.39 and 1.62 mg per 100 mg.

Table 4
Results of the ANOVA (*F*-values) for *A. arborescens* leaves weight and composition (in spikes, epidermis and gel), measured in two dates, according to substrate (within date), leaf position (within date), and their interactions.

Source of variability	DF	1 leaf		Spikes				Epidermis				Gel			
		FW (g)	DW (g)	FW (g)	DW (g)	% on leaf FW	% on leaf DW	FW (g)	DW (g)	% on leaf FW	% on leaf DW	FW (g)	DW (g)	% on leaf FW	% on leaf DW
Date	1	74.33***	47.80***	72.77***	19.79***	16.48***	<1 n.s.	75.30***	52.22***	1.03 n.s.	4.27*	22.91***	<1 n.s.	9.47**	22.94***
Substrate (Date)	4	8.26***	4.03**	4.28**	1.58 n.s.	1.65 n.s.	<1 n.s.	7.67***	3.81**	<1 n.s.	<1 n.s.	4.75**	1.64 n.s.	1.32 n.s.	<1 n.s.
Leaf position (Date)	4	16.05***	9.34***	2.89*	2.38 n.s.	2.75*	<1 n.s.	16.53***	10.36***	3.49*	1.64 n.s.	11.21***	3.06*	5.13**	2.47*
Substrate x Leaf position (Date)	8	4.05***	2.56*	1.35 n.s.	<1 n.s.	1.54 n.s.	1.86 n.s.	4.00***	3.22**	<1 n.s.	1.93 n.s.	2.42*	1.31 n.s.	<1 n.s.	<1 n.s.
Error	90														
Total	107														

n.s.: not significant; FW: fresh weight (g); DW: dry weight (g).
* Difference significant at $0.01 < P \leq 0.05$.
** Difference significant at $0.001 < P \leq 0.01$.
*** Difference significant at $P \leq 0.001$.

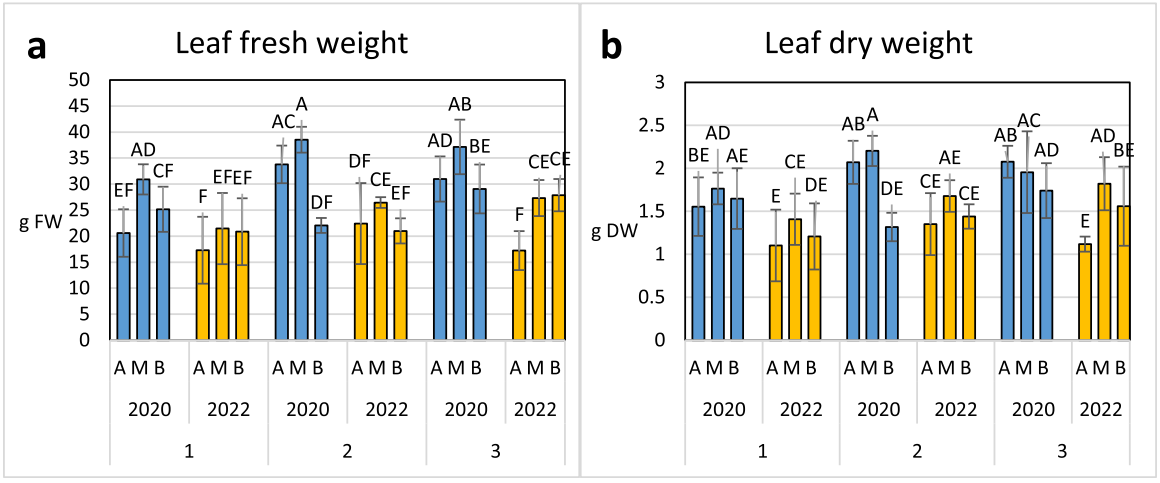

Fig. 7. Mean values of fresh (FW) and dry (DW) weight of one leaf of *A. arborescens*, according to the date of measurement (2020 and 2022), the used substrate (1, 2, and 3), and the position along the plant axis (A: apical; M: median; B: basal). For each bar, vertical lines represent the standard deviation of mean. Values marked by the same letter (including unreported intermediates) indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

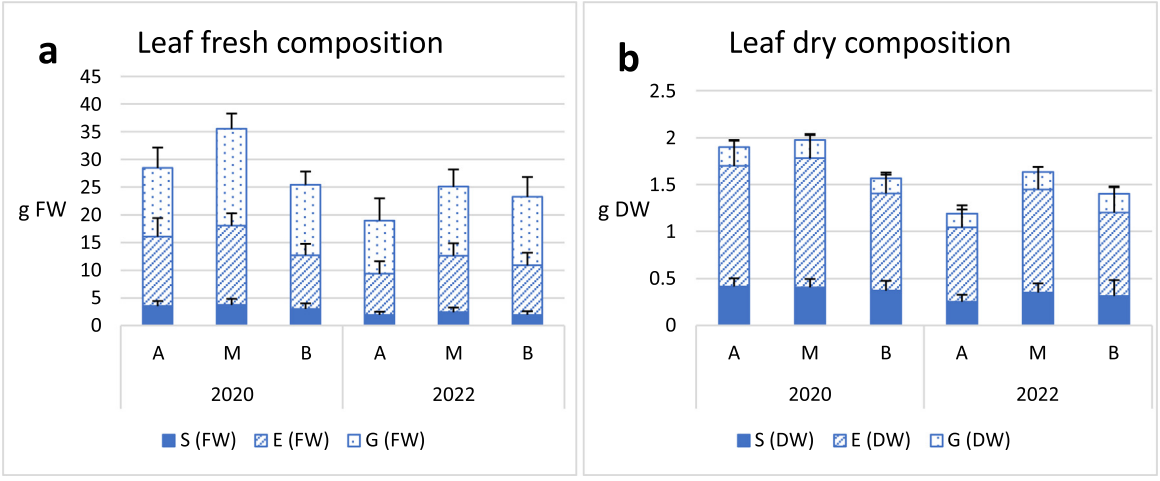

Fig. 8. Fresh (FW) and dry (DW) weight of *A. arborescens* leaf components (spikes, epidermis and gel) measured in two dates, according to leaf position (Apical: A; Median: M; Basal: B). For each value, vertical lines represent the standard deviation of mean.

Similar to aloesin, no significant differences were found at ANOVA (Table 5). On average, though, a different pattern emerged among the three substrates. In the second and third substrates, the average aloenin A content was lower in the apical leaves, while in the substrate 3, all three leaf positions had relatively similar values (Fig. 9).

The content of isoaloeresin D ranged between 0.19 and 7.31 mg per 100 mg across all samples. In the case of the third substrate, this compound was one of the few where the ANOVA yielded significant results (Table 5; Fig. 9). The apical leaves had the lowest average amount (1.03 mg per 100 mg), which was statistically different from

Table 5
Results of the ANOVA (*F*-values) for selected secondary metabolites in *A. arborescens* leaves, according to the different substrates and the position of leaf. Each value is expressed in mg 100 mg^{-1} of lyophilized plant material.

Source of variability	DF	Aloesin	Aloenin A	Isoaloeresin D	Aloin A	Aloin B	Aloin A + B	Total detected compounds
Substrate 1 (100 % commercial substrate)								
Leaf position	2	<1 n.s.	<1 n.s.	<1 n.s.	<1 n.s.	1.77 n.s.	1.26 n.s.	<1 n.s.
Error	15							
Total	17							
Substrate 2 (biochar+commercial substrate 50:50)								
Leaf position	2	2.62 n.s.	3.17 n.s.	<1 n.s.	1.86 n.s.	<1 n.s.	<1 n.s.	1.05 n.s.
Error	15							
Total	17							
Substrate 3 (100 % biochar)								
Leaf position	2	2.15 n.s.	3.29 n.s.	4.80*	5.94*	3.53 n.s.	4.94*	4.77*
Error	15							
Total	17							

n.s.: not significant.
* Difference significant at $0.01 < P \leq 0.05$.

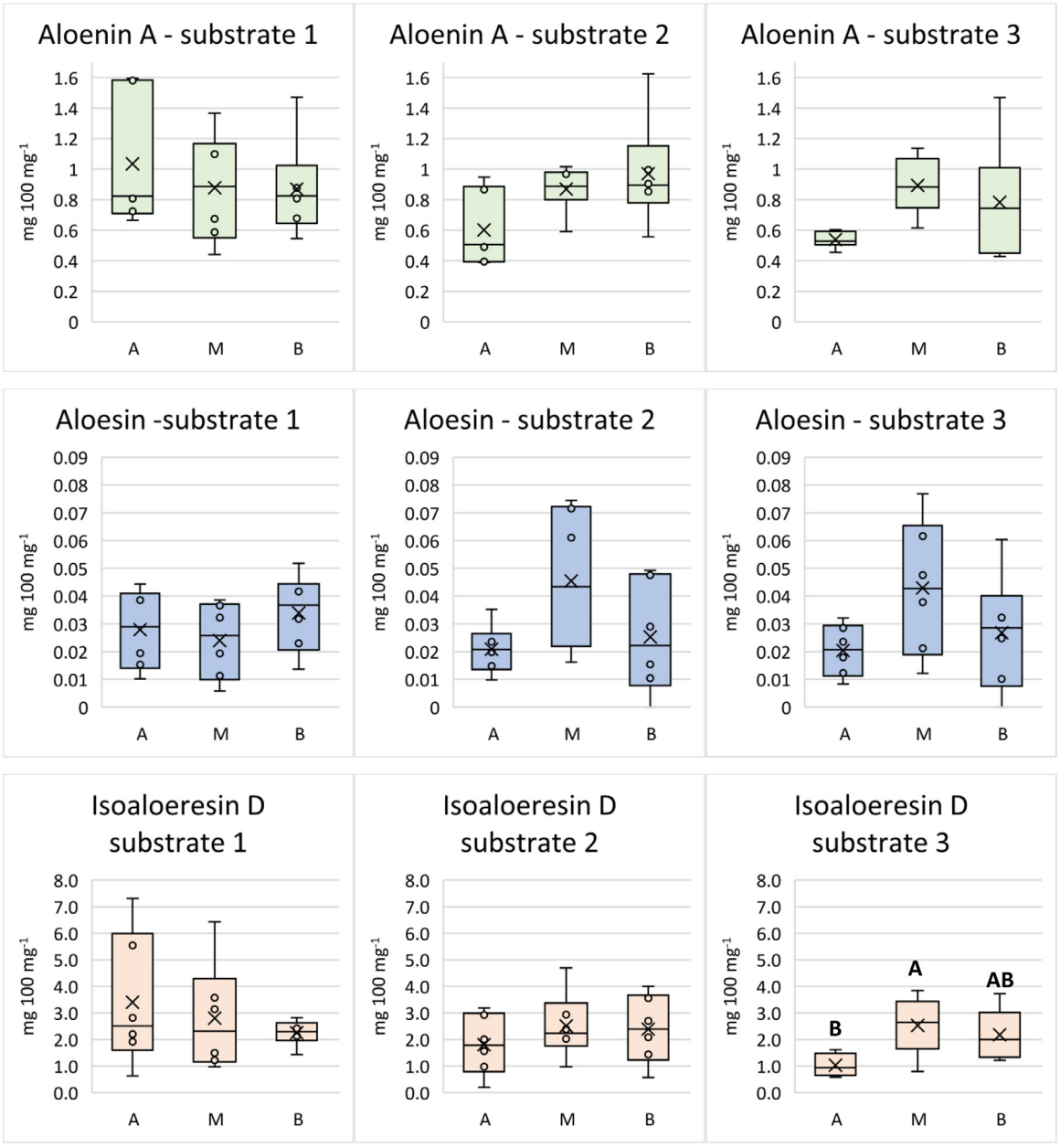

Fig. 9. *A. arborescens.* Box plots of the detected amounts (mg 100 mg^{-1} of lyophilized plant material) of aloesin, aloenin A and isoaloeresin D according to substrate (1, 2, and 3) and leaf position (Apical: A; Median: M; Basal: B). Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

the median leaves (2.52 mg per 100 mg). The same trend (A<B<M) was also observed in substrate 2, although in this case it was not significant at the ANOVA. The trend changed in substrate 1 (A>M>B), where apical leaves had the highest levels, followed by median and basal leaves (3.40, 2.80, and 2.26 mg per 100 mg, respectively).

According to the ANOVA (Table 5), aloin A and the cumulated amount of aloin A + aloin B in the third substrate showed significant differences ($P \leq 0.05$) between leaf positions. The average values of aloin were 0.40, 0.31, and 0.24 mg per 100 mg in median, basal, and apical leaves, respectively.

Aloin B was detected in lower amounts than its isomer aloin A, with levels ranging from 0.12 to 0.71 mg per 100 mg across all samples, and no significant differences were observed due to experimental factors at the ANOVA.

The average amounts of aloin A and aloin B (Fig. 10) followed a similar trend (M>B>A) in both substrates 2 and 3, whereas in substrate 1, their order was A>M>B. Remarkably, in substrate 1, the samples from apical leaves, despite having the highest mean values, also exhibited the widest interquartile range (0.463 for aloin A, 0.396 for aloin B, and 0.860 for the sum of both), hence showing the highest variability among all leaf positions in that substrate (Fig. 10).

4. Discussion

This work was driven by the hypothesis that biochar could influence the growth and phytochemistry of *A. arborescens*. Therefore, increasing amounts of biochar were mixed with the plants' growing media, and the response of growth, development, and the content of selected secondary metabolites was evaluated.

According to the performed statistical analysis, the most significant experimental factor was the date (year), which influenced almost all measured characters. Over the 3-years observation period

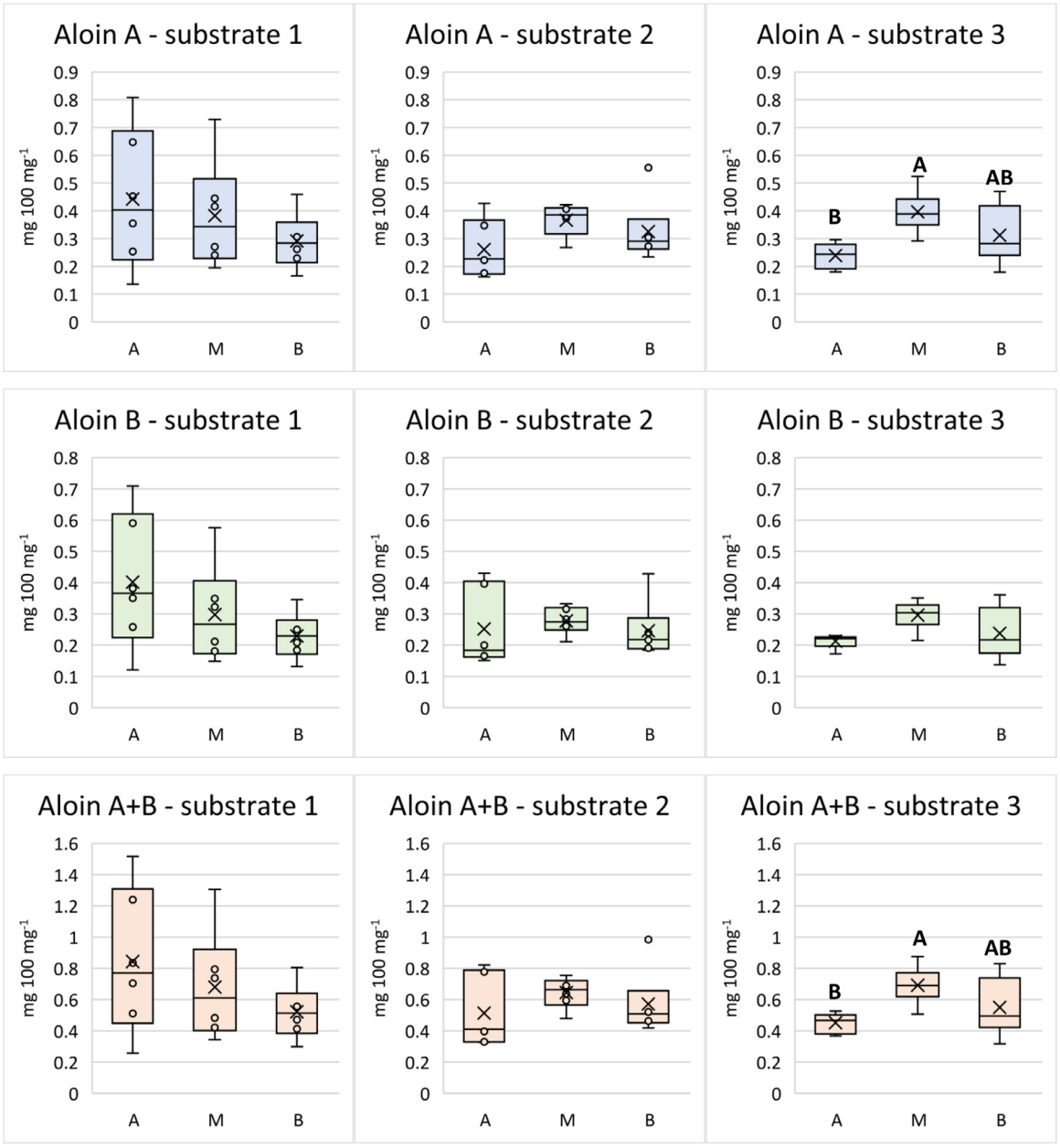

Fig. 10. *A. arborescens.* Box plots of the detected amounts (mg 100 mg^{-1} of lyophilized plant material) of aloin A, aloin B and their sum (aloin *A* + aloin B) according to substrate (1, 2, and 3) and leaf position (Apical: A; Median: M; Basal: B). Values marked by the same letter indicate no statistically significant differences at $P \leq 0.05$ (Tukey's test).

(2020–2022), the plants experienced an increase in height. However, constrained growth conditions, likely due to the small size of pots, led to a reduced emission of new leaves and a decrease in their size. On the positive side, these conditions fostered both the number of suckers and the weight of roots.

The different growing substrates significantly influenced many plant characteristics, such as the number and fresh weight of suckers per plant, fresh and dry weight of leaves, weight of the aerial part (stem+leaves), and fresh weight of roots. In general, the addition of biochar to the growing substrate favored the growth of larger leaves, with the enhancement of size being proportional to the amount of biochar used in the mixture. This effect was especially noticeable in the leaves from the median section of the plant. A study by Dispenza et al. (2016) found similar results with *Euphorbia lomi*, where the content of biochar in the growing substrate significantly influenced leaf production, leaf area, and weight. Higher values were recorded with 60 % biochar, whereas lower performances were observed with a 100 % peat-based substrate. Interestingly, the addition of biochar also allowed *A. arborescens* plants to produce larger root biomass by the end of the experiment. The improved physical and chemical properties of the growing substrates, such as hydraulic conductivity and Cation Exchange Capacity, as reported by various authors (Dispenza et al., 2016; Fascella et al., 2020b), can likely explain the positive outcomes observed in the biochar-enriched substrates. Moreover, in line with our results, Fascella et al. (2020a) noted that the root weight of potted Lavender plants increased when biochar was used at 50 % and 75 % in the growing substrate, whereas lower root weights were measured with a commercial substrate (100 % peat) and with 100 % biochar. The positive effects of adding inert and porous materials to the soil/substrate for *A. arborescens* potted plants were also confirmed by Prisa (2019) who reported that the use of zeolites could enhance plant growth, root development, and metabolites production.

Sampled leaves showed significant differences in fresh and dry weight values depending on their position along the stem. Leaves collected from the median section of the plants were larger and had a higher fresh weight of spikes, epidermis, and gel in leaves, as well as a higher incidence (%) of these components on fresh weight of leaves. As a matter of fact, median (M) leaves are the fully mature ones, whereas apical (A) leaves are probably still growing, and basal (B) have likely entered the senescence phase. In the available literature, several works have considered the influence of leaf age on the overall performance of *Aloe* plants, especially from a phytochemical perspective. For example, in *A. arborescens,* the content of aloenin A was found to decrease as the plant aged from 1 to 4 years old (Pawlowicz et al., 2021). Concerning intra-plant variability, Gajbhiye and Maiti (2010) reported that in *A. vera,* the maximum yield of aloin A was obtained by harvesting fully mature (but not senescent) leaves, i.e., the 9–12 month-old leaves collected from the intermediate position.

Furthermore, in *A. arborescens* leaves, the relative amounts of aloin A, aloenin, and aloeresin increased from the leaf base to the apex (Gutterman and Chauser-Volfson, 2000a, 2000b). Since aloin is predominantly found in the outer rind of the aloe leaf (Li et al., 2003; Patel and Patel, 2013), the higher proportion of epidermis in the dry matter can likely explain the greater amounts of aloin observed in the median leaves in this study.

The evaluation of the plant's response to different growing substrates provides some general insights as well. As a general trend, in plants cultivated within substrate 3 (100 % biochar), the highest quantity of secondary metabolites was found in the leaves from the intermediate position (M), although only isoaloeresin D, aloin A and the sum of aloin A + aloin B (collectively termed "aloin") confirmed this tendency at the ANOVA. All detected metabolites maintained the same concentration pattern also in the substrate 2 (trend visible but not confirmed by the ANOVA). In substrate 1 (100 % commercial soil) the highest average amount of all metabolites was in the apical leaves (A), except for aloesin, where the trend was B>A>M. The influence of growing substrates on the content of *Aloe* bioactive compounds was confirmed by Salighehdar et al. (2016), who reported higher concentrations of antioxidants, total phenols and aloin in *A. vera* plants grown in peat and perlite substrate (3:1 v/v) compared to those grown in other mixtures.

5. Conclusions

This experiment aimed to assess the suitability of biochar in mixed substrates for the containerized cultivation of *A. arborescens,* shedding light on various aspects of plant growth and development in substrates with increasing amounts of biochar. Biochar induced significant modifications in leaf size, especially in the median section of the plant and, interestingly, it led to a larger root biomass. Additionally, it demonstrated the potential to alter the yield of important secondary metabolites, such as isoalesin D and aloin A, particularly in the mature leaves collected from the median section of the plant. At the end of the trial, the plants showed reduced suitability for long-term containerized cultivation. However, this issue could potentially be addressed allowing for greater plant expansion through the use of larger pots. On the other hand, even though small-sized pots may not be the best choice for maximizing plant biomass for phytochemical production, their ability to stimulate sucker production could be beneficial for nursery purposes.

Conducting additional experiments encompassing a broader spectrum of biochar-to-soil ratios would be beneficial in elucidating the growth and biochemical responses of *A. arborescens* across different growth substrates, with the ultimate objective to enhance the plant's content of valuable secondary metabolites. Furthermore, studies on the variability of a larger number of secondary metabolites hold the potential to significantly enhance the plant's market and industrial value.

Author contributions

S.L., R.M. and G.F. performed the pots experiment, collected, and analyzed data; E.N.: performed chemical analyses; A.C., S.L., M.S. and G.F. designed the research and wrote the original draft of the manuscript; A.C., M.S., G.F. and R.M. analyzed data and supervised the experiment. All Authors contributed to the reviewing and editing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Silvia Lazzara: Methodology, Formal analysis, Data curation, Conceptualization, Writing – original draft, Writing – review & editing. **Alessandra Carrubba:** Conceptualization, Writing – original draft, Formal analysis, Supervision, Writing – review & editing. **Giancarlo Fascella:** Methodology, Formal analysis, Data curation, Conceptualization, Writing – original draft, Supervision, Writing – review & editing. **Roberto Marceddu:** Methodology, Formal analysis, Data curation, Supervision, Writing – review & editing. **Edoardo Napoli:** Formal analysis, Writing – review & editing. **Mauro Sarno:** Conceptualization, Writing – original draft, Formal analysis, Supervision, Writing – review & editing.

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