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Reusing Coir-Based Substrates for Lettuce Growth: Nutrient Content and Phytonutrients Accumulation

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Abstract: This research aimed to assess the influence of reusing coir-based substrates on growth, nutrient content, and phytonutrients accumulation in lettuce. The experiment included a new coir pith and four coir-based mixes (1) coir, biochar, and perlite; (2) coir, compost, and perlite; (3) coir, biochar, and pine bark; and (4) coir, compost, and pine bark. All mixes had been previously utilized to grow transplanted spinach and possessed identical ratios of 78:12:10% (*v*/*v*) among their components. Lettuce (*Lactuca sativa* L. cv. 'Godzilla') seedlings were transplanted into Styrofoam plant boxes. Each day, the planting boxes received a nutrient solution via drip irrigation. Plants grown in reused mixes had similar macronutrient concentrations as those grown in coir for the first time, except for N and K in the third mix. Plants grown in reused mixtures had similar yields as those in new coir. Lettuce heads yielded 4.6–4.9 kg/m^2, while plants grown in reused mixtures had equal or higher total phenols than those in new coir. Ascorbic acid content was higher in plants cultivated in reused mixes. Coir-based growing media can be reused for another short-cycle crop, like lettuce, without yield loss or phytonutrients decrease.

Keywords: *Lactuca sativa*; soilless system; short-cycle crops; municipal compost; biochar; total phenols; flavonoids; ascorbic acid; circular economy

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1. Introduction

In soilless culture, the reuse of substrates for cultivation is becoming a crucial issue due to the scarcity of resources, the need to reduce environmental impacts, continuous restrictions on the use of peat, rising demand for growing media components, and the increase in costs [1]. Maximizing the effective utilization of available resources offers a means to tackle their scarcity and diminish agriculture's carbon footprint [2]. One of the main objectives for agriculture in the coming ten years is to boost production system efficiency, promote sustainability, and optimize resource use efficiency [3]. Substrate culture, a widely adopted technique in vegetable crop production, is expected to increase in the future due to its numerous advantages over open-field-grown methods. These advantages include increased water and nutrient use efficiency, higher yields, and more precise pest and disease control [2]. Additionally, substrate culture offers a solution to address challenges such as reducing arable land and more frequent climate extremes [4,5]. One disadvantage of this technique is the disposal of the growing medium at the end of cultivation [6,7]. Therefore, reusing growing media is the most environmentally friendly approach [4,6,8]. Furthermore, considering the ongoing restrictions on peat usage, reusing substrates becomes even more crucial.

The number of growing cycles during which a substrate can be reused depends on the substrate's nature and the crop [7]. The spent growing media's stability is an essential indicator for its reuse [8]. Organic substrates are subject to decomposition, interfering with their physical and chemical properties [9]. The rate of the degradation reactions largely determines the useful life of an organic substrate. It depends on the substrate type, irrigation management, time-dependent action of roots, microbial activity [10], etc. In previous studies, [11,12] reported that coir could be an alternative to peat to produce spinach. Ref. [13] reported that spinach production and quality in coir-base substrates (78%, v/v) with municipal solid organic compost, or biochar, in the percentage of 12 by volume and either with perlite or pine bark, at a volume ratio of 10%, were similar to those obtained in coir. The spinach growth period in these mixes was 32 days. Spinach is a short-cycle crop of 25 to 50 days. Thus, the time for the substrate to lose its physical stability is short. Physical stability is defined by [9] as the ability of a product to maintain its physical dimensions and properties. On the other hand, these substrates were predominantly composed of coir, whose stability varies but is generally good [14–16]. It still has the advantage of having relatively low shrinkage [17] and being slightly hydrophobic.

In mixes with biochar, due to its recalcitrant nature [18–20], decay may occur at a slower rate. On the other hand, perlite and pine bark also had good stability [14–16]. According to Lemaire et al. [21], the pine bark remained stable for eight months. Ref. [22] reported that the reutilization of substrates did not pose a problem when fertigation parameters were adjusted to the reused substrate properties.

It is of the utmost importance to meticulously evaluate and contemplate the possible hazards associated with substrate reuse, specifically in relation to the dissemination of soil-borne ailments [23]. It is imperative to adopt suitable procedures to guarantee that any reused substrate is thoroughly sterilized and devoid of any detrimental pathogens that may threaten plant growth or the ecosystem. Adding compost to mixes can help prevent soil-borne diseases. Compost has disease-suppressing properties that can be an effective strategy for reducing risk [24–26]. Biochar can also suppress plant diseases [27–29], reported that biochar mildly improved the survival of beneficial microorganisms in a mix with peat.

Strategies to reduce potential issues related to substrate stability, improve sustainability, and reduce production costs are key to reusing substrates for planting other short-term crops. Lettuce is a short-season vegetable intensively produced in Mediterranean countries and is known to be a good source of health-promoting compounds like phenols [30,31].

We hypothesize that coir-based substrate, used for spinach cultivation, can be reused to grow other short-cycle crops. We predicted that these substrates would maintain their physical and chemical properties adequate for growing lettuce, another short-cycle crop. Here, we investigated the impact of reused coir-based substrate on lettuce growth, nutrient content, and the accumulation of phytonutrients.

2. Materials and Methods

2.1. Growth Conditions and Substrates

The experiment was conducted in a greenhouse located at the "Herdade Experimental da Mitra" (38°57′ N, 8°32′ W), University of Évora, Portugal. The greenhouse was covered with polycarbonate and had no supplemental lighting or heating. Diurnal changes in air temperature inside the greenhouse at the plant canopy level ranged from 8 to 27 °C. Solar radiation ranged from 34 to 248 $W{\cdot}m^{-2}{\cdot}d^{-1}$.

The experiment included five substrates, coir (the control, used for the first time), and four mixes already used to grow transplanted spinach, whose cycle lasted 32 days.

The coir (100% coir pith) (Projar S.A., Spain) had a pH of 5.5–6.0, electrical conductivity (EC) > 1.5 dS m^{-1}, granulometry = 0–10 mm, total porosity = 95%, air (%, v/v) = 25, and CEC (meq/100 g) = 60–120.

The mixes described in detail in Machado et al. [11] were coir-based (78 percent coir by volume, 12 percent biochar or municipal organic compost collected selectively, and 10 percent perlite or pine bark). The initial physicochemical characteristics of these mixes (pH, EC, mass wetness, moisture content, total porosity, and bulk density) were also presented in [11]. Before lettuce planting, the EC in mixes with compost averaged 2.3 mS/m ± 0.3, and in other mixes and in coir, it averaged around 1.9 d*S*/m. The pH in the mix of coir, compost, and perlite was 6.9. In the other mixes and in coir, it averaged 6.3 ± 0.5. It was observed that there was no noticeable shrinkage in the mixes.

Lettuce (*Lactuca sativa* L. cv. 'Godzilla') type Batavia seedlings with green leaves were transplanted into Styrofoam plant boxes (100 × 25 × 10 cm) on 8 April 2021, 35 days after emergence. After the spinach *(Spinacia oleracea* L. cv. Tragopan) harvest, the reused mixes remained undisturbed in the boxes. The reused substrates were not subject to sanitation. The seedlings were spaced at 20 cm in a row in the center of the box, with a plant density of 16 plants m^{-2}.

Treatments were arranged in a complete randomized block design with five replicates. Two 8 L/h pressure-compensating and anti-drain emitters were placed in each planting box. The emitters were attached to four fine tubes with a 70 cm length and 5 mm diameter. Thus, two water emission points were inserted into the substrate near the plant base, one on each side of the row crop.

The irrigation schedule was optimized for coir. It was based on substrate volumetric water content at Styrofoam box control (coir), measured using a soil moisture probe (SM105T Delta devices, Burwell, UK), and the volume of water drained. The nutrient solution was applied three to eight times daily and averaged 10 to 20% drainage (leaching fraction) for each application.

A nutrient solution was injected continuously into the irrigation system throughout the growing cycle. The nutrient solution used contained 14 mmol L^{-1} NO_3-N, 6.3 mmol L^{-1} NH_4-N, 1.32 mmol L^{-1} P, 11 mmol L^{-1} K, 3.5 mmol L^{-1} Ca, 3.5 mmol L^{-1} Mg, 1.31 mmol L^{-1} S, 46 µmol L^{-1} B, 7.86 µmol L^{-1} Cu chelated by EDTA, 8.95 µmol L^{-1} Fe chelated by EDTA, 18.3 µmol L^{-1} Mn chelated by EDTA, 1 µmol L^{-1} Mo, 2 µmol L^{-1} Zn chelated by EDTA, 2.1 mmol L^{-1} Cl, and 0.7 mmol L^{-1} Na. The EC-value of the nutrient solution ranged over time: 2 ± 0.2 dS m^{-1} ((from transplanting to 12 days after transplanting (DAP)) and 2.5 ± 0.3 d*S* m^{-1} ((from 13 DAP until harvest (29 DAP)).

2.2. Measurements

The pH and EC_W of the drainage water from each box were measured weekly using a potentiometer (pH Micro 2000 Crison) and a conductivity meter (LF 330 WTW, Weilheim, Germany).

Lettuce plants (heads) were harvested at 29 DAP. Two lettuce plants (heads) from each box were washed, oven-dried at 70 °C for 2–3 days, weighed, and ground so that they would pass through a 40-mesh sieve. The ground samples were analyzed for N, P, K, Ca, Mg, Na, Fe, B, Mn, and Zn. The total N was analyzed using a combustion analyzer (Leco Corp., St. Josef, MI, USA). The K and Na were diagnosed by flame photometry (Jenway, Dunmow, UK). The P and B were analyzed using a UV/Vis spectrometer (Perkin Elmer Lamba 25). The remaining nutrients were analyzed using an atomic absorption spectrometer (Perkin Elmer, Inc., Shelton, CT, USA).

The leaf area of two plants was measured using a leaf area meter (LI-COR Model LI-3000A).

The head samples, including inner, middle, and outer leaves, were collected in a 2 cm thick disc obtained by cross-cutting at a height of 6 cm from the base and cutting with a knife. Samples of lettuce leaf discs weighing 1.000 g were macerated in a mortar and, then homogenized for 1 min in 8 mL of a methanol/water solution (80:20 (*v*/*v*), MW80 extract) [32] to determine the total content of phenolic compounds (TPC) [33], flavonoids [34], anthocyanins [35,36], ascorbate (AsA) [37], proline [38], and FRAP antioxidant activity [33].

After that, samples were centrifuged at 4 °C at 6440× *g* for 5 min in a centrífuge Hermle Z323 K. Aliquots of the methanol extracts were kept at −20 °C for further use.

Samples of lettuce leaf discs weighing 1.000 g from each treatment were macerated in a mortar and then homogenized in 8 mL of methanol:water solution (90:10 (*v*/*v*), MW90 extract) for 1 min. They were then centrifuged at 4 °C at 6440× *g* for 5 min. to determine the amount of photosynthetic pigment present. Chlorophyll a and b and carotenoids were quantified in aliquots of MW90-extract by UV-vis spectrophotometry [38].

Total phenolic compounds (TPCs) were determined following Bouayed et al. [33], using the Folin–Ciocalteau phenol reagent by reading the absorbance at 760 nm. TPC content was estimated using a calibration curve (GAE, *n* = 6 concentrations from 0 to 50 mg/L) and expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight (FW).

A reaction mixture of 100 µL of MW80 extract, 20 µL of 10% $AlCl_3$, 500 mL of 1 M potassium acetate, and 380 µL of distilled water was prepared to determine the flavonoid content. After that, this combination was incubated for 30 min at 25 °C. Total flavonoid content was determined by measuring the absorbance at 420 nm, using an extinction coefficient of 0.004 μM^{-1} cm^{-1}, and expressed in mg of quercetin equivalent (QE) per 100 g of fresh weight [34].

A reaction mixture composed of 500 µL of MW80 extract, 500 µL of 50% ethanol (*v*/*v*), and 84 µL of 37% HCl was used to determine the total anthocyanin content [35]. After 30 min of incubation at 60°C, the absorbance of the mixture was measured at 530, 620, and 650 nm, and the absorbance of cyanidin-3-glycoside was estimated. The total anthocyanin content, expressed as mg of cyanidin-3-glycoside equivalent (C3GE) per 100 g of fresh weight, was calculated using the molar extinction coefficient of 34,300 M^{-1} cm^{-1} and the molar mass of 449.2 $gmol^{-1}$ [36].

For the determination of AsA content, each sample (extracts or standards suitably di-luted) was incubated in a mixture containing 5% TCA in ethanol, 0.4% H_3PO_4, 0.5% β-phenanthroline in ethanol, and 0.03% $FeCl_3$ in ethanol and warmed at 30 °C, for 90 min [37]. The absorbance of the Fe (II)–β-phenanthroline complex formed was read at 534 nm. AsA concentration was calculated from a calibration curve (ascorbic acid, *n* = 6 concentrations from 0 to 30 mg/L) freshly prepared.

The Free Pro content of MW80-extracts was determined using the acid ninhydrin reaction with the amino acid and reading the absorbance of the formed formazan at 546 nm. The concentration of proline was calculated using a calibration curve prepared from standard solutions of pure proline (L-proline, *n* = 6 concentrations between 0 and 20 mg/L) [38].

To determine the ferric-reducing antioxidant power (FRAP) of the lettuce extracts, a reaction mixture of 0.050 mL of the sample (plant extracts) or standards was mixed with 0.950 mL of the FRAP reagent. The absorbance change was read at 593 nm at 37 °C for 180 s. FRAP reagent was freshly prepared by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution (10:1:1, *v/v/v*) at 37 °C. The antioxidant activity reported as milligrams of Trolox equivalents per 100 g FW was calculated using a calibration curve (Trolox solution, *n* = 8 concentrations from 0 to 1120 mg L^{-1}) [33].

Samples of 0.2500 g of spinach leaves were macerated in liquid N_2 and homogenized in 50 mM phosphate buffer pH 7.0 to determine the proline dehydrogenase (PDH) en-zyme activity. This extract was centrifuged for 15 min at 15,000× *g* at 4 °C to obtain the supernatant, which was then collected and kept in aliquots at 20 °C (PB-extract) for later use [39,40]. PDH enzyme activity was measured following the reduction of NAD^+ at 340 nm at 30 °C for 180 s [40]. A reaction mixture containing 100 mM Na_2CO_3-$NaHCO_3$ buffer pH 10.3, 10 mM NAD^+, and PB-extract was used during the assay. The addition of 2 mM L-proline was used to initiate the reaction. Enzyme activity was estimated from the reaction curve slope (A_{340} vs. t) using the extinction coefficient of 6.22 mM^{-1} cm^{-1}. PDH activity was expressed in nmol min^{-1}/mg protein. According to the Lowry method [41], the amount of water-soluble protein in the PB extract was evaluated using a calibration curve (bovine serum albumin, BSA; *n* = 6 concentrations from 0 to 200 mg/mL).

A Genesys 10S UV-Vis spectrophotometer was used for all spectrometric measurements.

2.3. Data Analysis

Data were analyzed using the analysis of variance (ANOVA I) using SPSS Statistics 25 software (Chicago, IL, USA), licensed to the University of Évora. Means were separated at the 5% level using Duncan's new multiple-range test.

3. Results and Discussion

3.1. Leachate pH and EC

The nutrient solution, irrigation scheduling, and substrate may affect the leachate fraction's pH and electrical conductivity (EC_W). In the present study, the differences in hydronium ions concentration and EC_W of drained water were related to substrate (Figure 1). It can affect the volume of water drained, cation exchange capacity, and pH buffering capacity. The average leachate pH was higher in mixes with perlite than in other substrates during the first three sample dates. The highest pH occurs in the coir, compost, and perlite mix.

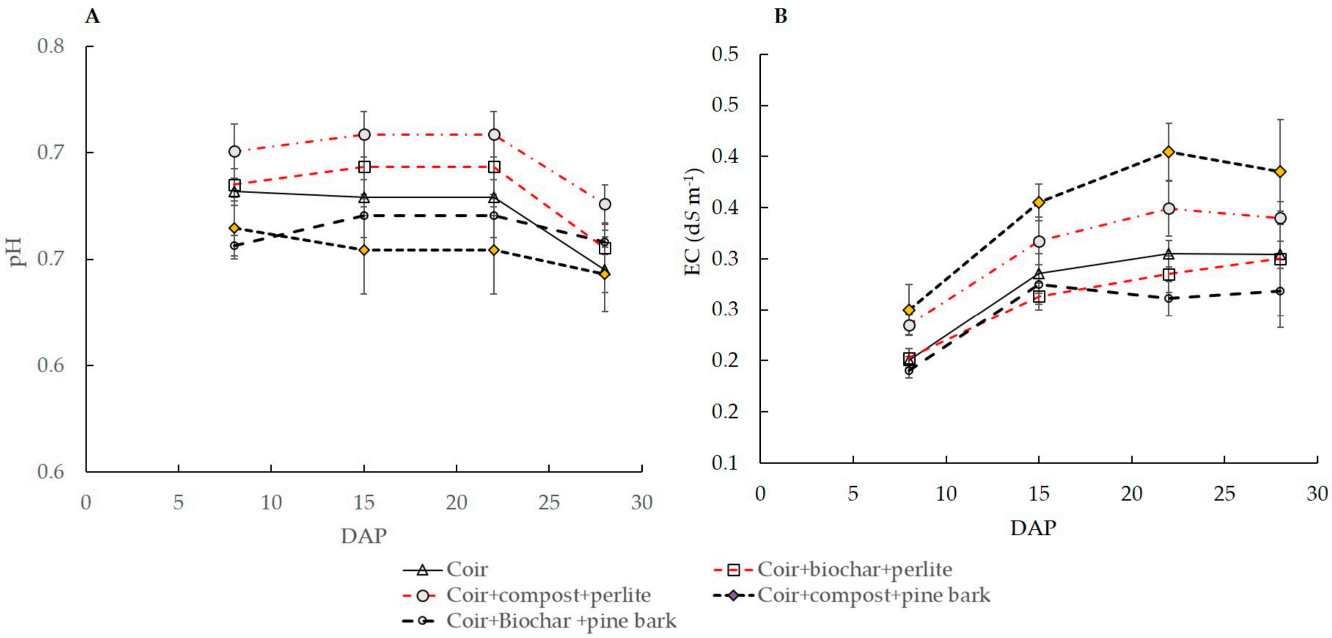

Figure 1. Effect of reused substrates on pH (**A**) and EC_W (**B**) in the drainage water. Each symbol represents the mean of five replicates, and the error bars represent ± 1 SE. DAP- days after transplanting.

The pH of this mixture ranged from 7.0 to 7.1, which is slightly higher than the nutritional solution's pH of 6.4 ± 0.3 and may negatively affect plant nutrition. In coir leachate, the pH was slightly higher than in the incoming nutrient solution. On the last sampling date, the pH of the drained water was not significantly affected by the mixes and ranged from 6.4 to 6.7.

The EC_W of leachate in the first sampling (8 DAP) was slightly higher in mixes with compost than in other mixes and coir (Figure 1). In the mix of coir, compost, and pine bark, the EC_W was 2.5 d*S* m^{-1}, 0.3 d*S* m^{-1} higher than the incoming solution (2 ± 0.2 d*S* m^{-1}). This slight increase could be attributed to this mix containing additional residual nutrients from the previous crop and/or a lower volume of water drainage. On the last three sampling dates in mix coir, compost, and pine bark, the EC_W was always higher than the EC of the nutrient solution (2.5 ± 0.3 d*S* m^{-1}). The EC_W in this mix reached high values between 3.33 and 4.05 d*S* m^{-1}, i.e., 0.7 to 1.24 d*S* m^{-1}, which is higher than the EC of the nutrient solution (2.5 ± 0.3 d*S* m^{-1}).

This can indicate a tendency for salt buildup in the substrate. The EC_W was slightly lower on mixes with biochar than in coir or the incoming solution. In coir, the EC_W was 0.34 to 0.55 units higher than the nutrient solution, which may be considered adequate. As the differences may be related to the volume of water drained, the irrigation schedule must be adjusted for each mix.

3.2. Photosynthetic Pigments

Total chlorophyll, chlorophyll a and b contents of lettuce were different for different mixtures (Table 1). Plants grown in mixtures with pine bark had lower total chlorophyll and chlorophyll a content than those grown in mixtures with perlite (Table 1). Leaf chlorophyll b of the plants grown in reused substrates was not significantly different from those grown in coir. Leaf average chlorophyll b concentrations in the various plants ranged from 7.5 to 8.6 mg per 100 g of fresh weight (FW). Chlorophyll b levels were higher than chlorophyll a, which is not typical. However, this has also been observed in green and red lettuce cultivars by [42]. The Chla/Chlb ratio was high in coir, which may suggest that the plant is optimizing its photosynthetic capacity. The total chlorophyll content within this spectrum surpassed the findings of [43], who observed 1.0 to 1.5 mg.100 g^{-1} FW in lettuce cultivated via a floating culture system under varying nitrogen levels. However, the chlorophyll content remained notably inferior to the results of [44], who recorded a range of 26.8 to 52.3 mg 100 g^{-1} FW for lettuce exposed to diverse light intensities and nutrient solution concentrations. This could be due to various factors such as growing conditions, season, and genotype. For instance, the nutrient solution composition affected the chlorophyll content. [45].

Table 1. Effect of substrates on leaf photosynthetic pigments content and Chl a/Chl b ratio.

Substrate	Photosynthetic Pigments (mg 100 g^{-1} FW)				
	Total Chl	**Chl a**	**Chl b**	**Cc**	**Chl a/Chl b**
Coir	14.67 † a	6.51 a	8.17 ab	5.51	0.80 a
Coir + biochar + perlite	13.79 ab	5.18 ab	8.61 a	3.51	0.60 b
Coir + compost [1] + perlite	13.43 ab	5.37 b	8.06 ab	3.64	0.68 ab
Coir + biochar + pine bark	11.33 c	3.86 c	7.47 b	5.07	0.52 b
Coir + compost + pine bark	13.06 b	4.64 bc	8.42 ab	5.45	0.54 b
Significance	**	**	**	NS	**

† Means followed by different letters within a column are significantly different at $p \leq 0.05$. NS—nonsignificant, ** significant at $p < 0.01$ level, [1]—municipal solid organic compost collected selectively. FW—fresh weight. Total Chl—total chlorophyl; Chl a—chlorophyl a; Chl b—chlorophyl b; Cc—carotenoids; Chl a/Chl b—chlorophyl a/chlorophyl b ratio.

Leaf carotenoid content was not significantly affected by treatments. The average carotenoid content in the leaves ranged from 3.51 to 5.54 mg/100 g FW. Thus, the carotenoid content was lower than those reported by [46] (6.1–7.3 mg/100 g FW). As chlorophyll, the carotenoid content may be affected by several factors, including growing conditions, light intensity, temperature, genotype, leaf age, and position. The outer leaves generally have higher carotenoid levels than the inner leaves, which are exposed to higher light intensity, promoting carotenoid biosynthesis [47].

The lower carotenoid content observed in this study may be due to the dilution effect caused by the sample, which included inner, middle, and outer leaves.

3.3. Shoot Nutrient Concentration

Shoot macronutrient concentrations of plants from the reused mixes, except for N and K in the coir, biochar, and pine bark mix (4.34%), were not significantly different from those of plants grown in the new coir (Table 2). The low content of N and K may contribute to lower levels of Chl a and b in mix coir, biochar, and perlite than the other substrates (Table 1). Shoot B, Zn, and Na content in plants grown in reused growing media were not significantly different from those grown in new coir (Table 2).

Table 2. Effect of reused substrates on shoot lettuce nutrient concentrations.

Substrate	Shoot Macronutrients (%)					Shoot Micronutrients ($\mu g \cdot g^{-1}$)				
	N	P	K	Ca	Mg	Fe	B	Mn	Zn	Na [1]
Coir	4.89 ab †	0.73	5.37 a	1.15	0.42	110.0 ab	23.3	34.4 b	59.4	0.62
Coir + biochar + perlite	4.93 a	0.68	5.57 a	1.07	0.38	107.5 ab	23.6	50.0 a	50.6	0.74
Coir + Compost [2] + perlite	4.72 ab	0.68	5.45 a	1.08	0.35	135.0 a	21.0	37.5 b	165.6	0.62
Coir + biochar + pine bark	4.78 b	0.77	4.48 b	1.20	0.42	91.3 b	23.9	55.0 a	84.4	0.74
Coir + Compost + pine bark	4.82 ab	0.74	5.57 a	1.25	0.41	58.8 c	20.9	29.4 b	45.6	0.62
Significance	*	NS	*	NS	NS	*	NS	***	NS	NS

† Means followed by different letters within a column are significantly different at $p \leq 0.05$. NS—nonsignificant. * and *** significant at $p < 0.05$ and 0.001 levels, respectively. [1]—Although sodium is not a micronutrient, it is included here for convenience. [2]—municipal solid organic compost collected selectively.

Plants grown in mixes that contained biochar had higher levels of Mn. Spinach, grown for the first time in these mixes, also increases shoot Mn content, as reported by [13]. Biochar may increase Mn availability in substrate solutions. Extractable Mn in biochar depends on the feedstock and the particle size [48]. Extractable Mn is high in particles smaller than 1 mm [48], and in the biochar used in this experiment, 42% of the particle, expressed as a percentage by weight, was <1 mm [49]. It could contribute to the Mn increased availability in the root medium. This emphasizes the significance of evaluating nutrient availability in the root medium of the blends for customizing nutrient solutions.

Pine bark in mixes led to a significant decrease in shoot iron content (Table 2). This could be due to iron immobilization caused by an increase in microbial activity resulting from the decomposition of pine bark, as highlighted by [50]. As previously mentioned, in future studies measuring nutrient availability in root medium is necessary. The shoot iron content was lower in plants cultivated in the coir, compost, and pine bark mix (58.8 $\mu g \cdot g^{-1}$). Despite the differences in the nitrogen, potassium, iron, and manganese concentrations, the plants grown in the different media did not show any visible signs of nutrient deficiency or toxicity.

Thus, the study suggests that reusing growing mixes can maintain shoot nutrient concentrations similar to those in coir used for the first time, with minor exceptions.

3.4. Plant Growth and Yield

Despite the low chlorophyll a in reused mixes with pine bark and low shoot K and Mn content in mix coir, biochar, and pine bark, the shoot dry weight, leaf number and area, and fresh yield were similar (Table 3). Thus, in terms of yield, the reuse of the mixes allowed yields similar to those obtained when coir was used for the first time. This is advantageous because pine bark can be locally sourced in Portugal. On the other hand, it may reduce the need for importing perlite, whose manufacturing process is resource-intensive and requires significant energy consumption [51], as well as lessen transportation-related greenhouse gas emissions. Refs. [22,52,53] also reported that the yields of some horticultural crops grown on reused organic substrates were comparable to or greater than those grown on new substrates. Lettuce plants grown on the different substrates exhibited no signs of disease during the growing cycle. Fresh yield average values ranged from 4.6 to 4.9 kg/m^2. These yields were similar to or higher than those obtained when lettuce was grown in a floating system [44], and greater than those obtained in soil in an open field and a greenhouse [54]. This finding indicates that, in terms of yield, the reuse of the mixes allowed yields similar to those obtained in coir used for the first time. On the other hand, carefully adjusting the nutrient solution and the irrigation schedule to each mix to control the pH, EC_W, and volume of the leaching fraction could potentially lead to an increase in yield.

Table 3. Effect of reused substrates on shoot dry weight, number of leaves, leaf area, and head fresh weight yield.

Substrate	Shoot Dry Weight		Leaves	Leaf Area	Head Fresh Yield
	(g/Plant)	(%)	(n°/Plant)	(cm^2/Plant)	(kg/m^2)
Coir	11.0	3.7	25.0	5286.0	4.9
Coir + biochar + perlite	10.8	3.7	26.0	5155.5	4.8
Coir + Compost [1] + perlite	12.0	4.0	27.3	5182.2	4.7
Coir + biochar + pine bark	11.2	3.8	26.0	5122.4	4.6
Coir + Compost + pine bark	11.7	3.8	26.8	5246.3	4.8
Significance	NS	NS	NS	NS	NS

NS—nonsignificant, [1]—municipal solid organic compost collected selectively.

3.5. Phytonutrients Accumulation

The leaf total phenols of the plants grown in reused mixes were higher or equal to those grown in coir, used for the first time (Table 4). This may be due to different water availability, salinity, and pH in the root medium, as indicated by the EC_W and pH of the drained water. Water availability and salinity generally affect the total phenolic content in plants [55–57]. In lettuce, the electrical conductivity of the nutrient solution is associated with the biosynthesis of secondary metabolites, such as phenolic compounds [58,59].

Table 4. Effect of reused substrates on total phenols, anthocyanins, flavonoids, and ascorbic acid.

Substrate	TPC (mg GAE 100 g^{-1} FW) [1]	Flavonoids (mg QE 100 g^{-1} FW) [3]	Anthocyanins (mg C3GE 100 g^{-1} FW) [2]	Ascorbic Acid (mg 100 g^{-1} FW)
Coir	65.48 c[†]	3.19	0.66	1.21 c
Coir + biochar + perlite	138.9 a	3.88	0.60	1.17 c
Coir + compost + perlite	54.52 c	3.41	0.65	1.82 b
Coir + biochar + pine bark	65.38 c	3.23	0.66	2.85 a
Coir + compost + pine bark	92.25 b	3.45	0.69	1.62 b
Significance	***	NS	NS	**

[†] Means followed by different letters within a column are significantly different at $p \leq 0.05$. NS—nonsignificant. ** and *** significant at $p < 0.01$ and 0.001 levels, respectively. [1] TPC—total phenolic compounds GAE—galic acid equivalent. [2] G3GE—cianidine-3-glicoside equivalent. [3] QE—quercetine equivalente.

The highest total phenols occurred in plants cultivated in mixes of coir, biochar, and perlite (138.96 mg GAE 100 g^{-1} FW) and coir, compost, and pine bark (92.3 mg GAE 100 g^{-1} FW).

The average leaf total phenol of plants ranged from 54.52 to 138.96 mg GAE/100 g^{-1} FW. Leaf total phenol content in lettuce varies with several factors such as genotype, growing conditions, harvest time, leaf position, etc. [31,60,61]. The outer leaves have the highest phytonutrient content and antioxidant properties [42,47,60]. Despite all leaves being mixed in the study samples, the leaf total phenols values were within the range reported by Kim et al. [31] (50–270 mg GAE g^{-1} FW), Llorach et al. [30] (18.2–571.2 mg GAE g^{-1} FW) for different lettuce varieties, and Petropoulos et al. [62] for green lettuce (18 to 203 mg GAE/100 g^{-1} FW).

Leaf averages of flavonoids and anthocyanin contents of the plants grown in reused substrates did not differ significantly from those grown in the coir used for the first time (Table 4).

The leaf ascorbic acid (AsA) content of the plants grown in the reused mixes was higher or similar to those grown in coir used for the first time. Leaf AsA in the different treatments ranged from 1.17 to 2.85 mg/100 g FW). These were lower or similar to the lower end of the range reported for lettuces with green leaves by Cozzolino et al. [61] (3.0–19.3 mg/100 g FW), Jibril et al. [63] (2.27–6.91 mg/100 g FW), and Llorach et al. [30] for lettuces of different leaf colors (2.8–9.5 mg/100 g FW). The lower values may be related to the genotype, growth conditions, and sampling method. Leaf AsA ranged with leaf position [64,65], and in the present study, leaf AsA represents the average of different leaves. The low AsA content may also be related to the environmental conditions in the greenhouse. Light intensity was low in the greenhouse, not only due to the time of year (early spring) but also because of the opacity of the plastic cover film used in our greenhouse. The condition in substrates affected leaf proline, which was lower in reused mixes than in coir used for the first time (Figure 2A).

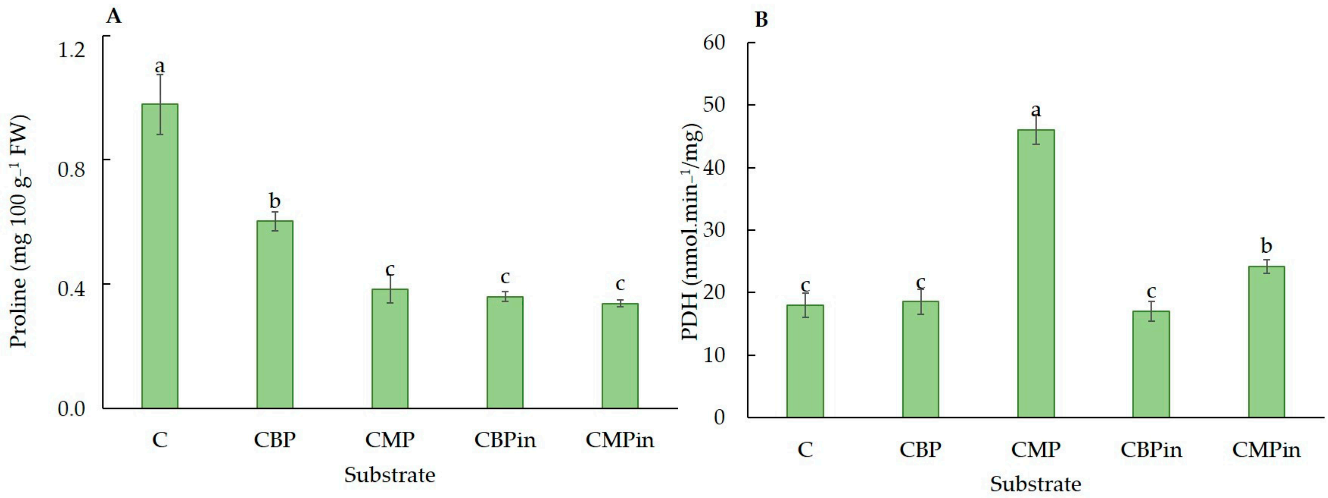

Figure 2. Effect of reused substrates on proline content (**A**) and proline dehydrogenase activity (**B**), (C), municipal compost (M), biochar (B), perlite (P), or pine bark (Pin). Each bar represents the mean of five replicates, and the error bars represent ± SE. Means with different letters are significantly different at $p < 0.05$.

Proline accumulation in plants [66], is an essential component of plant defense mechanisms [67]. One of the most effective osmoregulatory mechanisms at the molecular level involves the buildup of intracellular proline to lower water activity within the cytoplasm [68]. Overall, the water supply and the water condition in the substrate are significantly related to the proline content in plants [68,69]. In the present study, lettuce leaf proline content of the different treatments ranged from 0.38 to 1.00 mg/100 g FW (Figure 2A). These values were lower than those reported by Machado et al. [70] in leaf blades of spinach grown in the substrate (1.9 to 2.5 mg/100 g) and by Machado et al. [71] in coriander grown in soil (14.5–49.7 mg/100 g). The lower proline content in lettuce may be due to species since proline content is species-dependent [68,69]. The lower values of proline may also be linked to favorable growing conditions [72], indicating that the plants in the mixes were grown under favorable conditions.

Leaf proline dehydrogenase activity was higher in mixes with municipal compost, regardless of whether they had perlite or pine bark (Figure 2B). Proline dehydrogenase is an enzyme involved in the breakdown of proline into pyrroline-5-carboxylate (P5C). This process is part of the proline degradation pathway and is often associated with plant stress responses. Elevated proline dehydrogenase activity can also be triggered by variations in water availability and salinity [73]. Leaf PDH activity ranged from 18.00 to 47.00 nmol min^{-1}/mg protein.

3.6. Antioxidant Activity

The antioxidant activity measured by the ability of lettuce leaf extracts to reduce iron (Fe^{3+}) FRAP was higher in coir and in the mix of coir, compost, and perlite (Figure 3). Leaf FRAP values in the substrates range from 9.06 to 13.9 mg TEAC g^{-1} FW). These were much lower than those observed by Llorach et al. [30] (98.2 to 323.4 mg TEAC g^{-1} FW) in three green varieties of lettuce.

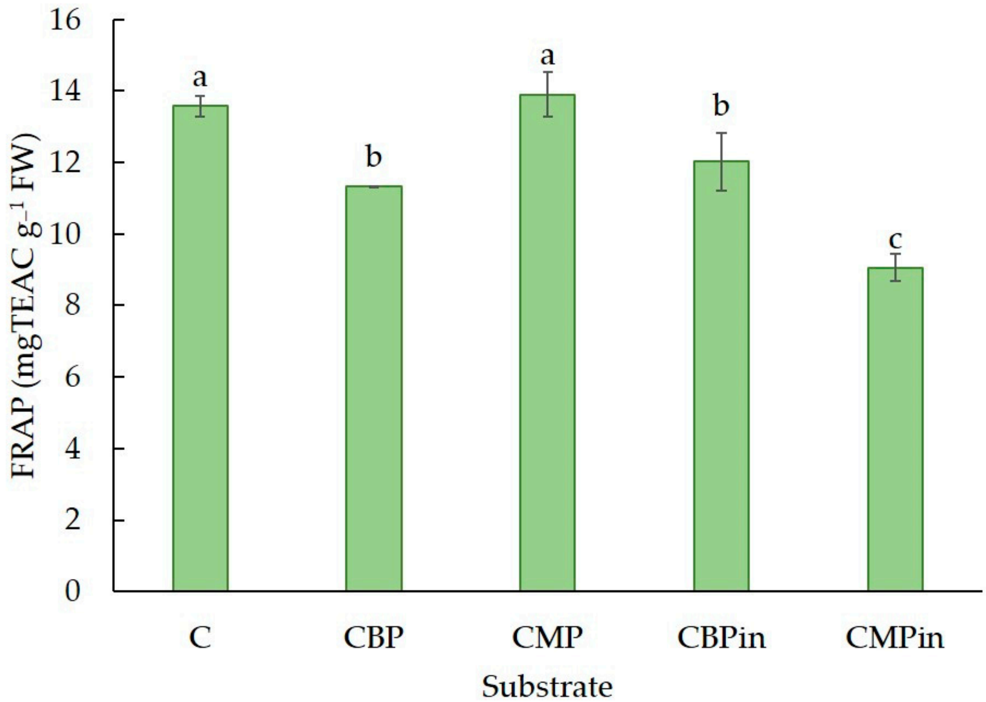

Figure 3. Effect of reused substrates on antioxidant activity estimated by FRAP, (C), municipal compost (M), biochar (B), perlite (P), or pine bark (Pin). Each bar represents the mean of five replicates and the errors bars represent ± SE. Means with different letters are significantly different at ($p < 0.05$).

Despite the observed effects on proline content, proline dehydrogenase activity, and antioxidant activity (FRAP), their magnitude was insufficient to affect the lettuce yield.

The differences observed in proline content, proline dehydrogenase activity, and antioxidant activity (FRAP) could potentially be reduced by optimizing irrigation scheduling and fertilization for each mix.

On the other hand, as the levels of total phenols, flavonoids, anthocyanins, and ascorbic acid in the lettuce leaves were either higher or comparable to those grown in coir used for the first time, this indicates that the reuse of coir-based substrates did not result in a decrease in yield and the product quality of lettuce.

4. Conclusions

The results show that coir-based growing media mixed with 12% compost or biochar and 10% perlite or pine bark, after its use in growing spinach, can still be successfully utilized for cultivating another short-cycle crop lettuce. Lettuce yield in reused substrates ranged from 4.6 to 4.8 kg/m^2, equal to the yield obtained in coir (4.9 kg/m^2) used for the first time.

The shoot nitrogen, phosphorus, potassium, calcium, and magnesium macronutrient concentrations, except in the coir, biochar, and pine bark mix, did not significantly differ between the reused and coir. Furthermore, the accumulation of total phenols, flavonoids, anthocyanins, and AsA in the leaves of plants grown on reused substrates was similar or even higher compared to those grown on coir used for the first time. Globally, the coir, biochar, and perlite mix allowed for better crop performance.

As the substrates reused did not undergo a sanitization procedure, it is strongly recommended that agricultural practitioners adopt a vigilant approach towards overseeing the growth and development of the prior crop and undertake thorough bioassays in case of uncertainties before considering substrate reuse. This proactive measure aims to preclude any unforeseen repercussions that might arise from the reuse, thereby ensuring the optimal outcome of subsequent cultivation.

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