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Agro-Physiological Performance of Iceberg Lettuce (*Lactuca sativa* L.) Cultivated on Substrates Amended with the Invasive Algae *Caulerpa prolifera* from the Mar Menor

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Abstract

The beneficial effects of algae on plant growth are widely known, so the combination of coconut fiber with algae waste from a species such as *Caulerpa prolifera,* which is an invasive species in the Mar Menor, could become an ideal substrate for leafy vegetable cultivation. The aim of this study was to evaluate the effect of an "algae waste + coconut fiber" combination in different proportions on the cultivation of iceberg lettuce. The proportions studied consisted of 0% algae waste + 100% coconut fiber (0% AW), 50% algae waste + 50% coconut fiber (50% AW), 75% algae waste + 25% coconut fiber (75% AW), and 100% algae waste + 0% coconut fiber (100% AW). Physiological parameters were evaluated. The results obtained showed that the mixture of algae waste and coconut fiber is a great alternative in the production of iceberg lettuce, since the proportion of 50% AW considerably improved the size of the lettuce (54.4%), the total phenol concentration (24.8%), the antioxidant activity (28.2%), the total sugars (14.1%) and reduced its nitrate concentration (24.6%), with respect to the 0% AW plants. These findings support the feasibility of reusing *Caulerpa prolifera* as a bio-enriched substrate for high-quality lettuce production.

Keywords: *Caulerpa prolifera*; algae waste; leafy vegetable; lettuce iceberg; nutritional qualities; circular economy framework; antioxidant activity

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1. Introduction

Algae such as *Caulerpa prolifera* (hare's ear) are currently considered an invasive species in one of the largest hypersaline lagoons in Spain, the Mar Menor lagoon, in Murcia (Spain) [1]. During the warm summer months, new stolons of *Caulerpa prolifera* grow very quickly and spread massively everywhere. In early autumn, senescence occurs, almost completely degrading the structure of the algae. Then, much of its biomass rots, and post-summer storms drag the degraded leaves towards the coast, which produce a foul smell as they decompose. In recent years, approximately 7 tonnes of waste have been extracted daily, representing a significant economic cost for the regional administration, more than €300,000 in 2021, according to the Directorate-General for the Environment of the Region of Murcia, Spain (personal communication).

Recently, the use of algae as biofertilizers has gained popularity due to their beneficial effects on plant growth. Algae provide a large number of compounds such as cytokinins, auxins, betaines, gibberellins, carbohydrates, vitamins, polysaccharides, alginates, amino acids and trace elements (Fe, Cu, Zn, Co, Mo, Mn and Ni), which are involved in plant functioning and development [2–4]. However, we propose an innovative approach compared to previous studies that have used other algae such as *Ulva lactuca* or *Sargassum* spp., which are generally non-invasive and have primarily been used mainly as biofertilisers or in liquid extracts [5]. Unlike these approaches, our work proposes the direct reuse of invasive algae in solid form as part of the substrate, which will not only allow us to take advantage of its potential agronomic benefits, but will also contribute to the recovery of problematic marine waste and reduce the environmental impact associated with its proliferation. In comparison with conventional organic substrates such as plant-derived compost, peat, or manure, *Caulerpa prolifera* exhibits additional advantages, including a higher content of bioactive compounds, an improved nutritional profile, and antioxidant properties that may be transferred to the cultivated plants [6]. In this way, a substrate with a biostimulant effect per se would be obtained, promoting better growth and allowing growers to reduce their use of fertilizers or other substances during the growing cycle, which in turn would imply economic savings for them. Furthermore, this innovative substrate, composed of coconut fiber combined with algae, could contribute to the achievement of objective 12 by the United Nations, which promotes responsible consumption and production. Its implementation may support more efficient resource management, reduce dependence on synthetic inputs, and enhance the sustainability of agricultural systems by reutilizing biomass and improving substrate fertility [7]. *Lactuca sativa* L. is widely considered as the most important leafy vegetables in the world. It has beneficial properties for human health, due to its concentration of phenolic compounds, vitamins and fiber [8], which have been shown to have the ability to prevent cardiovascular diseases [9].

This study addresses the identification of the optimal proportion of the mixed substrate composed of *Caulerpa prolifera* residues and coconut fibre, and its regulatory mechanism in the growth, nutritional quality and physiological metabolism of iceberg lettuce (*Lactuca sativa* L.).

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Iceberg lettuce cv. Nahifa seedlings were acquired from a commercial nursery (Semillero El Jimenado, S.A., El Jimenado, Spain), when the plants were 7–9 cm in height. They were transferred into 5 L pots filled with coconut coir fiber (Cocopeat, Pelemix, Alhama de Murcia, Murcia, Spain), algae waste (*Caulerpa prolifera*), or a mixture of both. Coconut fibre was chosen because it is the substrate most commonly used by farmers in the area and it yielded the best results in a study conducted by our research group [10]. The characterization of the algae is detailed in Table 1, where its physicochemical properties and elemental composition are presented. For the experimental setup, all treatments were established with algal biomass levels above 50%, in order to specifically evaluate the performance of substrates under conditions of maximal replacement of coconut fiber:

(1) 0% AW: 5 L of coconut fiber
(2) 50% AW: Mixture of 2.5 L of algae + 2.5 L of coconut fiber
(3) 75% AW: Mixture of 3.75 L of algae + 1.25 L of coconut fiber
(4) 100% AW: 5 L of algae.

Table 1. Characterization of the algae.

Parameters	Algal Waste	Coconut Fiber
pH	6.5 ± 0.01	6.3 ± 0.1
CE (μS/cm)	17,400 ± 1200	4100 ± 50
Organic matter (%)	73.9 ± 0.1	80% ± 0.1
%N	2.51 ± 0.54	Trace levels

Concentration (mg/kg DW)		
Cl^-	5324.01 ± 257.95	Trace levels
NO_3^-	165.48 ± 15.85	Trace levels
PO_4^{3-}	317.65 ± 169.13	Trace levels
SO_4^{2-}	4315.69 ± 9.11	Trace levels
Na	6.87 ± 0.33	Trace levels
K	2.17 ± 0.18	Trace levels
Ca	51.40 ± 0.66	Trace levels
Mg	7.10 ± 0.19	Trace levels
P	0.91 ± 0.02	Trace levels
Fe	3278.43 ± 504.89	Trace levels
Cu	8.53 ± 0.19	Trace levels
Mn	324.93 ± 36.92	Trace levels
Zn	146.78 ± 21.38	Trace levels
B	133.59 ± 0.58	Trace levels

Before the treatments, the algae were washed with plenty of tap water to remove excess salt. To verify the removal of excess salt, the electrical conductivity (EC) of the washing water was monitored until it reached a value equal to or below 1500 µS/cm. Before being used, they were spread on a net for 2 days to eliminate excess water from washing. Each pot contained a plant with a pressure compensating emitter (2 L h^{-1}). The plants were irrigated with a modified Hoagland solution with the following composition: 10.0 mM NO_3^-; 1.0 mM $H_2PO_4^-$; 3.5 mM SO_4^{2-}; 10.0 mM K^+; 1.8 mM Ca^{2+}; 2.2 mM Mg^{2+}; 20 µmM Fe; 0.6 µM Cu; 10 µM Mn; 2.0 µM Zn; 40 µM B; 0.5 µM Mo. Throughout the crop cycle, an excess of nutrient solution was applied to produce a minimum of 30% drainage to avoid salt accumulation and nutrient imbalance in the rhizosphere [11]. This experiment was carried out in a polycarbonate greenhouse, located at the IMIDA Research Center (La Alberca, Murcia, Spain). The mean values of temperature (daytime average and night average) and relative humidity were recorded in the greenhouse during the growth cycle using NTC probes manufactured by ACOM and collected by PC (Nutricontrol, S.L. software v1.5). The readings were 28 ± 1.2 °C, 14.2 ± 0.8 °C and 45% ± 8.2 and 70% ± 11.4, respectively. And the average external radiation was 274.0 w/m^2.

Thirty-one days after transplanting into the substrates under study, corresponding to the period required for lettuce plants to attain commercial maturity, the lettuce plants were harvested and weighed to determine their fresh weight (FW) and head diameter.

2.2. Mineral Concentration

The concentrations of NO_3^-, SO_4^{2-}, PO_4^{3-}, and Cl^- were quantified from lyophilized lettuce leaf tissue (0.4 g), previously ground, by extraction with 20 mL of deionized water under continuous shaking for 30 min. Ion quantification was carried out using an ion chromatography system (METROHM 861 Advanced Compact IC coupled with METROHM 838 Advanced Sampler, Metrohm, Herisau, Switzerland) equipped with a Metrosep A Supp7 250/4.0 mm column (Metrohm, Herisau, Switzerland). Analyses were performed at a flow rate of 0.7 mL min^{-1} and a column temperature of 45 °C, employing 3.6 mM Na_2CO_3 as the mobile phase in isocratic mode [8].

Macronutrients and micronutrients (K, Mg, Na, Ca, Fe, Cu, Mn, Zn, and B) were determined after acid digestion of lyophilized and ground samples (0.1 g) using a microwave digestion system (ETHOS ONE, Milestone Inc., Shelton, CT, USA). Elemental concentrations were then quantified with an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian Vista MPX, Palo Alto, CA, USA) [8].

The nitrogen content was determined from freeze-dried and finely ground lettuce leaf material using a combustion-based nitrogen/protein analyser (LECO FP-528; Leco Corporation, St. Joseph, MI, USA) [12].

2.3. Total Phenolic Compounds and Antioxidant Activity

Total phenolic content was quantified from fresh lettuce tissues previously stored at −80 °C. For extraction, 5 mL of 80% acetone was added to the samples, followed by centrifugation at 10,000× *g* for 10 min at 4 °C. An aliquot of 100 µL of the resulting supernatant was mixed with 1 mL of Folin–Ciocalteu reagent (diluted 1:10 in Milli-Q water), 2 mL of Milli-Q water, and 5 mL of 20% sodium carbonate. The reaction mixture was incubated in darkness for 30 min, and absorbance was recorded at 765 nm following the protocol described by Kähkönen et al. [13]. Results were expressed as gallic acid equivalents (mg 100 g^{-1} FW).

Antioxidant capacity was evaluated through radical scavenging activity using the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay. Lyophilized lettuce tissues (0.5 g) were extracted with 10 mL of an acidified methanol/water mixture (80:20 *v*/*v*, containing 1% HCl), followed by sonication at 20 °C for 15 min and incubation at 4 °C for 24 h. The sonication step was repeated for an additional 15 min, after which the extracts were centrifuged at 10,000× *g* for 10 min. The ABTS assay was carried out following the procedure described by Re et al. [14]. A Trolox standard curve (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for calibration. Antioxidant activity was expressed as µM Trolox equivalents (TE) g^{-1} dry weight (DW), reported as mean ± standard error.

2.4. Lipid Peroxidation

Lipid peroxidation was quantified by determining thiobarbituric acid-reactive substances (TBARS) following the TBA assay [15]. Briefly, fresh frozen lettuce tissues (0.1 g) were homogenized in 3 mL of 20% (*w*/*v*) trichloroacetic acid (TCA), and the homogenate was centrifuged at 3500× *g* for 20 min. A 1.5 mL aliquot of the supernatant was mixed with 1.5 mL of 20% TCA containing 0.5% (*w*/*v*) TBA and 0.15 mL of 4% (*w*/*v*) butylated hydroxytoluene (BHT) in ethanol. The reaction mixture was incubated at 95 °C for 30 min, rapidly cooled on ice, and centrifuged at 10,000× *g* for 15 min. Absorbance was recorded at 532 nm, with non-specific turbidity corrected at 600 nm. TBARS concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} [16].

2.5. Extraction and Quantification of Total Soluble Sugars

Soluble sugars were extracted from 50 mg of lyophilized lettuce leaves by two sequential incubations in 1.5 mL of 80% (*v*/*v*) methanol at 4 °C for 30 min each [17]. The extracts were centrifuged at 3500× *g* for 15 min at 4 °C, and the resulting supernatants were passed through C18 Sep-Pak cartridges (Waters Associates, Milford, MA, USA) preconditioned with 20 mL of 80% methanol. Combined eluates were further filtered through 0.45 µm membranes (Millipore, Bedford, MA, USA). Glucose, fructose, and sucrose contents were quantified by ion chromatography using a Metrohm 817 Bioscan system (Herisau, Switzerland) equipped with a pulsed amperometric detector (PAD) and a gold electrode, with separation performed on a Metrosep Carb 1–150 IC column (4.6 × 250 mm) (Metrohm, Herisau, Switzerland) maintained at 32 °C. Total soluble sugars were expressed as g Kg^{-1} DW.

2.6. Polyamine Analysis

Polyamine extraction was performed following the protocol of Rodríguez et al. [18], with minor adjustments. Fresh tissue (5 g) was homogenized in 7.5 mL of 5% perchloric acid at 4 °C and maintained under refrigeration for 1 h with intermittent shaking. The homogenates were centrifuged at 5000× *g* for 8 min, and the resulting supernatants were subjected to UHPLC analysis, as described by Rodríguez et al. [18], with slight modifications. Separation was achieved on a reversed-phase ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm; Waters Corp., Wexford, Ireland) maintained at 40 °C, using an isocratic elution system of water/acetonitrile (58:42, *v*/*v*) at a flow rate of 0.55 mL min^{-1}. A 10 µL injection volume was used. Benzoylated polyamines were detected with a UHPLC-DAD system (Waters Technologies, Waldbronn, Germany) at 254 nm. Data acquisition and processing were carried out using Empower 2 software (Waters). The results are expressed as nmol g FW^{-1}.

2.7. Statistical Analysis

The study employed a completely randomized design with six biological replicates per treatment. Statistical analyses were performed using Statgraphics Centurion XVI (StatPoint Technologies, Inc.). Prior to inferential testing, data were assessed for homogeneity of variances and normality of distribution using Kolmogorov–Smirnov test. Subsequently, an analysis of variance (ANOVA) was conducted, and treatment means were compared using Duncan's multiple range test at a significance level of $p \leq 0.05$. The experimental setup included four levels of substrates with algae (0%, 50%, 75%, and 100% AW). Additionally, principal component analysis (PCA) was executed using R software, version 4.5.0. [19]. PCA was applied to reduce data dimensionality and identify patterns among agronomic and physiological variables of lettuce grown in different substrates. This helped to distinguish the effects of the *Caulerpa prolifera*-based substrate compared to controls.

3. Results

3.1. Physical Characterization of Iceberg Lettuce

Iceberg lettuces harvested after 31 days of cultivation in different substrate mixtures saw increases in both head weight and head diameter (Figure 1A,B). The greatest weight corresponded to lettuce grown with the 50% AW treatment, followed by the 75% AW treatment. In the case of the 50% AW treatment, the increases in weight and head diameter as compared to the 0% AW were 54.4% and 23.3%, respectively (Figure 1A,B). On other hand, for the 75% AW treatment, the increases were 40.2% and 15.5% (Figure 1A,B). For the 100% AW treatment, values were similar to those of the 0% AW plants for both parameters (Figure 1A,B).

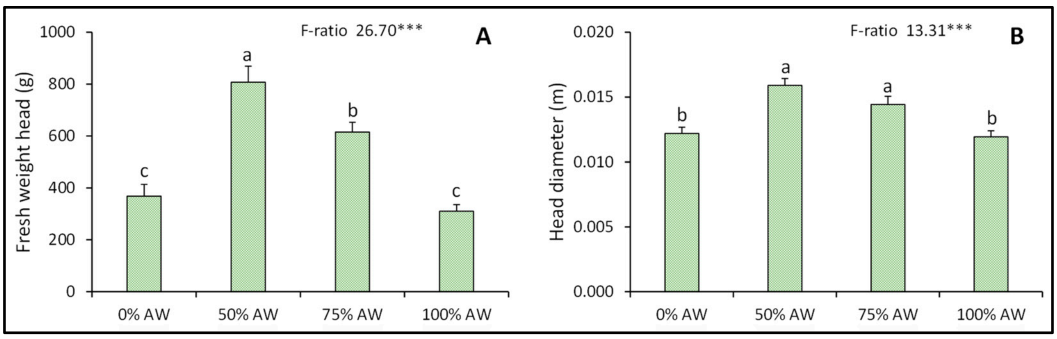

Figure 1. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on fresh head weight (**A**) and head diameter (**B**) of lettuce plants. Data are presented as means ± SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) between treatments. (*** F-ratio ≤ 0.01%).

3.2. Mineral Composition

The mineral concentration of lettuces was significantly affected by the use of algae as part of the substrate. All the treatments with algae reduced the accumulation of nitrates

(NO_3^-) in the lettuce leaves, although in the case of 100% AW, this reduction was not significant (Table 2). The reductions were 26.4% in the case of 50% AW, 32.3% with 75% AW and 11.0% with 100% AW (Table 2). On the contrary, the concentration of chlorides (Cl^-) increased in the treatments with algae, with this increase being around 14.4% in the 50% AW and 100% AW treatments, and 20.3% in the case of 75% AW (Table 2). Chloride accumulation, although not reaching toxic levels, emerged as a key finding due to its potential impact on nutrient uptake and plant development. This makes it a critical factor in assessing the suitability of the substrate for cultivation. On the other hand, the concentration of sulfates (SO_4^{2-}) showed a tendency to increase when algae were used as the substrate, but significant differences were only found in the 100% AW treatment (Table 2). However, phosphates (PO_4^{3-}) were not affected by any of the treatments applied (Table 2).

Table 2. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on mean concentrations of anions of lettuce plants.

Treatment	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
	g kg^{-1} DW			
0% AW	5.8 ± 0.4 ^{b}	32.3 ± 2.0 ^{a}	9.6 ± 1.22 ^{a}	4.1 ± 0.2 ^{b}
50% AW	6.8 ± 0.1 ^{a}	23.8 ± 1.1 ^{bc}	9.6 ± 2.45 ^{a}	4.1 ± 0.2 ^{b}
75% AW	7.3 ± 0.3 ^{a}	21.9 ± 1.9 ^{c}	10.0 ± 1.39 ^{a}	4.6 ± 0.2 ^{b}
100% AW	6.8 ± 0.2 ^{a}	28.7 ± 2.6 ^{ab}	9.6 ± 0.99 ^{a}	5.7 ± 0.1 ^{a}
F-ratio	5.1 *	5.3 **	1.5 ns	18.8 ***

Data are mean ± SE (n = 6). Different letters within a column indicate significant ($p \leq 0.05$) differences between treatments. (ns F-ratio > 5%, * F-ratio ≤ 5%, ** F-ratio ≤ 1% and *** F-ratio ≤ 0.01%).

Table 3 shows the concentration of cationic macro and microelements in the lettuce leaves. Potassium (K) was the most abundant cation of the measured macronutrients, while zinc (Zn) was the most abundant of the micronutrients (Table 3). Lettuces grown with the 50% AW treatment had a reduced concentrations of Mg and K (12.7% and 16.7%, respectively), and an increased concentration of Mn and Zn, with respect to 0% AW plants (58.4% and 59.1%, respectively) (Table 3). The greatest reduction of cations occurred with the 75% AW treatment, which reduced the concentrations of Mg, K, P, Ca and Fe (21.2%, 23.1%, 16.3%, 18.5% and 14.4%, respectively), and only increased the concentrations of Mn and Zn (72.8% and 63.1%, respectively) (Table 3). And on the contrary, the 100% AW treatment was the one that produced the greatest increase in cations, increasing the concentrations of Na, Cu, Mn, B and Zn (27%, 29.7%, 93.5%, 14.3% and 73.2%, respectively) and only reducing the concentration of Mg (11.8%) (Table 3). In all the treatments evaluated, an increase in foliar concentration of Zn and Mn is observed, demonstrating a direct correlation with the availability of these elements provided by the algae. Furthermore, a proportional rise in their concentration is recorded as the proportion of algae in the substrate increases.

Table 3. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on mean concentrations of cations of lettuce plants.

Treatment	K	Ca	Mg	P	Fe	Cu	Mn	Zn	B
	g kg^{-1} DW				mg kg^{-1} DW				
0% AW	49.2 ^{a}	3.6 ^{a}	2.3 ^{a}	8.1 ^{a}	103.0 ^{a}	4.5 ^{b}	4.8 ^{d}	59.0 ^{c}	16.0 ^{b}
50% AW	41.0 ^{bc}	3.3 ^{ab}	2.0 ^{bc}	7.5 ^{a}	95.2 ^{ab}	4.1 ^{b}	11.5 ^{c}	144.0 ^{b}	17.3 ^{ab}
75% AW	37.8 ^{c}	2.9 ^{b}	1.8 ^{c}	6.7 ^{b}	88.1 ^{b}	4.1 ^{b}	17.6 ^{b}	160.7 ^{b}	16.6 ^{b}
100% AW	44.8 ^{ab}	3.3 ^{ab}	2.0 ^{b}	7.5 ^{a}	96.1 ^{ab}	6.4 ^{a}	74.2 ^{a}	220.1 ^{a}	18.7 ^{a}
F-ratio	9.0 **	4.5 *	9.7 **	7.1 **	2.6 ns	32.3 ***	588.1 ***	107.1 ***	4.3 *

Data are mean ± SE (n = 6). Different letters within a column indicate significant ($p \leq 0.05$) differences between treatments. (ns F-ratio > 5%, * F-ratio ≤ 5%, ** F-ratio ≤ 1% and *** F-ratio ≤ 0.01%).

In the case of %N, the behavior was similar to that of the NO_3^- concentration, as expected (Figure 2).

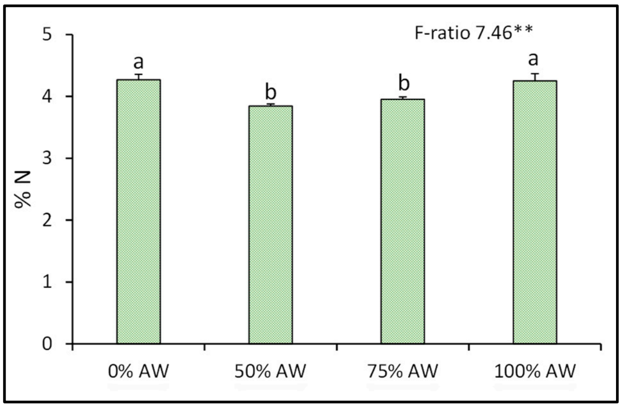

Figure 2. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on total nitrogen percent of lettuce plants. Data are presented as means ± SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) between treatments. (** F-ratio ≤ 1%).

3.3. Total Phenolic Compounds, Antioxidant Activity (ABTS+) and Lipid Peroxidation*

The highest values of total phenolic compounds were obtained in lettuce plants grown with the 50% AW treatment (118.2 mg 100 g^{-1} FW), and on the contrary, the lowest value was obtained in those grown with 75% AW (71.2 mg 100 g^{-1} FW) (Figure 3A). In this case, once again the lettuces grown with 100% AW presented concentrations of total phenolic compounds similar to those of the 0% AW plants (Figure 3A).

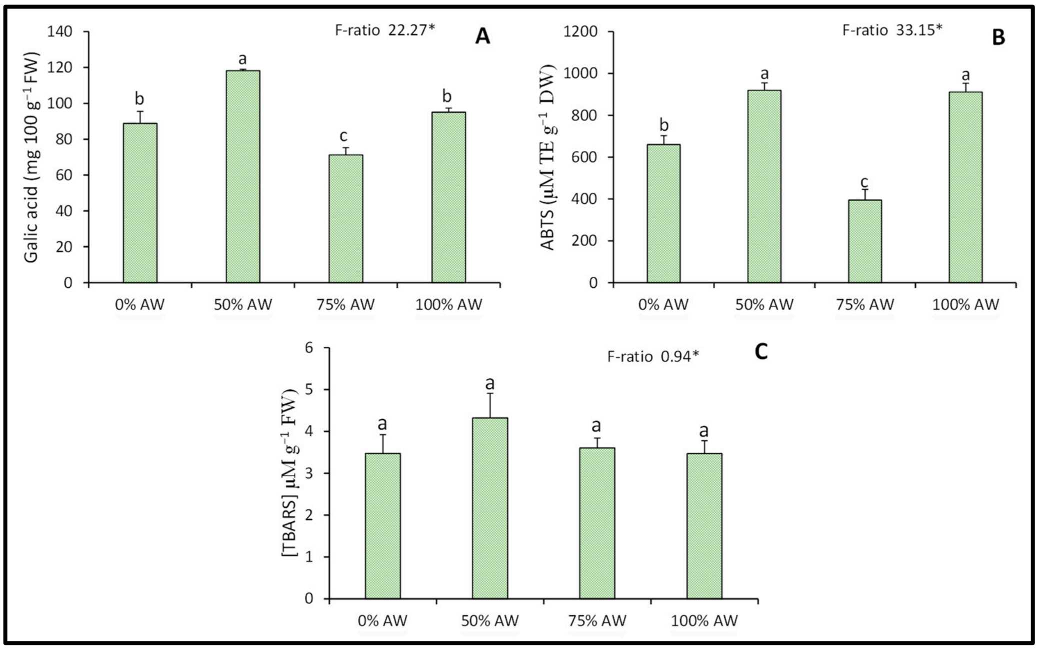

Figure 3. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on total phenolic (**A**), antioxidant activity (ABTS) (**B**) and lipid peroxidation ([TBARS]) (**C**) of lettuce plants. Data are presented as means ± SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) between treatments. (* F-ratio ≤ 5%).

However, in the case of antioxidant activity, the 50% and 100% AW treatments showed the highest values. These were 28.2% and 27.5% higher than the control plants, respectively (Figure 3B). In contrast, the 75% AW treatment caused a reduction of 40.2%

(Figure 3B). The highest values recorded in plants grown on a substrate composed entirely of AW could be attributed to a concentration effect.

Regarding lipid peroxidation, the lettuces presented values between 3.5 and 4.3 μM g^{-1} FW (Figure 3C); however, no significant effects were observed for any of the treatments applied (Figure 3C).

3.4. Total Soluble Sugars

The chromatographic profile of the sugars in the lettuce leaves was constituted by three free sugars (glucose, fructose, and sucrose). As it can be observed in Figure 4D, the total sugar concentration oscillated between 1.04 and 14.95 g kg^{-1} DW. In the case of individual free sugars, glucose was the most abundant ($p \leq 0.05$), while sucrose was found in the lowest concentration. These data show that the use of algae as substrates positively influenced the accumulation of sugars such as glucose and sucrose, and on the contrary, negatively influenced fructose (Figure 4A–C). In the case of glucose, the increase was similar in all treatments with algae, regardless of the percentage of algae used in the substrate, with this increase being between 15.3% and 17.3% as compared to the 0% AW plants (Figure 4A). In the case of sucrose, this increase was greater in lettuce plants grown in the 100% AW substrate, as compared to the 0% AW. Sucrose in the 50% AW and 75% AW treatments increased by 20.1% and 18.9%, respectively, as compared to the 0% AW, while in the 100% AW treatment, it increased by 31.8% (Figure 4B).

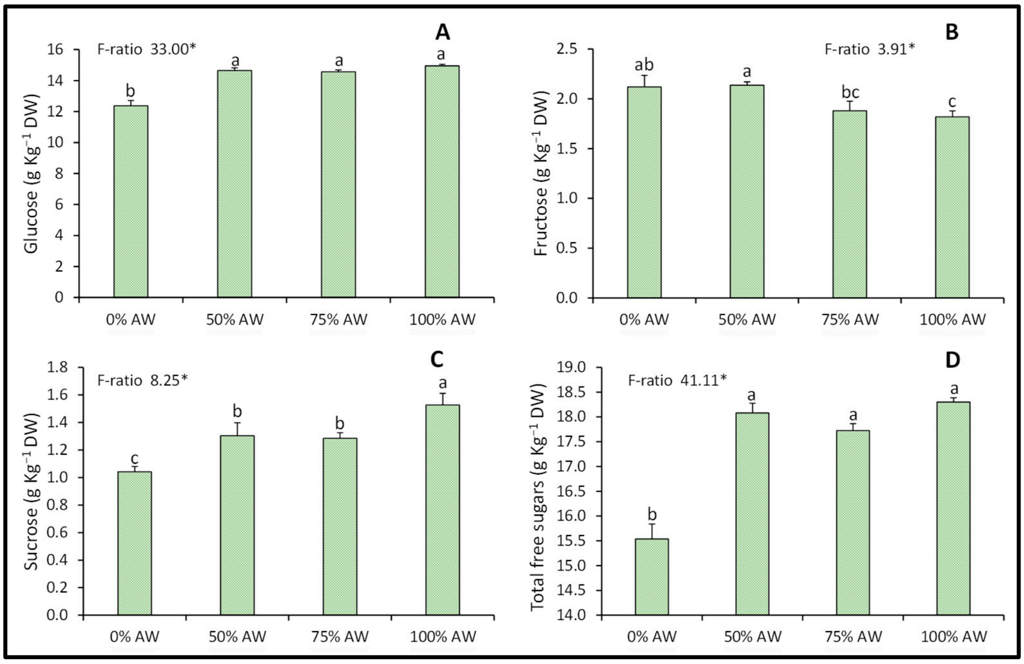

Figure 4. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on mean concentrations of sugars: glucose (**A**), fructose (**B**), sucrose (**C**) and total free sugars (**D**) of lettuce plants. Data are presented as means ± SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) between treatments. (* F-ratio ≤ 5%).

3.5. Polyamines

Figure 5 shows the concentrations of polyamines in iceberg lettuces leaves. Of the polyamines quantified, putrescine was found in the highest concentration, followed by spermine, cadaverine, and spermidine. All polyamines were affected by the applied treatments. In the case of the 75% AW treatment, as observed with total phenolic content and lipid peroxidation, there was a generalized reduction of all polyamines, except for cadaverine, which obtained values similar to the 0% AW plants (Figure 5A–D). The reductions observed corresponded to 43.2% in putrescine, 52.1% in spermidine, and 59.1% in spermine, as illustrated in Figure 5A,C,D. On the contrary, the 100% AW treatment caused an increase in putrescine and cadaverine, with the increase in cadaverine being the most important (73.5% as compared to the values of the 0% AW plants) (Figure 5A,B). Lastly, the 50% AW treatment caused a reduction in spermine concentration (Figure 5D).

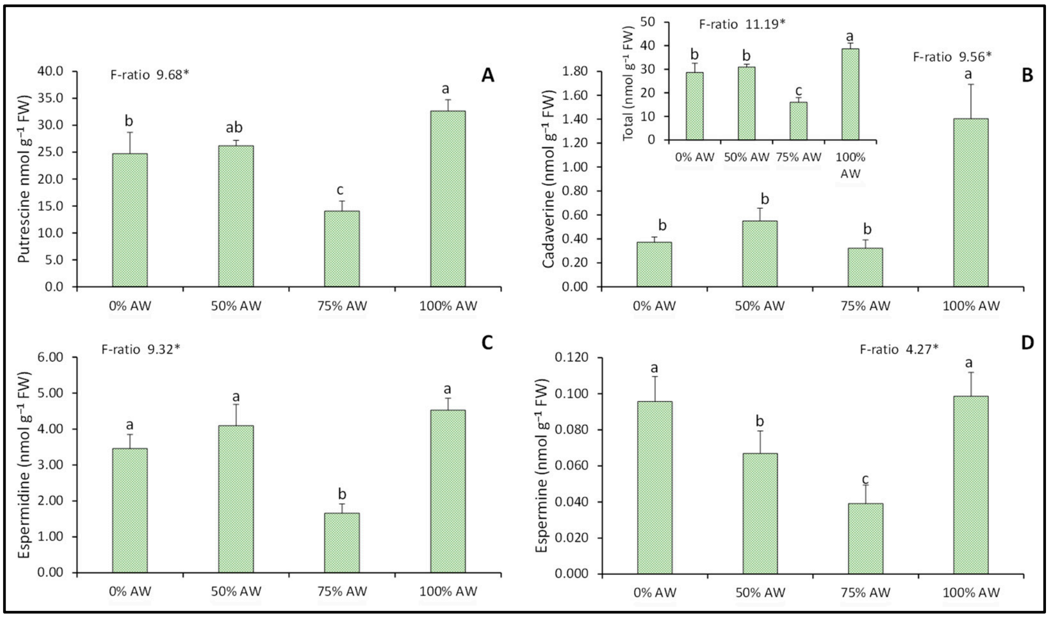

Figure 5. Effect of algae waste (AW) in the growing medium at different percentages (0%, 50%, 75%, and 100% AW) on mean concentrations of polyamines: putrescine (**A**), cadaverine (**B**), spermidine (**C**) and spermine (**D**) of lettuce plants. Data are presented as means ± SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) between treatments. (* F-ratio ≤ 5%).

Regarding the total polyamine concentration measured, it was observed that treatment with 75% AW resulted in a decrease of 44.1%, while treatment with 100% AW induced an increase of 25.9% compared to the 0% AW in both cases (Figure 5B).

3.6. Principal Component Analysis (PCA)

The first principal components (PCs) represented 29% and second PCs represented 24.9% of the population variability; therefore, both PCs accounted for a total variability of 53.84% observed across the 28 analyzed variables (Figure 6). The variables analyzed can be grouped into growth (fresh weight and head diameter), mineral composition, polyamines and antioxidant compounds.

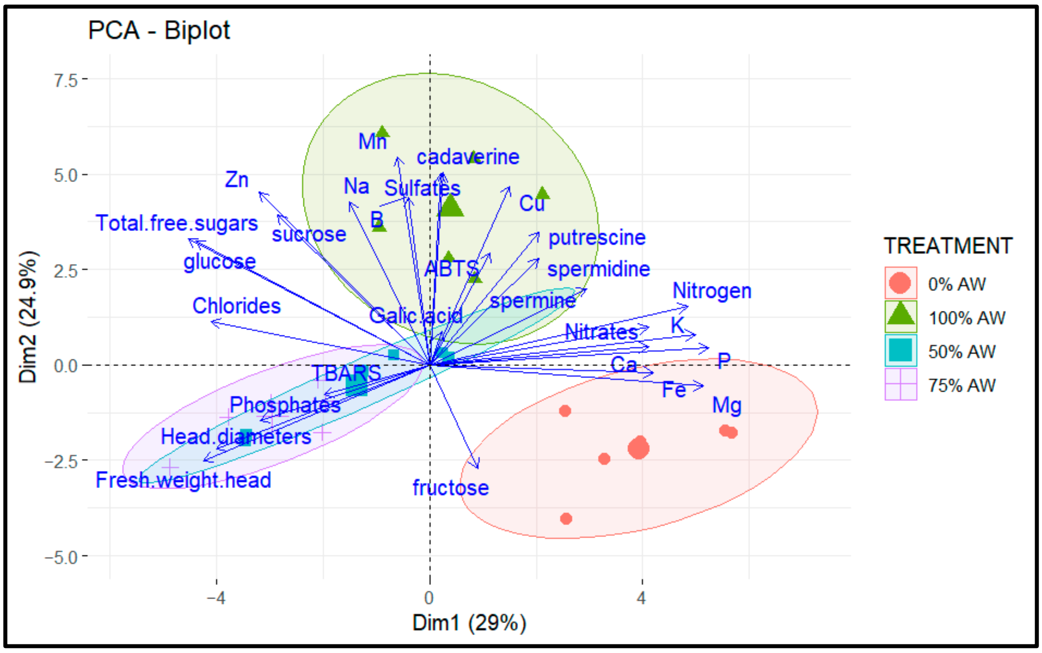

Figure 6. Principal component analysis (PCA) of the parameters analyzed in iceberg lettuce plants grown in different substrate mixtures (0% AW, 50% AW, 75% AW y 100% AW).

In the graph, the points represent the measured observations, while the arrows indicate the direction and magnitude of the variance explained by each variable, with their length reflecting the relative contribution of each to the overall variability. The PCA revealed a clear separation between 0% AW plants and plants grown on algae substrate (Figure 6). It is important to note that the 50% AW and 75% AW treatments even appear to overlap (Figure 6).

4. Discussion

4.1. Physical Characterization of Iceberg Lettuce

In recent decades, great efforts have been made on the use of algae as bio-fertilizers in crop production; however, to our knowledge, this would be the first work that addresses the use of algae as a substrate for growing crops. Algae are organisms rich in chemical compounds such as minerals, proteins, carbohydrates, polyphenols, pigments, polysaccharides, unsaturated fats, phlorotannins, and phytohormones [20]. Therefore, all of these chemical compounds could contribute to the nutrition of lettuce and, consequently, be responsible for the greater growth (increase in weight and size) observed in lettuce plants grown in the 50% AW treatment compared to plants grown on coconut fiber. However, when algae were used as the sole component of the substrate, the previously observed beneficial effects were no longer evident. This outcome may be attributed to the lower water retention capacity of the algae when used alone. These results are supported by authors such as Dasgan and Temtek [21], who reported an increase in the weight of lettuce plants by 47.2% as compared to the 0% AW, when they were treated with microalgae as a biofertilizer. However, the higher the proportion of algae in the substrate (100% AW treatment), the lower the beneficial effects observed, which could be due to the lower ability of algae to retain water. The synergistic use of coconut fiber and algae offers both physical and biological advantages in soilless cultivation. While coconut fiber optimizes root zone aeration and water balance, algae actively promote plant metabolic processes, root development and microbial interactions [3,4]. Together, they form a holistic solution that supports higher yields, improved resource efficiency and environmental sustainability in modern horticulture.

4.2. Mineral Composition

Algae is one of the most appreciated marine resources in agriculture, due to its effectiveness as an organic amendment, and the presence of macronutrients such as K, Ca and P and micronutrients such as Cu, Fe, B, Zn, Mn, Mo and Co [22]. Furthermore, authors such as Omar et al. [23] observed that algae extracts improved the morphological structure of plant roots, improved energy storage through the accumulation of non-structural carbohydrates, increased metabolism, water adjustment, and the accumulation of proline. Therefore, the greater contribution of nutrients, together with a better root system, could be responsible for the greater uptake of nutrients (Na, Cu, Mn, B and Zn) in the case of plants grown with 100% AW. On the other hand, the reduction in some nutrients in lettuce plants grown with the other two treatments could be due to a dilution effect, as they are larger plants. This phenomenon occurs when increased vegetative growth, mainly associated with greater water accumulation in the tissues, causes the absolute amount of nutrients absorbed by the plant to be distributed over a higher biomass volume. Consequently, although nutrient supply and absorption may be similar or even higher, the relative concentration of these compounds per unit of fresh weight decreases. This dilution effect is a process frequently described in fast-growing crops with high water content, such as lettuce, where an increase in size does not always correlate with a proportional increase in nutritional density.

Regarding nitrates, the current legislation has established the permitted nitrate concentration in lettuce to be between 3000 mg kg^{-1} FW (in lettuce grown in the field) and 5000 mg kg^{-1} FW (in lettuce grown indoors) [24]. Given that our lettuce is about 96% water, our highest values remain below 1500 mg kg^{-1} FW, and in the case of the 50% AW and 75% AW treatments, which presented the lowest concentration of nitrates, this was 950 mg kg^{-1} FW and 874 mg kg^{-1} FW, respectively. These findings are of great interest, because although nitrate itself is relatively non-toxic, high levels of nitrate are especially dangerous for infants and can cause methemoglobinemia [24]. In babies, nitrate can obstruct oxygen transport in the blood and prevent the adequate oxygenation of tissues. This is because they have low levels of the enzyme nitrate reductase [25]. On the other hand, its metabolic products, such as N-nitroso compounds, nitrite and nitric oxide, can also be harmful to health [25]. The lower NO_3^- concentrations observed in lettuce leaves grown in substrates enriched with algae (50% and 75% AW) could be attributed to the competitive and antagonistic effect between Cl^- and NO_3^- anions at the level of NO_3^- transporters in the root, as well as to the possible inactivation or decrease in the activity of these transporters due to osmotic and toxic stress generated by the relative high salinity of the substrate, which affects the absorption and translocation of NO_3^- [26]. According to González-Hernández et al. [27], these polyamines play a key role in enhancing tolerance to abiotic stresses such as salinity, potentially mitigating the negative effects of Cl^- accumulation on nitrate uptake and homeostasis.

In the PCA, a negative relationship was observed between quality parameters (fresh weight and head diameter) and NO_3^- and %N concentration. This could reflect a physiological balance in which the resources captured by the plant are allocated more to structural growth than to the accumulation of nitrogen compounds, which could have implications for nutritional quality and fertilisation management.

4.3. Total Phenolic Compounds, Antioxidant Activity (ABTS+) and Lipid Peroxidation*

The mixture of coconut fiber and algae can be a good tool to increase the phenolic concentration in plant tissues, in order to improve the nutritional quality of vegetables, in addition to increasing crop protection and reducing the use of pesticides [24]. However, the proportion in which they are combined is important, since in our case, the increase in the concentration of phenolic compounds was only observed in the treatment with the proportions 50% algae waste and 50% coconut fiber (50% AW treatment), with respect to the 0% AW. This result suggests that a balanced contribution of organic matter and mineral elements from the algae, combined with the structural and aeration properties of coconut fiber, may create an optimal environment for inducing secondary metabolism in plants. Excessive amounts of algae could lead to adverse effects, such as increased salinity or the accumulation of certain ions (e.g., Na^+, Cl^-), which may stress plants beyond a threshold and reduce their metabolic capacity, while very low amounts may not be sufficient to trigger significant metabolic changes.

The increase in phenolic compounds in vegetables is of great interest, since they are antioxidant compounds that can help prevent human diseases, such as cancer and heart disease [28]. Their accumulation in plants is often associated with moderate abiotic stress, which activates defense mechanisms and promotes the synthesis of secondary metabolites. In this context, the 50% AW treatment may have provided an intermediate stress level, sufficient to stimulate the phenylpropanoid pathway without impairing growth [29]. In contrast, treatments with lower or higher algae concentrations may not have generated the same metabolic response, either because the stimulus was insufficient or because excessive stress diverted plant resources toward primary metabolism and stress mitigation, reducing phenolic biosynthesis.

In the case of antioxidant activity, no direct relationship was observed with the concentration of phenolic compounds, which could be due to the fact that other families of compounds (such as carotenoids, glucosinolates, organic acids and amino acids) can contribute to the total antioxidant activity [30]. This indicates that antioxidant capacity is a multifactorial trait, and phenolics, while important, represent only one part of the antioxidant network in plants. It is also possible that some phenolic compounds synthesized in the 50% AW treatment had relatively low antioxidant capacity compared with other bioactive molecules, which could explain the absence of a strong correlation.

However, in the case of the 50% AW treatment, the antioxidant activity was slightly higher than that of the plants grown with the 0% AW, although it was not significantly different. This modest increase could be linked not only to the rise in phenolic concentration but also to the synergistic effects of other antioxidant families stimulated under this condition.

On the other hand, the highest values recorded in plants grown on a substrate composed entirely of AW could be attributed to a concentration effect. This phenomenon is explained by the reduced biomass developed by these plants, as observed in the determination of their weight, suggesting a greater relative accumulation of the evaluated compounds in relation to their smaller size.

4.4. Total Soluble Sugars

The addition of seaweed residues to the substrate was associated with an increase in glucose, sucrose and total free sugars, while the concentration of fructose decreased. This pattern can be explained by changes in enzyme activity induced by the presence of bioactive algal compounds. On the one hand, algae are a source of polysaccharides and mineral nutrients that can stimulate photosynthesis and carbohydrate biosynthesis, leading to increased accumulation of glucose as the primary photosynthetic product [31]. Part of this glucose is subsequently converted into sucrose, the main form of carbohydrate transport in higher plants, which explains the increase in both metabolites with increasing algal content. On the other hand, the reduction in fructose levels could be related to a change in the hexose balance: fructose, generally derived from sucrose cleavage compounds by invertases, can be rapidly metabolised in other pathways (glycolysis or secondary metabolism) under the influence of algal compounds, preventing its accumulation in leaf tissues. Authors such as Zhou et al. [32] observed that, specifically glucose and sucrose were involved in this response to stress and in the regulation of senescence. Thus, we observed that the increased bioavailability of free sugars, such as glucose and sucrose, plays a pivotal role in plant growth. Therefore serving as metabolic substrates and signalling molecules to generate energy and carbon skeletons for the synthesis of biomass and secondary metabolites, including antioxidants [33].

Furthermore, authors such as Chen et al. [34] have observed that a higher concentration of soluble sugars in lettuce leaves results in a better lettuce flavor. Therefore, increasing the sugar concentration in lettuce leaves grown in substrates with algae by 14.1%, as in the 50% AW treatment, would improve the flavor of these lettuces, making them sweeter than lettuces without algae, as well as more resistant to environmental stresses.

4.5. Polyamines

The PCA showed how the variables putrescine, cadaverine, spermidine and spermine were affected by the presence of algae in the substrate. The increase generated by the 100% AW treatment as compared to the 0% AW could mean that the lettuces grown with this treatment have a beneficial effect on the health of the consumer, as it is known that polyamines are responsible for regulating cell differentiation, the response to diabetes, inflammatory reactions, intestinal immunoallergic responses, and the prevention of food allergies in children [35]. Furthermore, the increase in the concentration of polyamines in foods is of great interest, since the endogenous production of polyamines is reduced with age, and these must be supplemented with the diet [36].

On the other hand, authors such as Liu et al. [37] have observed that polyamines have the ability to increase the activity of antioxidant enzymes in lettuce. Therefore, the increase that we previously observed in antioxidant activity could also be related to this higher concentration of polyamines. These results can be interpreted considering that both the accumulation of polyamines and the increase in antioxidant activity are typical plant responses to stress situations. In the 100% AW treatment, compared to 0% AW, significant differences were observed only in putrescine concentration, while spermidine and spermine levels did not show relevant changes. This response suggests that the stress generated by 100% AW was of low intensity, sufficient to activate the biosynthesis of putrescine—the first metabolite in the polyamine pathway—but insufficient to induce a significant accumulation of higher order compounds such as spermidine and spermine [38]. In the case of spermidine, an increasing trend was evident, although not statistically significant, and in the case of spermine, no variations were detected, which could indicate that the activation of the biosynthetic pathway did not fully progress to the final levels.

5. Conclusions

The results obtained in this work highlight the great benefits of using invasive algae that accumulate on the shores of the Mar Menor as part of the substrate in the cultivation of iceberg lettuce. Unlike other macroalgae such as *Ulva lactuca* or *Sargassum* spp., which have been extensively researched for their use as biofertilisers or sources of liquid extracts, the direct integration of algae as the predominant matrix in hydroponic or aquaponic substrates represents an innovative approach that is poorly documented in the current scientific literature. We have shown that lettuce plants grown in a substrate composed of 50% algae mixture and 50% coconut fiber increased their size by 54.4% and their nutritional qualities. Lettuce grown with this substrate, in addition to increasing its total phenolic concentration (24.8%), its antioxidant activity (28.2%), and its soluble sugar concentration (14.1%), had reduced NO_3^- concentration (24.6%). Given these good results, it would be interesting to study more varieties of crops and in different proportions. This would allow verification of whether the slight chloride accumulation observed in lettuce is consistently innocuous across other crops, or whether species-specific sensitivities may emerge.

In addition, this research contributes to the valorisation of waste generated by the accumulation of algae, the removal of which represents a considerable expense for public administrations in cleaning up the shores. The implementation of this organic substrate would, in turn, reduce dependence on imports of materials such as coconut fibre, promoting a more sustainable and economical approach to agricultural production.

6. Patents

Patent pending (P202530227/Substrate for soilless cultivation).

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