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Pretreated *Eucalyptus globulus* and *Pinus radiata* Barks: Potential Substrates to Improve Seed Germination for a Sustainable Horticulture

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Abstract: Commercial forest plantations in Chile are dominated by pine (*Pinus radiata*) and eucalyptus (*Eucalyptus globulus*). Tree bark is the main by-product of the forestry industry and has low value, but great potential for use as an agricultural substrate. However, the direct use of bark fibers may cause plant phytotoxicity due to the presence of polyphenolic and other compounds. This study aims to evaluate the physicochemical properties of *E. globulus* and *P. radiata* bark after water extraction treatments. The phytotoxicity of the resulting extracted bark alone and that mixed with commercial substrates (coconut fiber, moss, peat, and composted pine) at different ratios (25 to 75 wt%) were assessed using the Munoo-Liisa vitality index (MLVI) test. For all treatments, the seed germination and growth of radish (*Raphanus sativus*) and Chinese cabbage (*Brassica rapa*) species were evaluated and compared to a commercial growing medium (peat) as a control. The optimal mixture for seed growth was determined to be 75% extracted *E. globulus* bark fiber and 25% commercial substrates such as peat (P), coconut fiber (C), moss (M), and composted pine (CP), as indicated by the MLVI and germination results. Two phytostimulant products, chitosan and alginate-encapsulated fulvic acid, were added to the best substrate mixture, with the purpose of improving their performance. Encapsulated fulvic acid at 0.1% w/v was effective in promoting plant growth, while chitosan at all of the concentrations studied was effective only for mixture 75E-25CP. The mixture of *E. globulus* fiber and commercial substrates, containing a high proportion of water-extracted fiber (75%), shows the potential to be used in the growth of horticultural crops and in the plant nursery industry.

Keywords: growing media; agroforestry; biostimulants; sustainable agriculture; waste management; fibers; cultivation; germination; root growth

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1. Introduction

The total cumulative area of forest plantations in Chile in 2021 was estimated to be 2,321,257 hectares (ha). Radiata pine (*Pinus radiata*) is the main species planted, with 1,299,451 ha, representing 55.9% of the total area, followed by eucalyptus species (*Eucalyptus globulus* and *Eucalyptus nitens*), with 854,593 ha, representing 36.8% [1]. The Chilean forest sector has specialized and diversified in producing chips for the pulp and paper industry and timber for the sawmill and boards industry. This intensive economic and industrial activity generates significant quantities of lignocellulosic by-products (leaves, cones, seeds, and bark). These are considered to be interesting raw materials for the development of forest biorefineries, which would be in line with the circular economy demand for this sector. These biorefineries allow the production of biofuels, fine chemicals, and biobased materials [2–5]. The bark of *P. radiata* and *Eucalyptus* spp. species are the by-products that are obtained in the largest quantities, with both types of bark representing 10%–12% of the volume of the tree [6,7]. These forest resources are estimated to produce between two and three million tons of bark annually in Chile [8]. Pine bark is mainly used as a raw material for energy production, but it has also been used for composting in nurseries and as a landscaping material in public gardens [9]. Conversely, eucalyptus fiber bark has no large-scale commercial use.

Valuable resources such as peat and organic soils are often used as substrates for ornamental plants [10,11]. The global area and proportion of peat used for growing media is 0.05%. In particular, in Europe, peat is used as a substrate in 90% of ornamental plant production [12]. Unfortunately, the rapid depletion of wetlands caused by the increasing use of peat in horticulture has resulted in the loss of a non-renewable resource that is essential for CO_2 sequestration [13]. These materials could be replaced by organic waste such as sewage sludge, pine or eucalyptus bark, sawmill residues, etc., leading to environmental benefits by preventing ecosystem damage from soil or peat extraction and reducing the impact of residue accumulation [14–17]. Therefore, searching for new potted plant and nursery substrates has become essential [18]. Some commercially available substrates include coconut fiber, *Sphagnum (Sphagnum* spp.), and composted pine.

Coconut coir substrate is an organic, naturally occurring substance derived from the outer husk, or mesocarp, of the coconut fruit that provides environmental benefits due to its renewable nature. *Sphagnum* is used in horticulture as a soil amendment and potting substrate because of its water-holding capacity, air-filled porosity, and ability to regulate pH [19]. It also promotes root growth and increases nutrient availability [20].

Composted pine is a common horticultural substrate used in horticulture and landscaping. It comprises aged and composted pine bark, wood chips, and other organic materials to create a natural and nutrient-rich soil amendment. It is perfect for sandy soils because it helps to retain water and nutrients and improves soil quality [21,22]. In this sense, it has been reported that composting is an effective way to eliminate bark components (isoprenes, monoterpenes, tannins, phenols, and manganese, among others) that affect plant growth [23,24]. *P. radiata* bark, in particular, has been widely used as a substrate in agriculture, including forestry, horticulture, and ornamental crop production. The results indicate that it can support plant growth and improve soil structure [25–28]. It should be noted, however, that some bark substrates are susceptible to N immobilization, which affects the availability of this essential element to plants [29]. Despite this, pine and eucalyptus barks have gained attention recently due to their great availability of organic matter content and water retention [10,15,21,30,31].

For example, a study by Mupondi et al. [32] evaluated pine bark as a substrate for vegetable production and found that it could effectively support plant growth. Another study by Chemetova et al. [15], using treated *Eucalyptus* sp. bark as a substrate for vegetable plant growth, found an improvement in substrate aeration properties. Furthermore, Chinese cabbage growth, when using this substrate, was equal to, or higher than, that of a commercial substrate. However, previous research has shown that both types of bark contain high levels of toxic phenolic compounds, triterpenes, and other components [33–35]. In addition, wood biomass is often associated with phytotoxicity due to the presence of natural chemical barriers [36]. These chemicals serve to protect native plants from disease and infection, but can also be harmful to other crops when used as a growing medium [37]. Therefore, it is necessary to treat the bark with water below 150 °C (low-temperature hydrothermal treatment), as proposed by Chemetova et al. [31], in order to reduce its toxicity, especially for *E. globulus* bark, making it a potential component for horticultural growing media.

Improving soil fertility requires fertilizers, which are key to agricultural productivity and food security. Slow-release or controlled-release fertilizer products effectively provide a controlled release of nutrients to the soil. Still, they have drawbacks such as high cost, complex preparation procedures, and a lack of biodegradability [38]. Chitosan is a natural copolymer of β-(1-4)-linked glucosamine and N-acetylglucosamine units. It is a biodegradable and non-toxic material with plant-growth-promoting activity and plant defense elicitor. It also has bactericidal and antifungal properties. Therefore, it is a good candidate to be used to improve substrate quality and plant development [38,39]. Humic substances are complex heterogeneous mixtures that are formed by chemical and biochemical reactions during the transformation of plant, animal, and microbial residues in a process called humification. They contain polysaccharides, proteins, lipids, nucleic acids, and fine carbon particles. The wide variety of polar and non-polar functional groups in humic substances allows the formation of a broad range of chemical compounds that promote plant growth and development [38]. Therefore, chitosan and humic substances are considered to be plant biostimulants or phytostimulants [40].

The microencapsulation of humic substances is a method for designing controlled-release phytostimulants. Several polysaccharides, within the range of potential encapsulating matrices, are biodegradable and safe for soils. In this category are starch, chitosan, pectin, mucilages, cellulose derivatives, and algal polysaccharides (e.g., agar, alginate, fucans, carrageenans, etc.) [41].

In addition, alginate is one of the most widely used matrices in the biotechnological industry to encapsulate active molecules by the ionic gelation method, which is rather simple and inexpensive [42]. The ionic gelation technique is based on the ability of polyelectrolytes to crosslink in the presence of counterions to form hydrogel beads. These beads are hydrophilic polymeric gels that become crosslinked and spherical upon contact with a counterion (Mg^{2+} or Ca^{2+}). Such microspheres can be loaded with pesticides, soil microorganisms, and fertilizers [43]. Once they are spread in soils, the beads release bioactive compounds in a controlled manner [44]. This technique provides protection and stability to the active ingredient, therefore, preventing leaching.

Thus far, the effect of mixing extracted pine and eucalyptus bark with other commercial substrates on plant growth, as proposed in this work, has not been reported. Due to the low cost and huge availability of those by-products, there is an opportunity for the nursery industry and horticulture sector to develop and use cheaper mixtures of plant growth substrates. The finding of novel components will allow them to partially replace imported raw materials (coconut fibers and peat), as well as costly sphagnum. As a result, competitive and sustainable horticulture production could be developed. This study evaluates the physicochemical properties and the phytotoxicity of water-treated *E. globulus* and *P. radiata* barks, both alone and in a mixture with other commercial plant substrates. For this purpose, the Munoo-Liisa vitality index (MLVI) phytotoxicity test was used. As a complementary test to determine the best substrate combination for use in agriculture, the germination of two horticultural species, named radish (*R. sativus*) and Chinese cabbage (*B. rapa*), on the substrates was evaluated and compared with a commercial growing medium (peat). Once it was selected, the more promising mixture, comprising chitosan or alginate encapsulated fulvic acid, was tested as a plant biostimulant using the same bioassays. The biological tests allowed us to identify the higher proportion of treated pine and eucalyptus bark that can be used safely in a mixture for cultivating horticultural species. Collectively, those results are expected to contribute to adding value to a Chilean forest by-product using a simple and environmentally friendly process.

2. Materials and Methods

2.1. Raw Material

Eucalyptus (*E. globulus*) and radiata pine (*P. radiata*) bark were supplied by Forestal Collicura (Santa Juana, Bio-Bio region, Chile) and Forestal Arauco (Arauco, Bio-Bio region, Chile). The eucalyptus bark was sieved, first to a 20 mm size, to separate sticks and wood chips from the bark, followed by grinding with a hammer mill (Breuer model M8, St. Vith, Belgium). The fiber obtained was sieved to a 4 mm size to remove dust and small rocks and was successively named "eucalyptus fiber bark". In the case of the pine bark, it was first sieved to particle sizes of between 6 and 20 mm. The pine bark and eucalyptus fiber bark were dried at 60 °C for 48 h before being treated with water. Commercial substrates purchased from a local retail store, such as coconut fiber, sphagnum moss, and composted pine and peat, commonly used for these purposes, were used for comparison in the germination tests (see Figure S1). Table S1 shows some chemical properties of the commercial reference substrates.

2.2. Experimental Design: Water Extraction Treatment

An experimental design was created using Design-Expert® 11 software (Minneapolis, MN, USA) for the water extraction tests of the pine bark and eucalyptus fiber bark with the temperature (range 20–120 °C), extraction time (range 20–120 min), and pine bark content (range 25%–75%) as input variables. The yield of solids in the extract was considered to be the response variable. Table 1 shows the experimental design, consisting of 19 treatments.

Table 1. Experimental design for the extraction of eucalyptus and pine barks.

Nomenclature	Input Variables			
	Time (min)	**Temperature (°C)**	**% Pine**	**% Eucalyptus**
T1	70	120	50	50
T2	20	70	50	50
T3	70	70	50	50
T4	70	70	50	50
T5	120	70	50	50
T6	20	20	75	25
T7	120	120	75	25
T8	120	20	75	25
T9	70	70	25	75
T10	120	20	25	75
T11	20	20	25	75
T12	120	120	25	75
T13	70	20	50	50
T14	20	120	25	75
T15	20	120	75	25
T16	70	70	50	50
T17	70	70	75	25
T18	70	70	100	0
T19	70	70	0	100

Table 1 shows the input variables for the different treatments (T1 to T19) considered in the study. The input variables were time, temperature, and percentage of pine and eucalyptus in the mixture.

The yield of solids in the extract (% Y) was determined by Equation (1) as follows:

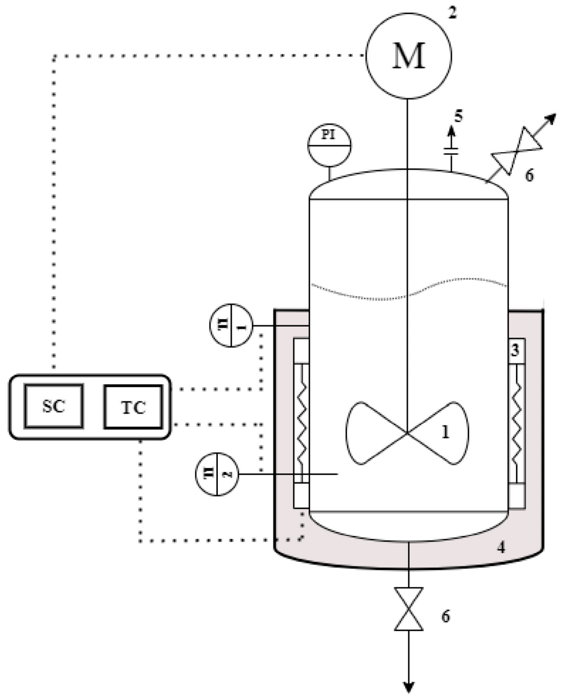
$$\mathrm{Y}(\%) = \frac{\text{mass of extracted liquor (g) \% solids in liquor(g)}}{\text{mass of dry feed (g)}} \tag{1}$$

2.3. Lab-Scale Extraction

Extraction was performed in a 0.75 L stainless steel reactor using 50 g of substrate mixture and 500 g of water (substrate/water ratio 1:10, *w*/*w*), and heating was performed on a hot plate. The airtightness of the system allowed temperatures of above 100 °C to be reached. In addition, this model included 100% pine and eucalyptus extraction at 70 °C for 70 min.

2.4. Pilot-Scale Extraction

The pilot-scale extraction was carried out under optimal conditions, as determined by the experimental design. A 25 L steel reactor was used for the extraction process. A schematic diagram of the reactor used for the pilot-scale extraction is shown in Figure 1. The reactor was heated with electrical resistance and equipped with a mechanical stirrer.



PI: pressure indicator, **TI**: temperature indicator, **TC**: temperature controller, **SC**: speed controller, **1**: Agitator, **2**: motor, **3**: electric heater, **4**: insulating material, **5**: rupture disc, **6**: valve.

Figure 1. Schematic diagram of the pilot-scale extraction reactor.

2.5. Physicochemical Characterization of Substrates

2.5.1. pH Determination

The pH of the samples was determined according to the standard method described in UNE-EN 13037 [45]. First, 60 mL of the material was mixed with 300 mL of water at 20 °C and stirred for 1 h. After sedimentation, the pH was measured using a PL-700PC pH meter (Gondo, Taipei, Taiwan).

2.5.2. Determination of Electrical Conductivity

The electrical conductivity was measured using the standard method described in UNE-EN 13038 [46]. The procedure followed was the same as that for the pH measurement, but the solution was filtered after stirring. The electrical conductivity was then measured with a PL-700PC pH meter.

2.5.3. Determination of Organic Matter and Ash

The organic matter and ash were determined according to the standard method described in UNE-EN 13039 [47]. First, a sample of 5 g of material was dried at 105 °C for 4 h in a previously calcined and weighed capsule. After drying, the capsule was allowed to cool in a desiccator before being weighed. The capsule containing the dried sample was then placed in a muffle and calcined at 450 °C for 6 h. After calcination, the capsule was allowed to cool in the desiccator before being weighed again. The moisture, organic matter, and ash contents were then calculated using Test Methods for the Examination of Composting and Compost (TMECC) 05.07 and 04.02, respectively.

2.5.4. Determination of Apparent Density

The apparent density was determined according to the standard method described in TMECC Standard 03.03 [48]. First, the weight of an empty 2000 mL beaker was recorded. An aliquot of the sample was then transferred to fill 600 mL of a 2000 mL beaker. This process was repeated twice, each time dropping the sample freely from a height of 15 cm until the beaker was filled to a volume of 1800 mL (the third time, the sample was not dropped freely from 15 cm).

2.5.5. Determination of $N\text{-}NO_3$, $N\text{-}NH_4$, and Chemical Elements

The quantification of $N\text{-}NO_3$, $N\text{-}NH_4$, P_2O_5, K_2O, CaO, MgO, and Na was performed according to the standard methodology of the TMECC [49–51]. The nitrate ion method (TMECC 04.02-B) determined the nitrate in the samples based on UV absorption at 220 nm and 275 nm. The determination of ammonium nitrogen in the compost beds was carried out using the colorimetric phenol hypochlorite composting method described in the standard methods [49]. This method involves reacting NH_3 with HClO and phenol to produce a strong blue compound (indophenol), measured spectrophotometrically at 635 nm.

2.5.6. SEM

Both before and after treatment, the morphological properties and surface characteristics of the pine bark and eucalyptus fiber bark were determined by scanning electron microscopy (SEM) using a JEOL JSM-6380 microscope (Tokyo, Japan). The microscope was operated at an accelerating voltage of 20 kV. The samples were coated with an approximately 150 Å thickness gold layer using an Edwards S 150 sputter coater (Agar Scientific, Standsted, UK) [52].

2.6. Phytostimulant Encapsulation

This study evaluated polysaccharide-based encapsulating matrices for their ability to encapsulate and gradually release fertilizers. Alginate was used as the encapsulating matrix using the ionic gelation method. Commercial fulvic acid was used as the phytostimulant, and sodium alginate (Sigma-Aldrich, reagent grade, St. Louis, MO, USA) was used as coating. First, a 2% (w/v) fulvic acid solution was prepared in milli-Q water with constant stirring until it was uniformly dissolved. Then, a 1.75% w/v sodium alginate solution was slowly added to the fulvic acid solution while stirring for 15 min at 40 °C to improve alginate solubility. The solution was then homogenized using an Ultra Turrax IKA® T25 digital (Staufen, Germany) at 10,000 rpm twice for 1 min (with a 1 min pause) and placed in a syringe. Next, this solution was dropped into a beaker containing an 8% $CaCl_2$ (w/v) solution at room temperature and stirred continuously at 200 rpm, waiting for the drops to gel as they fell into the beaker. Once the alginate–fulvic acid microspheres were formed, they were immediately filtered and washed with distilled water. Finally, the gel spheres were kept dry to prevent the fulvic acid from diffusing out of the coating. The system used for the ionic gelation of fulvic acid in alginate is shown in Figure S2.

Chitosan Dissolution

Crab chitosan (degree of deacetylation 86%, Mw 65 kDa) was previously obtained in our laboratory [39]. A biopolymer solution (1 wt%) was prepared by dissolving an appropriate amount (1 g) in acetic acid 1% (v/v), and stirred at 60 °C, until a complete homogenous solution was obtained. The pH of the solution was adjusted to 5 with NaOH (1 M) before use. From this solution, different concentrations of chitosan were prepared (0.05, 0.1, and 0.5 wt%). Each treatment was added to the corresponding substrate in an equivalent amount (dry weight base) of fulvic acid encapsulated in alginate.

2.7. Growth Evaluations

2.7.1. Phytotoxicity Test

Following the Spanish standard UNE-EN 16086-2 [53], a total of 3 radish (*R. sativus*) seeds were incubated in each plastic pot filled with a substrate (60 cm^3) at room temperature (25 °C) in the dark for 6 days. The experiment was performed in triplicate (n = 3), with a commercial substrate (peat) as the control (C). The phytotoxicity was evaluated by the Munoo-Liisa vitality index (MLVI; Equation (2)) using germination rate (GR; where GR 1–3 are triplicates and GRC is the control) and root length (RL; where RL 1–3 are triplicates and RL C is the control).

$$\text{Munoo} - \text{Liisa Vitality Index}(\%) = \frac{(\text{GR}_1 \cdot \text{RL}_1 + \text{GR}_2 \cdot \text{RL}_2 + \text{GR}_3 \cdot \text{RL}_3)}{3 \cdot \text{GR}_\text{c} \cdot \text{RL}_\text{c}} \cdot 100 \quad (2)$$

2.7.2. Growth Test

UNE-EN 16086-1, a Spanish standard related to soil improvers and growth substrates that determine plant response [54], was used to evaluate the growth test. Three seeds of Chinese cabbage (*B. rapa* subsp. pekinensis) were planted in each container (150 cm^3). Each 60 mL container was filled with 50% leaf soil and 50% of a mixture of substrate and peat (the proportions of the substrate-to-peat by volume were as follows: 10:90, 25:75, 50:50, and 25:75). In addition to 100% peat, different substrates, such as leaf soil, eucalyptus fiber bark, and pine bark, were investigated as controls. The experiment lasted for 14 days at 23 °C, with distilled water used for irrigation every other day.

2.8. Statistical Analysis

The mean and standard deviation of at least three values were used to report all data. Statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, version 8.0, San Diego, CA, USA). A two-way analysis of variance was used to analyze the data, and Tukey's and Holm–Sidak multiple comparison tests were used to determine the mean differences. The *p*-values of less than 0.05 were considered significant.

3. Results and Discussion

3.1. Characterisation of Raw Material

3.1.1. Chemical Properties of Substrates

Table 2 shows the chemical properties of the following four substrates: raw eucalyptus fiber bark, extracted eucalyptus fiber bark, raw pine bark, and extracted pine bark. These properties are important in understanding the characteristics and the suitability of each substrate for use as agricultural substrates or horticultural growing media.

One notable difference between the substrates is their pH levels. For example, eucalyptus fiber bark and pine bark have a lower pH level than their extracted samples, which is possibly due to the extraction of acid salts and water-soluble organic acidic compounds [30,55,56]. The lower pH levels of the extracted substrates make them more suitable for specific agricultural or horticultural applications where a lower pH is desired [57]. For example, plants such as tomatoes, peppers, and lettuce prefer a slightly acidic soil pH, and using a substrate with a lower pH may benefit these plants [58].

Another relevant difference between the substrates is their electrical conductivity. The extracted samples had the lowest electrical conductivity, indicating that the treatment processes promote a lower ionic content than the other substrates, which may be relevant for specific agricultural or horticultural applications. For example, substrates with high electrical conductivity, such as those with high available N, P, and K, may be more suitable for growing plants with high nutrient requirements, such as fruiting vegetables. In contrast, substrates with lower electrical conductivity, such as those with low available N, P, and K, may be more appropriate for plants with lower nutrient requirements, such as ornamental plants [59,60].

Table 2. Chemical properties of the substrates used in the experimental design.

Properties	Raw Material			
	Eucalyptus Fiber Bark		Pine Bark	
	Raw	Extracted *	Raw	Extracted *
pH	5.5	6.0	4.2	5.1
Electrical conductivity (µS/cm)	316	124	164	35
Organic matter (%)	93.71	94.52	98.33	98.53
Organic carbon (%)	52.80	53.30	54.40	54.70
Total nitrogen (%)	0.43	0.22	0.28	0.32
C/N ratio	123	242	194	171
Humidity (%)	11.07	6.60	8.26	7.39
Ash (%)	6.29	5.47	1.67	1.47
N-NH_4 (mg/Kg)	574	119	126	147
N-NO_3 (mg/Kg)	133	112	133	126
NH_4/NO_3 ratio	4.3	1.1	0.95	1.2
P_2O_5 (%)	0.18	0.09	0.09	0.09
K_2O (%)	0.38	0.12	0.12	0.07
CaO (%)	1.41	1.42	0.39	0.38
MgO (%)	0.32	0.22	0.08	0.07
Na (%)	0.32	0.12	0.14	0.31

* The table shows the pH, electrical conductivity, organic matter, organic carbon, total nitrogen, C/N ratio, moisture, ash, N-NH_4, N-NO_3, NH_4/NO_3 ratio, P_2O_5, K_2O, CaO, and MgO values of raw and extracted eucalyptus fiber bark and pine bark. Treatment T18 for pine and T19 for eucalyptus fiber bark were extracted at 70 °C for 70 min.

The organic matter (OM) and carbon contents of the substrates are also noteworthy. All of the substrates have a high organic matter and carbon content, and no relevant differences in these parameters were observed after treatment. The OM content would make these substrates particularly suitable for agricultural or horticultural applications that require a high OM or carbon content. A substrate with a high OM content can improve the soil structure, water-holding capacity, and nutrient availability, which can be essential for plant growth [61,62].

The total nitrogen content of all of the substrates is relatively low. Contrary to the pine bark, for the eucalyptus fiber bark, the extraction process affected the N content. Approximately 50% of the nitrogen in the eucalyptus fiber bark was removed by the heat treatment. The C/N ratio is also relatively high, indicating that the substrates contain more carbon than nitrogen. However, the quality of the carbon source, such as the proportion of lignin and cellulose, may also affect nutrient immobilization. This ratio could be relevant for specific agricultural or horticultural applications where a high C/N ratio is desired [63]. In addition, a high C/N ratio can inhibit the decomposition of organic matter, which can be beneficial for maintaining the soil structure and nutrient availability in a substrate that is used for long-term cultivation [64]. Compared to coconut fiber (C/N = 112), which is commonly used as a growth substrate, the samples that have been analyzed in this work have slightly higher C/N values. It has been reported that a high carbon (C) to nitrogen (N) ratio can affect the mobility of nutrients in plants [65].

The humidity of the eucalyptus fiber bark varies, especially after the treatment, which decreases the humidity of the substrate. The treatment of pine bark did not modify the moisture content. The ash content is relatively low for both the extracted and the non-extracted pine bark. These properties may be relevant for understanding the physical characteristics and handling of the substrates, since a lower humidity level may make a substrate easier to handle and store. At the same time, the ash content influences the electrical conductivity of the substrates [66].

The nitrogen content of the substrates in the form of NH_4 and NO_3 should also be considered. The NH_4/NO_3 ratio that was obtained for the eucalyptus fiber bark was the highest (4.3), but this value decreased after the treatment. For the pine bark substrates, this ratio was slightly changed with the treatment. The presence of these nitrogen species may be relevant for understanding the nitrogen availability of the substrates for specific agricultural or horticultural applications [67,68]. In addition, the availability of NH_4 and NO_3 can affect plant growth and development, with NH_4 being more readily available to plants than NO_3 [69,70].

Finally, the table shows the content of various macronutrients and micronutrients in the substrates, including P_2O_5, K_2O, CaO, MgO, and Na. These compounds may be relevant for understanding the nutrient content and their availability in the substrates for specific agricultural or horticultural applications [71]. It can be observed that the total nutrient content is lower than 3 wt% for *E. globulus*, and even lower for *P. radiata* (<1 wt%).

3.1.2. Surface Morphology: SEM Analysis

The results of the SEM analysis of the pine bark and the eucalyptus fiber bark before and after treatment are shown in Figure 2. The pine bark appears fibrous with a rough surface both before and after the water treatment and became slightly porous on the surface after the treatment (Figure 2A,B). The raw eucalyptus fiber bark also shows a rough fibrous surface, however, after the treatment, its surface became highly porous, and broken cells can be observed [72–74]. These results demonstrate the effectiveness of the treatment in altering the surface properties and morphology of both of the barks [75].

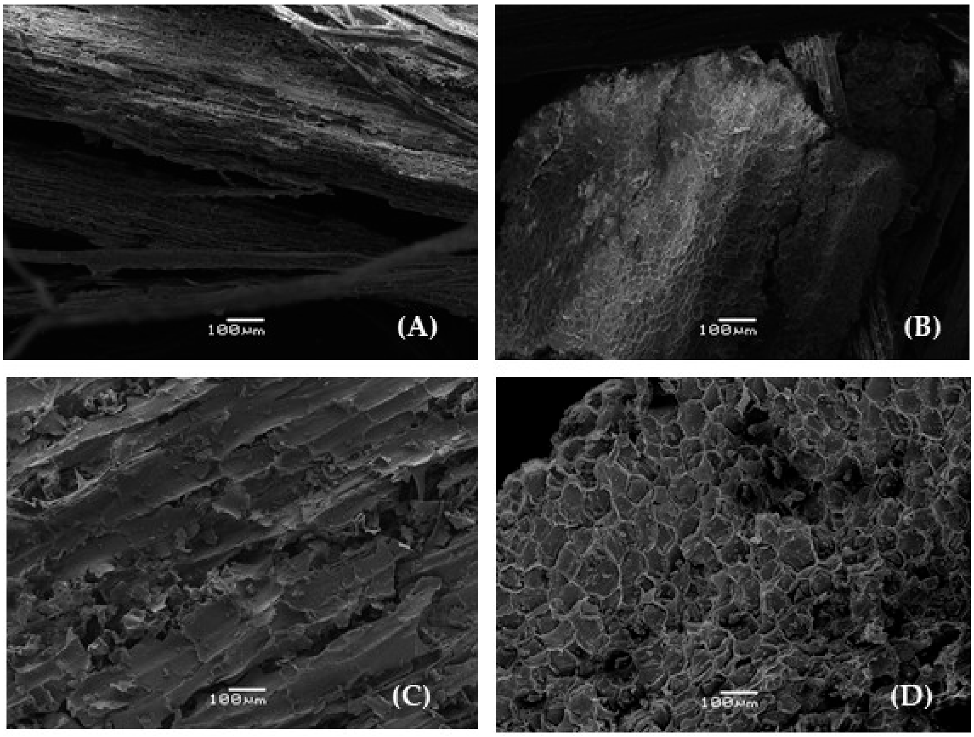


Figure 2. SEM images of raw and extracted pine bark and eucalyptus fiber bark. (**A**) Raw pine bark, (**B**) extracted pine bark, (**C**) raw eucalyptus fiber bark, and (**D**) extracted eucalyptus fiber bark.

3.1.3. Physical Properties of Substrates

Table 3 shows the physical properties of the following four substrates: raw eucalyptus fiber bark, extracted eucalyptus fiber bark, raw pine bark, and extracted pine bark. These properties are essential for understanding the characteristics and suitability of each substrate for a particular application.

One notable difference between the substrates is their particle size distribution. For example, whether extracted or not, the eucalyptus fiber bark has a particle size distribution concentrated of more than 50% in the <2 mm (37%) and 2.0–4.0 mm (28%) size range, respectively. On the other hand, the pine bark and the extracted pine bark have a coarser particle size distribution, with a higher percentage of particles (68%–77%) in the

8.0–16.0 mm size range. This difference in particle size may be relevant to understanding the physical properties and handling of the substrates, particularly for aeration and water-retention capacity [76]. In fact, several studies have shown that particles that are smaller than 1 mm can have a significant effect on the physical properties of substrates such as bark, peat, and coconut fiber [77].

Table 3. Physical properties of the substrates used in the experimental design.

Properties		Raw Material			
		Eucalyptus Fiber Bark		Pine Bark	
		Raw	**Extracted ***	**Raw**	**Extracted ***
Particle size (%)	>16 mm	0	0	0	0
	8.0 mm–16.0 mm	15	15	68	77
	4.0 mm–8.0 mm	20	19	27	20
	2.0 mm–4.0 mm	28	29	4	2
	<2 mm	37	37	1	1
Bulk density (g/mL)		0.030	0.028	0.227	0.203
Pore space (%)		98	97	49	52
Free air space (%)		39	32	43	49
Water-retention capacity (% v/v)		59	65	6	3

* Treatment T18 for pine and T19 for eucalyptus fiber bark (extracted at 70 °C, 70 min).

Another critical difference is the bulk density of the substrates. Eucalyptus and extracted eucalyptus fiber bark have a lower bulk density (~0.03 g/mL) than pine bark and extracted pine bark (~0.2 g/mL). The pore space and free air space of the substrates are also noteworthy. Eucalyptus and extracted eucalyptus fiber have a higher pore space and a lower free air space than pine bark substrate counterparts. These results agree with the previous SEM observations (Figure 2). Eucalyptus fiber (extracted and non-extracted) has the highest water-holding capacity, which may be related to its fiber morphology. This fact may be relevant in understanding the substrates' water-holding capacity and suitability for specific applications. Bark and wood-based materials increase the growing media components' aeration, porosity, and drainage capacity [30,31,78]. The fibers reduce shrinkage when they are combined with peat-based substrates by improving the re-wettability and water circulation [79]. In terms of bulk density, the prepared substrates also meet the requirements of the Chilean Standard 2880 (<0.7 g/mL) [80]. Overall, the table provides valuable information on the physical properties of the four different substrates that have been used in the study. This information can help us to understand the characteristics and suitability of each substrate for a particular application.

3.2. Plant Response to Different Substrates

3.2.1. Phytotoxicity of Eucalyptus and Pine Mixtures in Pot Trials with Radish

The Munoo-Liisa vitality index (MLVI) for radish grown in the substrates that were prepared according to the experimental design (Section 2) is shown in Figure 3. In addition, the treatments using pine and eucalyptus fiber bark (extracted and non-extracted) and their respective mixtures are included. The commercial substrate that was used as a control was peat (T0) because it is the most commonly used organic substrate [30]. It is worth noting that MLVI takes into account the seedling RL (root elongation) and GR (germination rate) regarding the peat control. Thus, the higher MLVI values obtained account for lower substrate toxicity.

A 100% GR was obtained for the evaluated samples, except for two treatments: (1) T3 and (2) T18. It was observed that the highest MLVI was reported for the substrates that were extracted at T $\geq$ 70 °C and the lowest pine content (25%). Concerning the substrates with 100% pine and eucalyptus, the highest MLVI was registered for the 100% extracted eucalyptus (108%). However, it is considered that this value is not significantly different ($p < 0.05$) to the control peat (T0). Nevertheless, this result is considered to be promising because of the possibility of using a forest by-product as a total or partial substitute for peat. The 100% non-extracted pine and the mixture of extracted pine–eucalyptus samples prepared with a pine content that was higher than 50% reported a lower MLVI, which would be related to phytotoxic compounds from the pine bark [33–36].

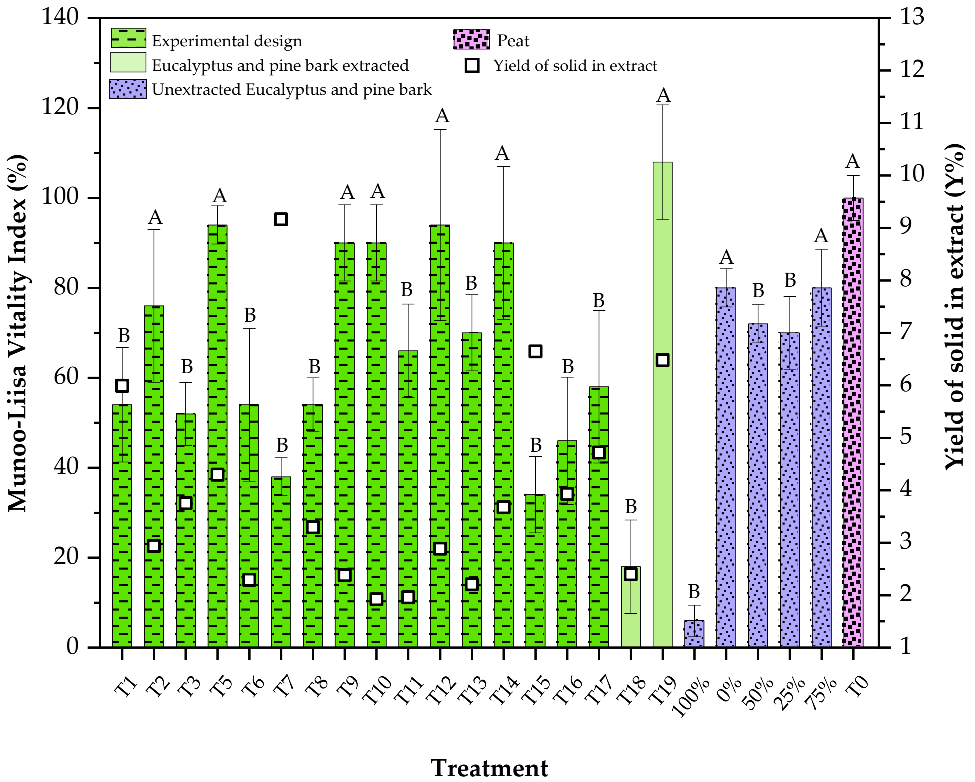


Figure 3. Munoo-Liisa index and yield of solids in extract (Y%) for radish growth in substrates prepared from the experimental design (see Table 1). The capital letters indicate whether there is a significant difference compared to peat (P), using ordinary one-way ANOVA multiple comparisons and Tukey's alpha = 0.05).

The results obtained agree with those that were reported by Chemetova et al. [30], who indicated that the hydrothermal treatment of eucalyptus bark at temperatures between 60 and 100 °C effectively removes the phytotoxic compounds from it, improving the germination percentage and root length when sowing cress seeds.

In order to better understand the effect of the extraction process on bark phytotoxicity, the solid content obtained in the liquid phase of the extracts was also quantified. Figure 3 also shows the result of the solid percentages (indicated as a square) for each treatment of the experimental design. It would be expected that the higher the solid content in the liquids phase, the more phytotoxic compounds would be removed. Thus, higher MLVI values of bark substrates would be observed, as well as seed germination. However, our results show that a higher solid yield in the extract does not correlate with the MLVI values. Maybe a deeper characterization of extracted solids composed of both organic and inorganic compounds would provide more clues on this matter. This result suggests that there would be a complex balance between phytotoxic compound extraction and substrate physicochemical properties (pH, water-holding capacity, electrical conductivity, etc.), that determines the seed germination and plant root growth improvement [81].

3.2.2. Phytotoxicity of Eucalyptus and Substrate Mixtures in *R. sativus* and *B. napa*

The previous results show that the best substrate is 100% extracted eucalyptus fiber bark, hence we decided to not use mixtures of pine and eucalyptus substrates in subsequent studies. Therefore, we decided to prepare a higher quantity of extracted eucalyptus fiber bark substrate to be used in forthcoming tests. For this purpose, the bark was treated in a 25 L reactor at 70 °C for 70 min in order to scale up the substrate production process (see Section 2.4).

Considering the previous results of the bark physicochemical properties (Tables 2 and 3), and those related to the MLVI reported in Figure 3, we decided to evaluate the behavior of the extracted eucalyptus fiber bark substrate in a mixture with commercial substrates. This proposal was based on the possibility that a highly available and cheap forest by-product may replace some commercial substrates. The search foralternative high-quality and low-cost growing media materials for horticulture is necessary due to the increasing demand and cost of peat and its uncertain availability due to environmental restrictions [11]. Therefore, radish and Chinese cabbage growth trials were conducted on extracted eucalyptus fiber bark substrates that were mixed with coconut fiber, sphagnum, peat, and composted pine at different ratios (25, 50, and 75% *v*/*v*). The germination rate results for both species are presented in Table 4.

Table 4. The germination rate for radish and Chinese cabbage growth in substrates prepared from extracted (EEB) and non-extracted (NEB) eucalyptus with coconut fiber (C), moss (M), peat (P), and composted pine (CP) mixtures.

Sample	Germination Rate, GR (%)			
	Radish		Chinese Cabbage	
	NEB	EEB	NEB	EEB
E			100	
75E-25C			100	
50E-50C			100	
25E-75C			100	
75E-25M			33	
50E-50M			33	
25E-75M			0	
75E-25P			67	
50E-50P	100	100	100	100
25E-75P			67	
75E-25CP			33	
50E-50CP			33	
25E-75CP			67	
C			100	
M			67	
P			100	
CP			67	

The germination rate of radish and Chinese cabbage in substrates prepared from extracted (EEB) and non-extracted (NEB) eucalyptus with coconut fiber (C), moss (M), peat (P), and composted pine (CP) mixtures. “E” refers to eucalyptus only. Germination rates are expressed in percentage (%).

The eucalyptus substrate, whether extracted or not, did not affect the germination rate of radish, which was 100% for both substrates, regardless of the mixture used or the commercial substrate. Nevertheless, this behavior was different for Chinese cabbage, which showed a different germination rate depending on whether the eucalyptus fiber bark was extracted or not, as well as on the mixture with a commercial substrate. With extracted eucalyptus, Chinese cabbage germination was 100% in all of the mixtures that were evaluated. However, using non-extracted eucalyptus significantly affected the germination of this species, especially in mixtures with moss (M), peat (P), and composted pine (CP). The germination results suggest that Chinese cabbage is more likely to be affected by the phytotoxins in non-extracted eucalyptus fiber bark than radish. Similar results have been obtained by other authors using the same plant model and heat treatment of *E. globulus* bark, showing a lower germination rate (95%) with *E. globulus* bark mixtures than with treated and commercial substrate (peat) (98%–100%) [30].

Figure 4 shows the MLVI value for radish in substrates prepared with the extracted and non-extracted eucalyptus mixtures and commercial substrates. As previously discussed, the raw eucalyptus fiber bark can negatively affect the MLVI values. An increase in MLVI values with the incorporation of extracted eucalyptus fiber bark into commercial substrates can be noticed. The mixture of extracted eucalyptus and composted pine (75E-25CP) registered the highest MLVI values (170%) compared to its pure counterpart (82%) and the remaining commercial substrates. The same trend was observed for the other mixtures, although to a lesser extent. This substrate synergic effect on enhancing plant growth is very important for farmers and the nursery industry worldwide. It also justifies the search for an optimal substrate combination for the potential development of a germination and plant growth device.

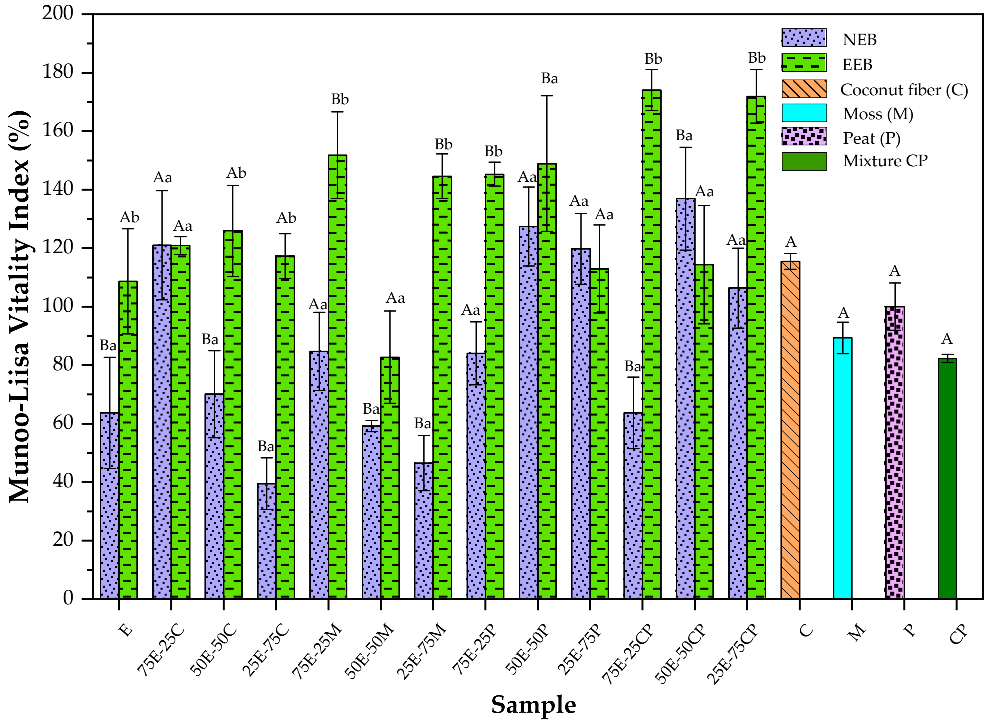


Figure 4. Munoo-Liisa index for radish growth in substrates prepared from extracted (EEB) and non-extracted (NEB) eucalyptus with mixtures of coconut fiber (C), moss (M), peat (P), and composted pine (CP). The capital letters indicate a significant difference compared to peat (P), and the lowercase letters indicate a significant difference between the extracted and non-extracted samples (two-way ANOVA Holm–Sidak's multiple comparisons test, with alpha = 0.05).

To the best of our knowledge, there are few studies related to mixtures of commercial substrates and extracted eucalyptus fiber bark that have reported a synergic phenomenon. In a recent study, the authors tested mixtures of extracted eucalyptus at different temperatures (60–100 °C) with peat in a 25/50% (v/v) ratio, respectively [30]. The results showed that the MLVI values decreased when 50% of the extracted eucalyptus fiber bark was used. These authors concluded that the mixture of 25% extracted eucalyptus and 75% peat is optimal for obtaining a substrate with excellent aeration properties and maintaining an adequate water content.

Our results indicate the possibility to expand the use range of extracted eucalyptus fiber bark as a substrate (75%), containing a smaller proportion of peat or other commercial reference substrates (25%). This result is promising as it reduces the use of peat, which is no longer considered to be a renewable resource due to its long regeneration times [12]. Under these mixed conditions, the aforementioned synergic behavior was observed, where aeration, porosity, and drainage capacity would be improved together with the presence of peat. Other work has indicated that eucalyptus bark could improve aeration when it is added to commercial peat-based substrates after hydrothermal treatments [31]. Regarding synergy, research has been carried out using mathematical approaches in search of models to predict this phenomenon in mixtures of regulating or promoting chemical substances for plant growth [82]. From the results that were obtained for radish, it can be concluded that 25% of the commercial substrate would be sufficient to achieve a significant improvement in MLVI.

Figure 5 shows the MLVI values for Chinese cabbage in different eucalyptus-based substrates and their mixtures with commercial substrates. For this species, it was observed that the 25E-75C, 25E-75M, and 50E-50P mixtures have a similar behavior to that of peat. In this sense, other researchers have conducted tests on the growth of Chinese cabbage and have concluded that peat helps to lower pH and increase the water-holding capacity, organic matter content, total nitrogen, and available nitrogen and phosphorus, which improves the growth of this species [83,84].

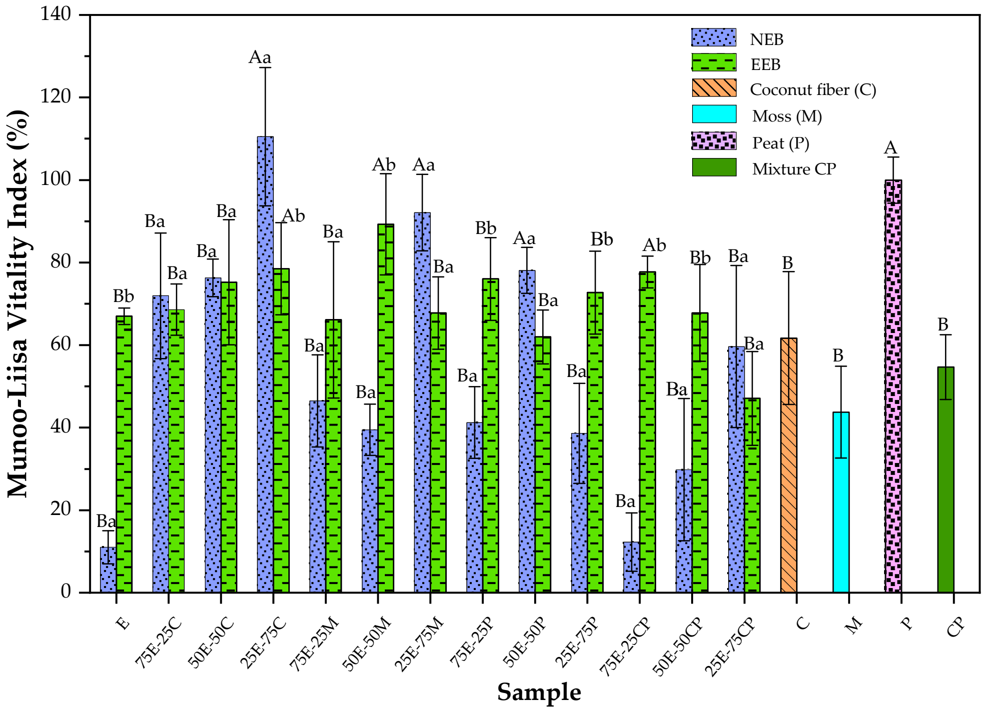


Figure 5. Munoo-Liisa index for Chinese cabbage in substrates prepared from extracted (EEB) and non-extracted (NEB) eucalyptus with mixtures of coconut fiber (C), moss (M), peat (P), and composted pine (CP). The capital letters indicate a significant difference compared to peat (P), and the lowercase letters indicate a significant difference between the extracted and non-extracted samples (two-way ANOVA Holm–Sidak's multiple comparisons test, with alpha = 0.05).

3.2.3. Effect of Phytostimulant on *R. sativus* and *B. rapa* Subsp. Pekinensis

The germination results showed that the optimal mixture for seed growth was 75% extracted eucalyptus fiber bark and 25% peat (sample 75E-25P). Therefore, germination tests with a phytostimulant were carried out with this mixture. In addition, other commercial substrates were mixed with extracted eucalyptus fiber bark and were included for comparison. Seed germination is the first step and the most sensitive period in the life cycle of plants [85]. It has been reported that using phytostimulants enhances the growth and development of plants and improves their metabolism, which is vital in adverse conditions (abiotic stress) such as drought or eroded site conditions that reduce their germination [86–88].

The availability and adsorption of phytostimulants can be improved through nanotechnology, specifically micro/nano encapsulation. This technique can be defined as a technology that allows the preparation of individualized micro- and nanoparticles composed of a coating material containing a central active ingredient. It can protect the encapsulated material and control its release or facilitate the use of liquid products. There are three types of encapsulation processes: (i) physical processes using mechanical techniques (e.g., pulverization and extrusion), (ii) physicochemical processes based on the regulation and control of factors such as pH, temperature, solubility, and precipitation of polymers, as well as the control of state changes in oligomers, and (iii) chemical processes based on the in situ formulation of the coating material by polycondensation, radical polymerization, or anionic polymerization [89].

This research uses fulvic acid (2% w/v) as a phytostimulant encapsulated in an alginate matrix (1.75% w/v). Table 5 shows the radish and Chinese cabbage germination rate in the mixture of extracted eucalyptus fiber bark substrates with coconut fiber, moss, peat, and composted pine. Only the radish in the composted eucalyptus–pine mixture showed 100% GR for all of the stimulants used. For the other mixtures, the germination rate ranged from 33% to 100%. The use of chitosan did not promote germination, especially for Chinese cabbage seeds.

Table 5. Germination rate for radish and Chinese cabbage seeds when applying microencapsulated fulvic acid (FA) and chitosan (CS) to substrates prepared from extracted eucalyptus (EEB) mixed with coconut fiber (C), moss (M), peat (P), and composted pine (CP) at a 75/25 ratio, respectively.

Sample	Germination Rate (%)							
	75E-25C		75E-25M		75E-25P		75E-25CP	
	Radish	Chinese Cabbage	Radish	Chinese Cabbage	Radish	Chinese Cabbage	Radish	Chinese Cabbage
Water	100	67	100	100	100	100	100	100
CS_1	67	0	0	0	67	0		100
CS_2	33	0	0	0	33	0		67
CS_3	67	33	0	0	33	0		100
FA_{1ue}	100	100	100	67	100	100		33
FA_{2ue}	100	100	100	100	100	100		100
FA_{3ue}	100	33	67	67	100	100		100
FA_{1e}	100	100	100	33	100	100		67
FA_{2e}	100	100	100	100	100	100		100
FA_{3e}	100	67	100	100	100	100		33

$CS_{1–3}$ represent different concentrations of chitosan application, while $FA_{1ue–3e}$ represent different microencapsulated fulvic acid applications.

Figures 6 and 7 show the MLVI values for radish and Chinese cabbage growth using phytostimulants. In these tests, water was used as a control, and commercial biostimulant, chitosan (CS), and unencapsulated fulvic acid were also included. Chitosan was included because it is used in agriculture as a plant root growth enhancer [90,91]. Fulvic acid is also widely used in agriculture because it stimulates root development and plant metabolism and increases plant resistance to abiotic stress. In addition, fulvic acid acts as a natural chelator, mobilizing nutrients in the soil and improving their availability and uptake by the plant [92]. Indeed, the use of fulvic acid favored growth, although no significant difference was observed whether it was encapsulated or not. The short growth period that was set for the experiment was probably not long enough to maintain a considerable effect.

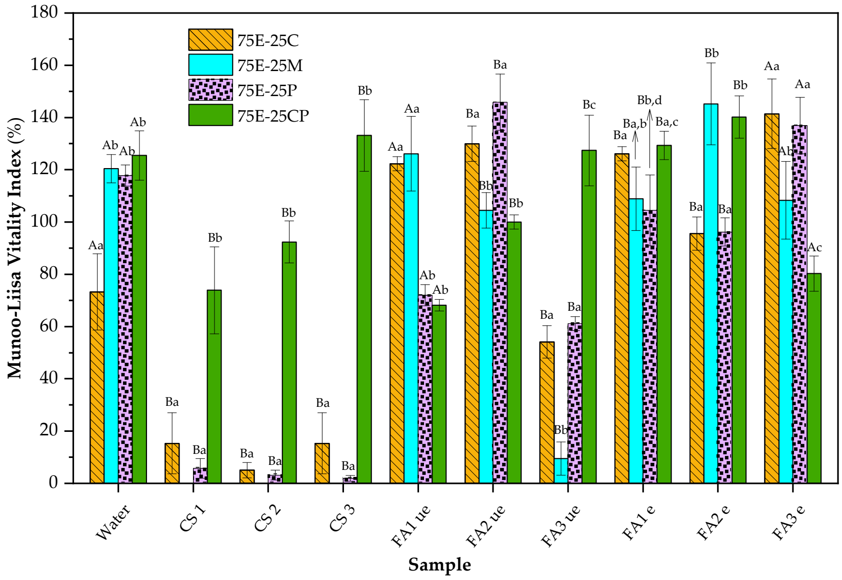


Figure 6. Munoo-Liisa index for radish growth in substrates prepared from extracted (EEB) eucalyptus fiber bark with coconut fiber (C), moss (M), peat (P), and composted pine (CP) mixtures, using phytostimulants (CS: chitosan; FA_{ue}: unencapsulated fulvic acid; FA_e: encapsulated fulvic acid. The numbers 1, 2, and 3 correspond to the concentration of the phytostimulant: 1 = 0.05% *w*/*v*; 2 = 0.1% *w*/*v*; 3 = 0.5% *w*/*v*). The capital letters indicate a significant difference between treatments compared to water (two-way ANOVA Holm–Sidak's multiple comparisons tests, with alpha = 0.05). The owercase letters indicate a significant difference between samples under the same treatment (two-way ANOVA Tukey's multiple comparisons tests, with alpha = 0.05).

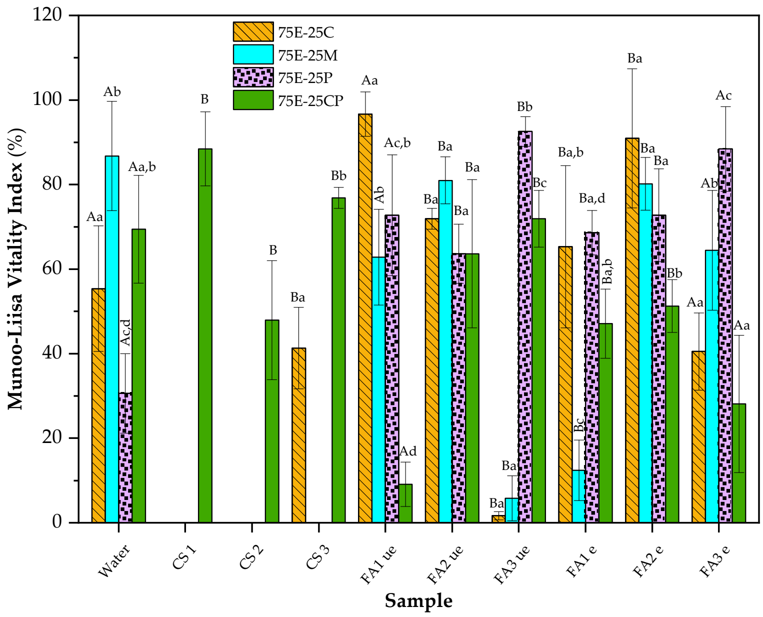


Figure 7. Munoo-Liisa Index for Chinese cabbage in substrates prepared from extracted eucalyptus fiber bark (EEB) with coconut fiber (C), moss (M), peat (P), and composted pine (CP) mixtures using phytostimulants (CS: chitosan; FA_{ue}: unencapsulated fulvic acid; FA_e: encapsulated fulvic acid. The numbers 1, 2, and 3 correspond to the concentration of the phytostimulant: 1 = 0.05% *w*/*v*; 2 = 0.1% *w*/*v*; 3 = 0.5% *w*/*v*). The capital letters indicate a significant difference between treatments compared to water (two-way ANOVA Holm–Sidak's multiple comparisons tests, with alpha = 0.05). The lowercase letters indicate a significant difference between samples under the same treatment (two-way ANOVA Tukey's multiple comparisons tests, alpha = 0.05).

The concentration of the phytostimulants used varied between 0.05% and 0.5%, indicating that an optimal concentration would be between 0.05% and 0.1%. In the case of the encapsulated phytostimulants, this optimum concentration is more evident for the mixtures of extracted eucalyptus fiber bark with moss (75E-25M) and composted pine (75E-25CP). Bülent et al. [93] investigated the effect of humic substances on wheat plant growth and mineral nutrient uptake. They concluded that 0.1% humic acid is the optimal concentration to promote growth and nutrient use. Plants consume more minerals when their root systems are better developed [94]. It has been found that the surface activity of humic substances results from the presence of both hydrophilic and hydrophobic sites, allowing these substances to interact with the phospholipid structures of cell membranes and act as a vehicle for nutrients through them [93]. A higher concentration of fulvic acid does not necessarily promote more significant plant growth. According to the investigations of Türkmen et al. [95], high concentrations of humic acid can either limit or reduce plant development and nutrient levels. This result was most clearly observed for the 75E-25C, 75E-25M, and 75E-25P mixtures when the phytostimulant was not encapsulated. The advantage of encapsulated fulvic acid is that the diffusion of the phytostimulant is gradual, and less aggressive degradation is not observed in the MLVI. On the other hand, the best performing substrate for the chitosan phytostimulant at all of the concentrations was the 75E-25CP combination, which increased the MLVI value.

The use of chitosan did not have the expected result of enhanced germination, except for the substrates that were mixed with composted pine (CP). A possible hypothesis for this result could be explained by the pH of the composted pine substrate and its mixture with extracted eucalyptus, which is slightly basic (pH = 7.7) compared to the other substrate mixtures (75E-25C = 6.5; 75E-25M = 6.1; 75E-25T = 6.9). In addition, the chitosan solution's pH was adjusted with an acetic acid solution until it reached a pH close to five. Therefore, it is likely that the combination of the chitosan solution with a more basic substrate created an optimal pH environment for the growth of radish and Chinese cabbage. Chitosan is a biopolymer that improves plant protection against various biotic and abiotic stressors. It can help to reduce the effects of stress by reducing the water content in cells, increasing the root length, reducing the transpiration rate, and improving plant growth. It can also act as a physical barrier against pathogens, increase the thickness of cell walls in the leaf epidermis, and can be used as a soil amendment. In addition, it can increase the germination rate, seedling growth, and shelf life [96]. For example, other studies have shown that Chinese cabbage plants that were treated with a chitin-based product grew faster than plants treated with a standard mineral fertilizer [97]. On the other hand, for the fulvic acid encapsulated phytostimulant, the optimum concentration was 0.1%, which is the same as that found for radish.

The MLVI and germination results showed that the mixture of the extracted eucalyptus fiber bark with commercial substrates such as peat or coconut fiber, using a more significant proportion of the extracted fiber (75%), has the potential to be used as a device for the growth of horticultural species.

4. Conclusions

In conclusion, our experiments have shown that treating eucalyptus (*E. globulus*) fiber bark with water at 70 °C for 70 min produces a suitable and sustainable substrate for the germination of horticultural crops such as radish (*R. sativus*) and Chinese cabbage (*B. rapa*). According to the MLVI and germination results, combining eucalyptus substrate with other commercial substrates has a positive effect on plant growth and development. Therefore, we recommend using a mix of 75% eucalyptus and 25% commercial substrates such as peat, coconut fiber, moss, and composted pine. In addition, encapsulated fulvic acid at a concentration of 0.1% was observed to enhance plant growth, while chitosan at all concentrations studied was effective only for the 75E-25CP mixture. Overall, our results support treating eucalyptus fiber bark as a substrate for plant propagation, providing an environmentally friendly and sustainable alternative to traditional substrates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14050991/s1, Figure S1: Pine bark (1) and eucalyptus fiber bark (2) used in the extraction tests. Reference samples: coconut fiber (3), sphagnum moss (4), peat (5), and composted pine (6); Figure S2: (A) Fulvic acid encapsulation system in alginate by ionic gelation technique. (B) Prepared microcapsules; Table S1: Chemical properties of the commercial reference substrates.

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