Citrus peel waste valorization through a biorefinery strategy for the production of succinic acid, ethanol, methane and fertilizer

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Abstract

Purpose: Citrus fruits constitute one of the most highly utilized food products worldwide. The industrial processing of the fruit generates half of its mass as citrus peel waste (CPW), which is rich in components that can be valorized. Herein, CPW has been applied for the production of succinic acid (platform chemical), ethanol and methane (biofuels) and a fertilizer through a biorefinery strategy.

Methods: The proposed biorefinery combined physicochemical and biological treatment of CPW to extract essential oils and pectin as added-value products preparing the material for subsequent production of succinic acid, ethanol, methane and fertilizer. The first step employed distillation for the collection of essential oils, followed by acid hydrolysis and pectin extraction. The hydrolyzate was microbially fermented for the production of succinic acid or ethanol, while the solid biorefinery residue was tested for application in agriculture as fertilizer and in anaerobic digestion for biomethane production.

Results: Succinic acid fermentations (*Actinobacillus succinogenes*) reached 14.43 g L^{-1} of succinic acid at 24 h through supplementation of vitamins and corn steep liquor. The highest yield in ethanol production was obtained in *Pichia kudriavzevii* KVMP10 fermentations that reached 0.42 $g_{ethanol}$ $g^{-1}_{total\ reducing\ sugars}$. Solid biorefinery residues were tested and compared against CPW and dry CPW in anaerobic digestion, where the latter achieved the highest methane production. The application of biorefinery residues as fertilizer demonstrated that the material imposes stress on plant growth.

Conclusions: The construction of the environmental friendly biorefinery offers an alternative management practice for CPW, which is valorized towards the production of a series of added-value products and zero-waste.

Keywords

Succinic acid, ethanol, anaerobic digestion, fertilizer, biorefinery.

1. Introduction

Citrus fruits belong to one of the most-utilized food categories classified under vegetables and fruits [1]. The global citrus production constitutes over 121 x 10^6 tons annually, while juice manufacturing generates 25 x 10^6 tons of citrus peel waste (CPW) [2]. CPW formed during the industrial processing of the fruit consists of peels, seeds and segment membranes, accounting for 50% of the whole fruit [3, 4]. Current management practices include the use of CPW as animal feed or disposal in landfills [5]. However, peels of citrus fruits contain a valuable composition that renders CPW a promising feedstock for valorization through the biorefinery platform.

The application of food waste as a feedstock for biorefineries consists a novel practice for the reduction of biodegradable waste, replacing the use of petroleum for production of high added-value chemicals and biofuels [6]. CPW could serve as a promising feedstock for valorization through the biorefinery strategy due to the high content of cellulose, hemicellulose, soluble sugars and pectin [5]. The isolation of pectin is technologically important due to its application in the food and pharmaceutical industries [7]. Moreover, CPW include 0.5% g $g_{wet\ mass}^{-1}$ of essential oils, consisting 90% of D-limonene [8], an antimicrobial agent that could be employed in food or pharmaceutical industries [9].

The valorization of CPW was previously investigated by several studies through bioprocessing for biofuels production (ethanol, methane) as well as added-value products (e.g. succinic acid) using different pretreatment approaches and microorganisms [4, 10-12]. Succinic acid constitutes a dicarboxylic acid, which is predicted to be one of the most important bio-based platform chemicals. The bio-based production of succinic acid entails a series of advantages, compared to its chemical production due to high theoretical yield and environmental friendly impact [12,13]. Thus, several studies have previously examined the production of succinic acid using various microorganisms. Among the different strains, *Actinobacillus succinogenes* holds the ability to valorize monosaccharides and to utilize CO_2 for the production of high succinic acid concentrations.

The current study developed a zero-waste CPW biorefinery (Fig 1.), which employed acid and enzyme hydrolysis for isolation of essential oils and pectin as well as generation of a hydrolysate. The latter comprised a high content of soluble sugars that was used as a nutrient rich fermentation feedstock for the production of ethanol or succinic acid. Moreover, the remaining solid biorefinery residues were tested in agricultural applications as fertilizer and in anaerobic digestion for the production of biomethane closing the loop in the management of CPW through development of a zero-waste process.

2. Materials and methods

2.1. Raw material

CPW used in the current work constituted citrus residues, which were obtained from a local juice factory (KEAN, Limassol, Cyprus) and stored at -20 ^{o}C until further use. CPW was thawed and ground to particles less than 2 mm in diameter using a laboratory blender (Waring Commercial, Texas, USA).

2.2. Isolation of D-limonene and pectin

The first step of CPW pretreatment required extraction and collection of essential oils through the addition of water to the raw material at a ratio of 6:1 (w/w) and boiling for 1 h. Essential oils were collected through distillation [8] and the residue was dried at 70 ^{o}C for 24 h [14]. An autoclave (SANYO MLS-3781L, Panasonic, Tottori, Japan) was employed for dilute acid hydrolysis. Acid hydrolysis for succinic acid fermentations was optimized in a previous study [12] and it was performed at 116 ^{o}C for 10 min using 5% (w/v) of dry raw material and 0.5% of H_2SO_4. Dilute acid hydrolyzate for ethanol fermentations was performed at 108 ^{o}C, 116 ^{o}C and 125 ^{o}C for 10 and 20 min using 5% (w/v) of dry CPW (diluted with distilled water). Experiments were performed in duplicate. Centrifugation and filtration followed dilute acid hydrolysis to obtain the supernatant, which was mixed with an equal volume of ethanol (96% v/v) to precipitate pectin at room temperature for 4 h [15]. Subsequently, the mixture was centrifuged at 3000 rpm for 30 min. The precipitate was washed five times with ethanol (45% v/v) followed by drying at 50 ^{o}C to obtain pectin [16].

2.3. Enzyme hydrolysis

Enzyme hydrolysis was applied as pretreatment prior ethanol fermentations. The procedure was performed as previously described, while cellulases and β-glucosidases were added at 30 IU g^{-1}_{drm} and 25 BGL g^{-1}_{drm}, respectively [12].

2.4. Microorganism and culture conditions

Pichia kudriavzevii KVMP10 was isolated in a previous work [10], while *Kluyveromyces marxianus* and *Actinobacillus succinogenes* were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Baker's yeast was used as a source of *Saccharomyces cerevisiae*. The strains were maintained at -80 ^{o}C in glycerol stock cultures.

Succinic acid fermentations were performed using *Actinobacillus succinogenes*. The inoculum of succinic acid fermentations was cultured in 30 g L^{-1} of tryptic soy broth (TSB), which was previously sterilized at 121 ^{o}C for 20 min. *Actinobacillus succinogenes* was incubated at 37 ^{o}C for inoculum preparation in an orbital shaker stirred at 100 rpm for 14 h. Succinic acid fermentations were performed in batch mode using 100 ml shake flasks with a working volume of 130 ml. The reducing sugars obtained from dilute acid hydrolysis were used as carbon source, while yeast extract, ammonium sulfate and corn steep liquor were evaluated as nitrogen source at 5 g L^{-1}. Additionally, the supplementation of vitamins (B_{12} 1 μg L^{-1}; biotin 20 μg L^{-1}; folic acid 20 μg L^{-1}; thiamine 50 μg L^{-1}; riboflavin 50 μg L^{-1}; niacin 50 μg L^{-1}; pantothenate 50 μg L^{-1}; p-aminobenzoate 50 μg L^{-1}; lipoic acid 50 μg L^{-1}; B6 100 μg

L^{-1}) [17] was also tested. The inoculum volume applied was 13% (v/v) and continuous sparging of CO_2 was supplied with a flow rate of 0.5 vvm. Stirring was controlled at 100 rpm in a rotary shaking water bath and temperature was maintained constant at 37 ^{o}C. All shake flask experiments were performed in duplicate, while two samples were analyzed for each replicate.

Pichia kydriavzevii KVMP10, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were used in ethanol fermentations, while precultured in 50 mM citrate buffer supplemented with 10 g L^{-1} of yeast extract and 20 g L^{-1} of peptone. Ethanol fermentations were carried out in batch mode using 100 ml flasks with working volume of 60 ml at 42 ^{o}C [10]. The reducing sugars obtained from dilute acid hydrolysis or combined acid and enzyme hydrolysis of CPW were used as carbon source for the experiments, while the supplementation of 10 g L^{-1} of yeast extract was examined in ethanol fermentation as nitrogen source. All experiments were performed in duplicate, while three samples were analyzed in each replicate.

2.5. Biomethane production

Solid biorefinery residues were tested and compared against the raw material (citrus peel) as well as dried raw material using equal contents of volatile solids (6 g L^{-1}) to produce biogas under mesophilic conditions (37 ^{o}C). Anaerobic digestion was performed in batch experiments using 250 ml flasks with working volume of 150 ml, which were flushed with 100% CO_2 gas to ensure anaerobic conditions. Each bottle was supplemented with 6 g of granular sludge, which was used as active inoculum (4%), obtained from a local dairy factory (Charalambides Christis Ltd, Limassol, Cyprus) as well as nutrient medium prepared according to Angelidaki et al [18]. Experiments were performed in triplicate and methane accumulation was determined for 71 d. Following 35 d, a re-feed of each material was applied in each digestion.

2.6. Assessment of biorefinery residues as fertilizer

The solid residue (SR) was initially dried at 40 ^{o}C in an air circulating oven until constant weight was achieved and then milled. Different ratios of the dried residue in commercial peat substrate were used to evaluate lettuce seedling production (0%, 1%, 2.5%, 5% and 10% w/w). The main physicochemical properties of the mixtures were determined. Organic matter content was determined after ashing in oven at 550 ^{o}C and organic carbon was then calculated accordingly. Total pore space (TPS), air-filled porosity (AFP), available water holding capacity (AWHC) and bulk density (BD) were also measured along with the EC and the pH, using the 1:5 dilution method as described previously [19].

For the seed germination tests, 6 serial dilutions (up to 10^{-6}) of the dried material in water (extract was stirred for 24 h at ambient temperature) were used. Filter paper was placed in Petri dishes and it was moistened daily (4 replicates/treatment, 15 seeds/replicate). Seeds were considered germinated upon radicle emergence. Mean shoot and root length was evaluated on the eighth day.

Substrate mixtures were placed in plastic seedling trays and 3 seeds of lettuce (*Lactuca sativa* var. Paris Island) were placed per module (40 cm^3 module capacity). Nine modules per treatment were used as replications. Trays were placed in walk-in

growth chambers (temperature of 25±1 ^{o}C max, 20±1 ^{o}C min; RH: 65-70%) and watered according to plant needs. Daily observations were recorded for seed emergence. After 25 days of cultivation, seedling growth was assessed and six plants per treatment were harvested. Plant height (in terms of longest leaf), leaf number, fresh weight and dry upper plant matter were measured. Maximum Fv/Fm photochemical quantum yields of PSII were measured using OptiSci OS-30p Chlorophyll Fluorometer (Opti-Sciences). Chlorophyll content was measured as described by Chrysargyris et al [20] using DMSO.

Following the hydrochloric digestion of plant ash, potassium (K) and sodium (Na) content was determined using microprocessor flame photometer (Lasany Model 1382) and phosphorus (P) content using the vanadate/molybdate (yellow method) as described by Gupta et al [21]. Nitrogen (N) content was determined through Kjeldahl method. The above methods were used to determine K, Na, P and N content into the substrate mixtures as well.

Plant stress level and damage indices were determined in terms of malonialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, as described by Chrysargyris et al [22], following homogenization of plant tissue with 0.1% trichloroacetic acid.

2.7. Analyses

Determination of succinic acid concentration: The concentration of succinic acid during fermentation was measured through High Pressure Liquid Chromatography (HPLC). Culture samples were centrifuged at 13,000 rpm for 5 min and filtered with 0.22 mm syringe filters. A Shimadzu LC-20AD liquid chromatograph (Shimadzu, UK) equipped with a Shimadzu SPD- 20A UV/VIS detector, a Shimadzu SIL-20A HT auto sampler and a CTO-10AS VP column oven was used. The column was eluted isocratically at a rate of 0.3 ml min^{-1} from an organic analysis column (Rezex ROA-Organic Acid column, Phenomenex, USA) with 5 mM H_2SO_4 at 80 ^{o}C. The injection volume was 15 μl.

Determination of ethanol concentration: Gas Chromatography was employed for the determination of ethanol concentration. A Shimadzu GC-2014 (Shimadzu, Milton Keynes, UK) using a flame ionization detector and a 30 m long Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK) with 0.25 mm internal diameter was employed. The mobile phase applied was nitrogen, while the stationary phase of the column was 5%-phenyl and 95% dimethylpolysiloxane. Samples were centrifuged for 5 min at 13,000 g and the supernatant was filtered through 0.45 μm filters. Ethanol was extracted into hexane by vortexing 1 ml of the filtered sample with 2 ml of the solvent for 1 min. About 1 μl of the extract was injected and the temperature of the column was kept constant at 40 °C for 3 min.

Determination of reducing sugars' concentration: During succinic acid as well as ethanol fermentations the content of reducing sugars was analyzed by the phenol-sulfuric acid method [23].

Determination of biogas composition: The biogas composition was determined using a gas chromatograph (Agilent Technologies, 7820OA) equipped with ShinCarbon ST 50/80 mesh column (2 m length, 2.2 mm ID) (Restek Corporation, USA) and thermal conductivity detector.

3. Results and Discussion

3.1. Succinic acid production

Actinobacillus succinogenes is predicted to be an industrially important microorganism because of its high efficiency in succinic acid production. Hydrolyzates from wheat, bread, cotton stalk and macroalgae have been previously tested for the production of succinic acid by *Actinobacillus succinogenes* through supplementation of CO_2 as well as nitrogen source (yeast extract) and vitamins obtaining 62.1 g L^{-1} (1.02 $g_{succinic\ acid}$ $g^{-1}_{total\ reducing\ sugars}$), 47.3 g L^{-1}, 63 g L^{-1} (0.64 $g_{succinic\ acid}$ $g^{-1}_{total\ reducing\ sugars}$) and 33.8 g L^{-1} (0.63 $g_{succinic\ acid}$ $g^{-1}_{total\ reducing\ sugars}$) of succinic acid respectively [24-27].

The current study tested the production of succinic acid in *Actinobacillus succinogenes* fermentations, evaluating the effect of different nitrogen sources (ammonium sulfate, yeast extract and corn steep liquor) as well as the addition of vitamins in CPW hydrolyzates generated following acid hydrolysis and pectin recovery. The highest succinic acid yield was achieved with the use of corn steep liquor and yeast extract (0.49 and 0.48 $g_{succinic\ acid}$ $g^{-1}_{total\ reducing\ sugars}$ respectively), while the highest succinic acid concentration reached 17.79 g L^{-1} at 36 h when corn steep liquor was applied (Fig. 2a). Furthermore, corn steep liquor and yeast extract combined with the addition of vitamins were tested in *Actinobacillus succinogenes* fermentations obtaining 0.48 and 0.47 $g_{succinic\ acid}$ $g^{-1}_{total\ reducing\ sugars}$ respectively, where the concentration of succinic acid reached 14.43 g L^{-1} and 10.28 g L^{-1} respectively at 24 h reducing the duration of fermentation (Fig. 2b).

3.2. Ethanol production from CPW hydrolyzates

CPW was previously tested as raw material to produce ethanol using different pretreatment approaches and microorganisms. Specifically, bioethanol production was investigated in a citrus peel derived hydrolyzate following steam explosion as well as in a hydrolyzate generated following dilute-acid hydrolysis and pectin recovery using *Saccharomyces cerevisiae*, where the product yield reached 0.43 g g^{-1} [15, 28]. Furthermore, the production of bioethanol has been evaluated using *Mucor indicus* and *Pichia kudriavzevii* in an enzymatically derived hydrolyzate of orange and mandarin peel waste obtaining 15 g L^{-1} and 33.87 g L^{-1} of ethanol respectively [29, 30].

Herein, a thermotolerant microorganism, *Pichia kudriavzevii* KVMP10, was tested and compared against two industrial microorganisms, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*, for bioethanol production from six different hydrolyzates which were pretreated at three different temperatures (108 ^{o}C, 116 ^{o}C, 125 ^{o}C) for 10 and 20 min. Optimal conditions for CPW saccharification were identified as 116 ^{o}C for 10 min through the addition of 0.5% (v/v) H_2SO_4 in 5% (w/v) dry raw material based on the concentration of ethanol that was produced and the final product yield during fermentations. Ethanol concentration and product yield reached 5.83 g L^{-1} and 0.48 $g_{ethanol}$ $g^{-1}_{total\ reducing\ sugars}$ respectively using *Pichia kudriavzevii* KVMP10. The highest ethanol concentration (6.69 g L^{-1}) obtained was achieved during fermentation of the hydrolyzate generated at 125 ^{o}C for 20 min and the product yield reached 0.32 $g_{ethanol}$ $g^{-1}_{total\ reducing\ sugars}$. The effect of nitrogen source as well as the application of

enzyme hydrolysis were also examined. Although the addition of yeast extract as nitrogen source did not demonstrate any noticeable effect on the production of bioethanol, the use of enzyme hydrolysis substantially enhanced the final concentration of ethanol (9.21 g L^{-1}) increasing the product yield at 0.42 $g_{ethanol}$ $g^{-1}_{total\ reducing\ sugars}$.

3.3. Biomethane production from biorefinery residues

Biomethanization of CPW was previously evaluated, following pretreatment to extract D-limonene, under thermophilic and mesophilic conditions [9]. Also, fragments (peel, pulp and seed) of fresh as well as dried citrus were investigated for biomethane production under mesophilic conditions [31, 32]. In the present study, solid biorefinery residues were tested and compared against the raw material as well as dried raw material using equal contents of volatile solids to produce biogas under mesophilic conditions. The dry raw material demonstrated the highest production of biogas (437 ml) and methane (303 ml) as compared to the other two materials tested. The solid biorefinery residues demonstrated biogas inhibition for the first 15 d of the process accumulating acetic acid at 9.7 g L^{-1} in the medium, while the raw material also showed lower inhibition in biogas production. Following 35 d, a re-feed of each material was applied in each fermentation and the inhibitory effect was substantially reduced indicating adaptation of the sludge to each material.

3.4. Assessing the applicability of biorefinery residues as fertilizer

The effects of the solid residue on the substrate's properties are presented on Table 1. Addition of the material in peat gradually increased the conductivity and reduced the pH of the substrate mixture. This response is due to the low pH and the high EC of the solid residue, which were measured at 1.52 and 14.77 mS cm^{-1} respectively. Potassium, phosphorus and nitrogen increased in content in the mixture, as the partition of the residue in the substrate increased. That is a result of the higher content of these elements in the organic residue as compared to the control (100% peat). Total and air filled porosity have been recorded higher at the substrates using 5% and 10% of the residue, because of the texture of the solid fine grinded residue.

Seed germination decreased at 10^{-1} dilution for the first 4 d, exhibiting zero germination for the pure SR extract (Fig. 3). Similar effects were observed for the shoot and root length *in vitro* for seeds in Petri dishes. *In vivo*, while increasing the percentage of SR in the mixture, plants appeared shorter and developed a lower number of leaves, resulting to lower fresh and dry weight (Table 2). This effect was apparent even at low SR concentrations of 1%. Plants appeared stressed following addition of the residue in the substrate. The chlorophyll content gradually declined, while stress indicators (as the overproduction of H_2O_2 and MDA levels) increased, supporting the high abiotic stress that the SR triggered to the plants. Seed emergence has been delayed as well, adding 3.5 d to seeds developed at 10%.

4. Conclusions

The proposed biorefinery evaluated the production of succinic acid from CPW through *Actinobacillus succinogenes* fermentations obtaining 14.43 g L^{-1} of succinic acid with the supplementation of corn steep liquor and vitamins. The application of

the feedstock in ethanol fermentations was also investigated obtaining a yield of 0.42 $g_{ethanol}\ g^{-1}_{total\ reducing\ sugars}$ in *Pichia kudriavzevii* KVMP10 fermentations. Solid biorefinery residues were tested and compared against untreated CPW and dry CPW for biomethane production demonstrating that dry CPW generate the highest cumulative methane production that reached 303 ml. The applicability of biorefinery residues as fertilizer was examined investigating that the addition of the residue in the substrate stressed plant growth. In conclusion, the construction of the environmental friendly biorefinery offers an alternative management practice for CPW, which is valorized towards the production of a series of added-value products and zero-waste.

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Figure Captions

Fig. 1 Simplified process flow sheet of the biorefinery used for CPW valorization

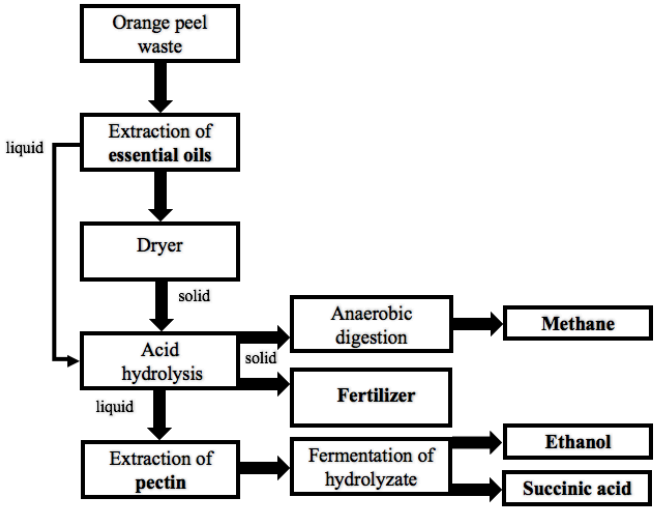
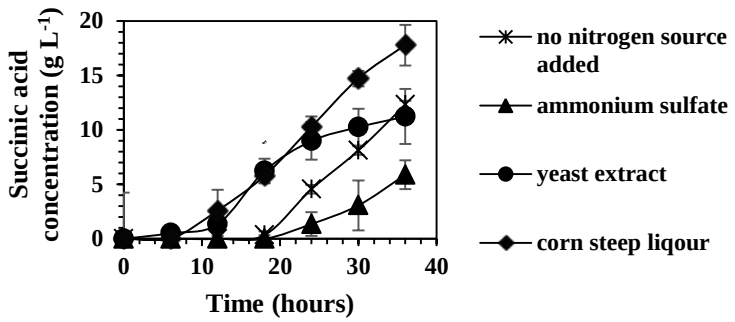


Fig. 2 Concentration of succinic acid achieved in *Actinobacillus succinogenes* fermentations of hydrolyzates obtained through dilute acid treatment of CPW with the addition of: ammonium sulfate —▲—, yeast extract —●—, corn steep liquor —◆—, and without the addition of nitrogen source —✱—. a) Without vitamins addition, b) with the supplementation of vitamins

a)



b)

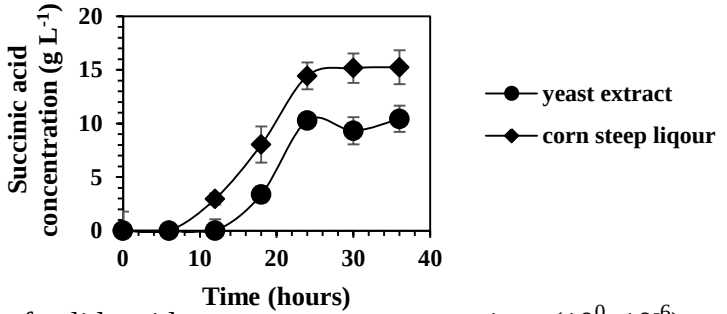


Fig. 3 Effects of solid residue extracts at concentrations (10^{0}–10^{-6}) on (A) cumulative seed germination and (B) on shoot and root length of lettuce *in vitro*. Values represent mean (±SE) of measurements made on four Petri dishes (15 seeds and 5 radicles/dish) per treatment. Mean values followed by the same letter do not differ significantly at P=0.05 according to Duncan's MRT

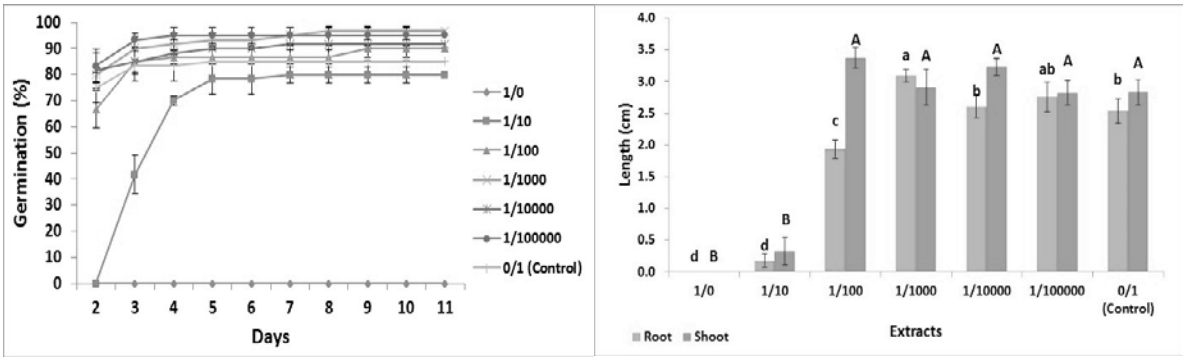


Table 1. Physicochemical properties of the substrate mixtures. The different rations of the solid residue in the peat mixture resulted in 5 different substrates.

	0%	1%	2.5%	5%	10%
pH	5.59 ± 0.0318a[Y]	5.10 ± 0.0289b	4.85 ± 0.0289c	4.03 ± 0.0231d	3.31 ± 0.0203e
EC (µS cm^{-1})	1215 ± 7.10e	1374.1 ± 8.03d	2085.9 ± 12.15c	2778.9 ±16.19b	3970.9 ± 23.15a
Organic matter %	97.87 ± 0.372ab	98.57 ± 0.248a	98.18 ± 0.003ab	97.84 ± 0.003b	97.99 ± 0.121ab
Organic C%	56.77 ± 0.217ab	57.18 ± 0.147a	56.95 ± 0.000ab	56.75 ± 0.000b	56.84 ±0.069ab
Total N (g kg^{-1})	4.60 ± 0.087d	6.02 ±0.318b	5.13 ± 0.044cd	5.55 ± 0.193ab	6.65 ± 0.017a
K (g kg^{-1})	0.62 ± 0.003e	0.69 ± 0.015d	0.76 ± 0.002c	0.83 ± 0.025b	1.10 ± 0.003a
P (g kg^{-1})	0.402 ± 0.0364b	0.357 ±0.0165b	0.396 ± 0.0069b	0.414 ± 0.0147ab	0.473 ± 0.0040a
Na (g kg^{-1})	0.172 ± 0.0012b	0.182 ± 0.0038b	0.179 ± 0.0009b	0.180 ± 0.0121b	0.215 ± 0.0090a
Total porosity % (v/v)	85.4 ± 0.84bc	82.3 ± 1.06c	82.7 ± 0.10c	85.9 ± 1.59ab	88.8 ± 0.31a
Air filled porosity % (v/v)	11.8 ±1.01bc	9.3 ± 0.21d	10.6 ± 0.20cd	12.7 ±0.61ab	14.2 ± 0.70a
Container capacity % (v/v)	73.6 ± 0.17ab	73.0 ±0.85ab	72.1 ±0.29b	73.3 ± 0.97ab	74.6 ± 0.39a
Bulk density (g cm^{-3})	25.0 ± 0.33a	24.5 ± 0.16a	23.6 ± 0.26b	24.5 ±0.24bc	25.2 ± 0.28a

[Y] values (n=3) in rows followed by the same letter are not significantly different, $P \leq 0.05$,

Table 2. Effect of the different mixtures tested on plant growth, mineral analysis and plant stress condition on lettuce seedlings, after 24 days of cultivation.

	0%	1%	2.5%	5%	10%
Plant height (cm)	8.16 ± 0.120a,[Y]	6.03 ± 0.070b	4.38 ± 0.177c	1.96 ± 0.095d	1.00 ± 0.047e
Leaf number	7.00 ± 0.365a	5.66± 0.333b	5.00 ± 0.000b	2.16 ±0.166c	2.01 ± 0.009c
Fresh weight (g)	2.48 ± 0.556a	1.09 ± 0.049b	0.78 ± 0.133bc	0.03 ± 0.002c	0.01 ± 0.000c
Dry weight (g)	0.27 ± 0.061a	0.11 ± 0.009b	0.08 ±0.026b	n.d.	n.d.
Total N (g kg^{-1})	18.30 ±0.262a	15.37 ±0.235b	15.89 ± 0.511b	n.d.	n.d.
P (g kg^{-1})	4.21 ± 0.019a	4.04 ± 0.059b	4.30 ± 0.017a	n.d.	n.d.
K (g kg^{-1})	27.63 ± 1.166b	30.68 ±0.321a	31.73 ± 0.924a	n.d.	n.d.
Na (g kg^{-1})	11.61 ± 0.0447a	11.09 ± 0.479a	11.00 ± 0.038a	n.d.	n.d.
Chlorophyll Fluorescence	0.821 ± 0.002a	0.809 ± 0.006a	0.0810 ± 0.003a	n.d.	n.d.
Chrorophyll a (mg g^{-1} FW)	1.070 ±0.160a	0.910 ± 0.076ab	0.673 ± 0.059b	0.237 ±0.003c	n.d.
Chrorophyll b (mg g^{-1} FW)	0.243 ±0.035a	0.230 ± 0.020ab	0.160 ± 0.015b	0.06 ±0.000c	n.d.
Total Chlorophyll (mg g^{-1} FW)	1.350 ±0.200a	1.143 ± 0.096ab	0.833 ± 0.074b	0.293 ±0.003c	n.d.
MDA (nmol g^{-1} FW)	17.11 ± 0.665c	27.54 ±0.506b	21.52 ±0.201c	60.27 ± 0.375a	n.d.
H_2O_2 (µmol g^{-1} FW)	0.17 ± 0.005d	0.46 ±0.006b	0.39 ± 0.000c	1.42 ±0.009a	n.d.
Mean germination time (days)	4.39 ± 0.104c	4.43 ±0.108c	4.93 ± 0.338c	5.98 ±0.513b	7.36 ±0.322a

[Y] values (n=6 for plant growth and n=3 for minerals/plant physiology) in rows followed by the same letter are not significantly different, $P \leq 0.05$. nd: (not detected)