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Potential applications of biochar and terpene-enriched bio-oil produced from a semi-arid native Asteraceae

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ABSTRACT

Biomass of *Flourensia oolepis*, a native shrub of the semi-arid central region of Argentina, was treated by vacuum pyrolysis to investigate the potential of this species to be used as a source of energy and chemicals. In this study we determined the influence of temperature on the product yields in different plant organs (leaves and stems), characterized the bio-oil, and assessed the bioactivity of biochar aqueous extracts through germination and growth bioassays with *Lactuca sativa*. The pyrolysis oils showed a predominance of long chain, cyclic and aromatic hydrocarbons in the leaves pyrolysate. The sesquiterpene spathulenol was the major compound in these reactions. Pyrolysis of stems produced mainly phenolic compounds. The effect of phosphoric acid pretreatment on leaves and stems was also evaluated in order to improve bio-oil yield and selectivity to any interesting compound. The results showed that acid treatment enhanced the liquid formation in pyrolysis of leaves giving a high amount of long chain hydrocarbons compared with the untreated organ. The biochar water extracts from leaves exhibited a hormetic type of response, with a promoting growth effect on roots and shoots up to 225%, and only a transient inhibition of germination at higher doses (≥7.5% w/v). Biochar water extracts from stems did not affect seed germination and showed a remarkable promoting effect, stimulating growth at all concentrations tested up to 330%. Although additional testing is required, overall results show *F. oolepis* as a promissory species for the production of bio-oil and biochar with a wide range of applications, including the potential use as growth regulator.

1. Introduction

Bio-fuels and biomass-based energy have the potential to become major contributors to the global primary energy supply in both developed and developing nations [1]. So far, market demands on bio-fuels have been largely met by traditional crop species that are also used for food [2] posing the question as whether this could be in any way sustainable [3–5]. In the last decade, many countries have invested in programs aimed at developing new bioenergy crops, and new or more efficient processing technologies that would allow plant biomass or alternative feedstocks to be converted into bio-fuels, gas, biochar and other valuable sub-products that are used in different industries. However, prospection has rarely been focused on species from arid environments, which host the largest pool of marginal lands that could be used for energy purposes without competing for food crops. In this sense, research aimed at evaluating the potential energy uses of native species from arid and semi-arid areas is mandatory.

The Asteraceae are among the largest families of flowering plants [6] and many species are well known for their use in traditional and western medicine [7]. The genus *Flourensia* (Family Asteraceae; Subfamily Asteroideae; Tribe Heliantheae; Subtribe Enceliinae) comprises 25 species of resinous shrubs distributed throughout America, twelve of them native to Argentina [7,8]. *Flourensia oolepis* (chilca), hereinafter referred to as FO, is an endemic species of the semi-arid central region of Argentina [7,9] and is found growing at high densities in almost pure stands [10]. FO is a long-lived shrub, with a woody perenneting structure (xylopodium) from which it sprouts profusely after fires [11], and traditionally used as fuel in rural areas [12]. Our ecophysiological studies on species of the genus, including FO, show an array of adaptations to xerophytic environments [11], as the production of high

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levels of compatible solutes [13] and a diversity of secondary compounds produced in glandular hairs and resin ducts [11,14] that serve as defense against biotic stressors (microbes and insects), or play a role in allelopathic interactions [15]. Altogether, the fact that FO naturally exhibits most of the traits recognized as desirable for bioenergy production and sustainable yield in perennial species [2] and the feasibility of obtaining plants derived from seeds [11], makes this species an interesting target for its development as energy crop.

Among the different technologies available for biomass conversion, pyrolysis is receiving worldwide attention [16] as a promising pathway to sustainability, as it allows for the complete utilization of the biomass yielding bio-oil, bio-syngas and biochar. In this process, where organic material undergoes rapid thermal decomposition in the absence of oxygen, the relative contribution of each product is influenced by feedstock properties and operation parameters [17]. The obtained bio-oil can be directly used in diesel engines, turbine and furnace with some modification of equipment [18,19] or as transportation fuel after an upgrading process [20]. The gas and char products can be burnt for heating, reducing the additional production cost for heating the system. In addition, the bio-oil, being a rich mixture of organic compounds, may be the source of some pure chemicals such as anhydrosugars [21] and furans among others [22].

Prior to pyrolysis, pretreatment of lignocellulosic feedstocks has been carried out in order to increase the efficiency of the process and the production of high-valued compounds. Within the chemical methods, the use phosphoric acid has proved to be effective in dissolving the crystalline structure of cellulose [23] facilitating its hydrolysis into fermentable sugars and other bio-based products that could be used as chemical intermediates or platform chemicals.

On the other hand, biochar has been the focus of increased research due to its major potential benefits in relation to carbon sequestration, bioenergy, enhanced soil fertility, and waste management owing to their low cost [24–32]. Biederman and Harpole [33], in a comprehensive meta-analysis on the effects of biochar on multiple ecosystem functions, including plant productivity and nutrient cycling, conclude that biochar indeed may be regarded as a promissory solution to energy, carbon sequestration and ecosystem function; although caution should be taken in considering biochar impacts on yet unexplored, non-target environments [29,33,34]. Negative effects may derive from VOCs and other adsorbed compounds that may be released to the environment [32–35], which may interfere with biological signaling within the rhizosphere [36] affect the soil biota [37] and produce phytotoxic effects [38].

The objectives of the present study were: (i) to determine product yields in the vacuum pyrolysis of leaves and stems (untreated and phosphoric acid-treated) of *Fluorensia oolepis* (FO), (ii) characterize the liquid product obtained under optimum pyrolysis conditions to explore its possible uses as fuel and/or chemical feedstock and (iii) characterize the solid product (biochar) and evaluate its potential to be used as soil amendment through studying the effects of biochar water leachates on germination and growth bioassays in *Lactuca sativa*.

2. Materials and methods

2.1. Plant materials

Plant material was collected in a natural area corresponding to the Punilla Valley, Córdoba province, Argentina, in a typical shrub community (total plant cover 70- 90%), dominated by the evergreen shrub *Flourensia oolepis* S.F. Blake (FO). Aerial shoots from 15 specimens of FO were collected in “Dique El Cajón” Capilla del Monte (S 30°51′43″ W 64°33′39.2″, 900-1000 MASL) in early summer (January) of 2015. Harvested shoots were air dried, separated in two fractions: leaves and stems, and stored at ca. 20 °C. A voucher specimen (BAA 26.498) was deposited at the Herbario “Gaspar Xuárez” of the Facultad de Agronomía, Universidad de Buenos Aires, Argentina.

2.2. Sample preparation and characterization

Elemental analysis for carbon (C), hydrogen (H) and nitrogen (N) was carried out using a Perkin Elmer 2400 Series II analyzer. Carbon, hydrogen and nitrogen content (wt% on dry basis) were analyzed in duplicate and average values taken. Oxygen (O) was calculated by difference.

The higher heating values (HHV) of feedstocks and biochars, expressed in MJ/kg, were calculated using the Eqs. (1) and (2) described by Friedl et al. [39] and an average taken from these two values:

$$HHV_{(OLSmodel)} = 1.87C^2 - 144C - 2820H + 63.8C \times H + 129N + 20147 \quad (1)$$

$$HHV_{(PLSmodel)} = 5.22C^2 - 319C - 1647H + 38.6C \times H + 133N + 21028 \quad (2)$$

The lignin, hemicellulose and cellulose content were determined by the Laboratorio de Servicios de Nutrición Animal (Faculty of Agronomy, University of Buenos Aires) using an ANKOM 200 fiber analyzer (ANKOM Technologies, USA), following the original method described by Goering and Van Soest [40] and its modifications (Van Soest et al. [41]) as adapted by ANKOM ® 2005 [42]. Oven dried samples (40 °C for 72 h) ground to 1–3 mm particle size, were used to determine neutral detergent fiber [NDF], acid detergent fiber (ADF), and acid detergent lignin (ADL). NDF accounts for all cell wall constituents (hemicellulose + cellulose + lignin), ADF yields cellulose + lignin, and ADL accounts for lignin content (gravimetrically measured). Thus, the concentrations of cellulose and hemicellulose were calculated as: cellulose = ADF − ADL; hemicellulose = NDF − ADF. NDF and ADF are expressed ash free. Other organic fractions, including non-fiber carbohydrates, organic acids, fats, waxes, resins, essential oils, glycosides, and proteins are not determined by this method.

2.3. Pyrolysis experiments

The vacuum pyrolysis reactions were conducted in a tubular reactor under inert atmosphere. The quartz reactor with a length of 25.00 cm and an inner diameter of 2.50 cm was heated externally by using a tube furnace with a temperature-controller device. The reactor was connected to a high vacuum pump where pressures were in the range of 0.05–0.1 Torr. Crashed leaves and chopped stems samples of FO (1.00 g) were placed in a sliding ceramic boat, which was fed into the pyrolysis furnace when temperature (200–300 °C) and vacuum conditions of the system were reached. The sample was subjected to pyrolysis conditions for 20 min, and due to the vacuum system, contact times of the generated products were very short (< 0.5 s). Oxygen-free dry nitrogen, at a flow rate of 0.1 mL s^{-1}, was used as inert carrier gas to improve the transportation of the volatile products to the condensation region. Liquid products were trapped at liquid air temperature (−196 °C) immediately after they escape the hot zone, while gaseous products were not trapped. The liquid pyrolysate was extracted with acetone and subjected to different analyses. After the experiment was finished, the system was led at atmospheric pressure (in an inert medium) and the condensation trap was removed. In order to recover all bio-oil from this trap, acetone was added at room temperature and the solution was subjected to different analyses. After evaporation of acetone, the liquid phase consisting of oil was weighed. The solid char was removed from the ceramic boat and also weighed, and the gas yield was calculated by difference. All yields are expressed as the average of at least three experiments to confirm the reproducibility of the reported results.

The first set of pyrolysis experiments were performed with FO leaves at 200, 250, 280 and 300 °C. FO stems were only tested at the temperature at which bio-oil yield was maximized (i.e., 280 °C). To compare the effect of acid-treatment of starting material on the bio-oil composition, leaves and stems were impregnated with phosphoric acid

(Anedra) before the pyrolysis experiments. Thus, 5 g of plant materials were mixed with a phosphoric acid solution 5% (w/w) and kept at 70–80 °C during 24 h. After that, the mixture was filtered under vacuum, washed until pH 6 and the material was dried at 40 °C under vacuum overnight [22].

In order to quantify the amount (percentage by mass) of the different classes of compounds: long chain hydrocarbons (LCH), cyclic hydrocarbons (CyH), aromatic hydrocarbons (ArH) and other minority products, the pyrolysate obtained from pyrolysis was chromatographed on silica gel column with cyclohexane, cyclohexane/chloroform (5:5), chloroform and chloroform/ethyl acetate (5:5) to afford the different products separated by polarity. GC–MS analysis of the collected fractions allowed grouping them according to the above classification.

2.4. Bio-oil characterization

The components of the bio-oil from the pyrolysis reactions of leaves and stems of FO (untreated and acid-treated) were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) using a Shimadzu GC–MS-QP 5050 spectrometer. The injector temperature was kept at 300 °C and the separation was performed using a VF–5 ms capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas with a constant flow rate of 0.5-1.0 μL min^{-1}. The oven temperature was programmed from 80 °C (3 min) to 280 °C (15 min) with a heating rate of 10 °C min^{-1}. The temperature of the GC/MS interface was held at 280 °C, and the mass spectrometer was operated at 70 eV. The mass spectra were obtained from 40 to 400 *m/z* with the scan rate of 500 amu s^{-1}.

Compounds were tentatively identified based on the comparison between mass spectra experimentally obtained with those from the National Institute of Standards and Technology (NIST) library (match > 88%).

^{1}H spectra of bio-oils were recorded in acetone-d_6 with a Bruker Avance II 400 MHz spectrometer Chemical shifts are reported in parts per million (ppm) downfield from TMS. The spectra were measured at 22 °C.

2.5. Biochar characterization

Biochar elemental composition and higher heating values (HHV) were determined using the methods described above (Section 2.2). Proximate analysis of the biochars from FO leaves and stems were conducted using the standardized techniques. Samples, in a covered ceramic crucible, were oven dried at 100 °C for 2 h, and then heated to 550 °C for 4 h. The cover of crucibles was removed and the samples held at 550 °C for 1 h to combust the fixed carbon, leaving the residual ash. Moisture, volatiles and ash were calculated by direct weight loss and fixed carbon content calculated by difference.

2.6. Extraction of biochar water-extractable substances

Extraction of water-soluble substances from biochar was performed at 10% (w/v) by soaking the solid sample material in distilled water. The biochar/water mixture was vortexed and placed at 22 °C for 24 h. This mixture was transferred to 15 mL centrifuge tubes and centrifuged at 1500 rpm and 15 °C for 5 min using a Labofuge 400R (Heraeus). The supernatants and pellets were collected separately. Then, the biochar/water mixture was filtered through a – Whatman grade 1- qualitative filter paper (11 μm pore size) via a 12-cm diameter Buchner funnel. After extraction, the remnant biochar was dried (50 °C) yielding ca. 90% of the original weight. The average (mean ± SE, N = 5) pH, conductivity (μS cm^{-1}) and DTS (ppm) of 10% aq. extracts of the leaves biochars were 11.4 ± 0.02 > 3999 μS cm^{-1} and > 2000 ppm, respectively. In the case of biochar ethanolic extracts, 3 g of biochar were extracted in 30 mL of ethanol at r.t. The biochar/ethanol mixture was filtered in the same way as the biochar/water mixture. The resulting solution was evaporated, dried and re-suspended in acetone for GC–MS analyses. Using this procedure the total amount of organic material extracted from biochar was 1.5–2.0% (w/w).

2.7. Bioassay of biochar water-extractable substances

The bioactivity of biochar aqueous extracts was evaluated on seeds of lettuce (*Lactuca sativa*, Grand Rapids). Twenty five seeds were placed in a 5.0 cm Petri dish lined with one sheet of filter paper previously moistened with each test solution (1.5 mL) or distilled water in the case of controls, and allowed to germinate in a growth chamber in the darkness at 22 °C. Three different bioassays with three replicates for each concentration were performed. Aqueous extracts were bio-assayed at 10% (w/v) as well as serial dilutions with water at 7.5, 5, 3.0, 2.5 and 1.5%. Seed germination was assessed at 24 h interval during three days as previously described [15]. A seed was considered germinated when root protrusion was evident (ca. 1 mm). At day 3, lengths of roots and shoots (hypocotyls) of 60% randomly chosen lettuce seedlings per Petri dish were determined; relevant morphological features were also observed and annotated. Controls showed (mean ± SE) 99 ± 0.33% germination and 0.44 ± 0.02 cm of root and 0.55 ± 0.03 cm of shoot growth. Germination and growth responses, expressed as percentage of the controls were plotted against treatment concentrations. Effective concentrations capable of inhibiting 50% of germination, root growth or shoot growth were calculated as ECg50, ECr50 and ECs50, respectively. Non germinating seeds were tested further to explore whether the inhibitory effect was long-lasting or transitory, and if the leachates may have caused damage to the seeds. In the case of a treatment causing inhibition, once the final count was done at day 3, half of the seeds of each Petri dish that have not germinated were transferred to new Petri dishes lined with filter paper embedded in distilled water and placed at 22 °C; germination was counted daily for three days. Additionally, seed viability was tested in three to four seeds per Petri dish by means of the Tetrazolium test [43]. The results were analyzed by ANOVA (REML) and DGĆs test ($p < 0.01$), using InfoStat (National University of Córdoba, Argentina).

3. Results and discussion

3.1. Feedstock characterization

In lignocellulosic biomass feedstocks the structural carbohydrates (cellulose and hemicellulose) and lignin polymers are the main organic constituents of plant cell walls. Table 1 displays the content of cellulose, hemicellulose, and lignin of the two biomass samples of *Flourensia oolepis* (FO) used in this study: leaves and stems. Elemental analyses, O:C and H:C molar ratios, and calculated HHV values of these samples are also shown.

Table 1
Chemical composition of leaves and stems of FO used in the pyrolysis experiments.

	Leaves[a]	Stems[a]
% C	45.44 ± 0.05	45.67 ± 0.06
% H	5.71 ± 0.01	3.26 ± 0.01
% N	2.47 ± 0.05	1.56 ± 0.03
% O[b]	38.4 ± 0.1	42.5 ± 0.1
% Ash	8.00 ± 0.03	7.00 ± 0.03
O:C molar ratio	0.6	0.7
H:C molar ratio	1.5	0.9
HHV (MJ/Kg)[c]	18.3	18.0
Lignin[d]	3.70 ± 0.04	6.75 ± 0.07
Cellulose[d]	10.51 ± 0.08	30.3 ± 0.1
Hemicellulose[d]	6.40 ± 0.06	18.85 ± 0.09

[a] Dry material of *Flourensia oolepis*.
[b] Calculated by difference.
[c] Calculated values as described by Friedl et al. [39].
[d] Biopolymer content determined by the reported method (wt% dry basis).

Biopolymer differences were detected between both fractions. In leaves, these components represented only 21% of the dry material while in stems ca. 56% of the weight is accounted for the three polymers, being cellulose the main component. The content of lignin in leaves was almost half of that found in stems, and fall within the range observed for other biomasses such as *Lolium* and *Festuca* grasses [44], while the cellulose content was lower than the obtained for wheat straw, switchgrass (*Panicum virgatum*) and *Miscanthus*, among others [45].

The relative low representation of cell wall structures relative to total dry biomass in the leaves is similar to that found in forage leguminous species, such as alfalfa, in which other constituents, as proteins, may account for more than 35% of the biomass [46,47]. In the case of shrubs, and particularly resinous species of arid and semi-arid environments like *Flourensia* spp., a large proportion of the total biomass is accounted for non-structural carbohydrates and the production of secondary metabolites, including waxes and essential oils, among others. In *Flourensia*, crude resins may represent between 20 and 40% of leaves dry weight; values depend on leaf developmental stage being higher in not fully expanded leaves [11]. These values are similar to those found in *Larrea* spp. (20–30%) [48], *Larrea tridentata* [49], *Grindelia chiloensis* (17–40%) [50,51], *Euphorbia lathyris* (24.5%, latex) [52], *Haplopappus baylahuen* (up to ca. 28% in leaves, 16% in stems) and *H. rigidus* (up to ca. 31% in leaves, 21% in stems) [53].

From the ultimate analysis, leaves and stems exhibited similar high content of carbon and oxygen. These values are in accordance with those observed in switchgrass, *Miscanthus*, wheat straw, and other biomasses [44,45].

Conversely, the nitrogen content of leaves, and particularly stems, was high relative to those previously reported for a diverse group of forestry and agricultural biomasses [54]. For leaves, similar values were detected in some *Lolium* and *Festuca* grasses (e.g., 2.24% and 2.33–2.48%, respectively) [43]. The high nitrogen content found in FO may be at least partly due to the abundance of compatible solutes in this species. We have demonstrated that FO and *F. campestris* are quaternary ammonium compound accumulators [13]. Specifically, in FO, glycine betaine can account for as much as 62 and 38 μmol g^{-1} DW in the leaves and stems, respectively [13].

Leaves and stems biomass of FO gave analogous heating values, which can be explained by the similar carbon content in these two plant organs. These calorific values are in the range of values obtained for grasses and *Miscanthus* but lower than for hardwood biomasses [44,45,55].

3.2. Vacuum pyrolysis of Flourensia oolepis (leaves and stems)

The vacuum pyrolysis of leaves of FO was carried out at 200, 250, 280 and 300 °C, while pyrolysis of stems was performed at 280 °C. In the case of materials impregnated with phosphoric acid, the reactions were also carried out at 280 °C. The different fractions (liquid, solid and gas) were determined at each temperature and the results are displayed in Fig. 1. The pyrolytic decomposition of leaves biomass samples started at about 200 °C, followed by a great loss of mass at 250 °C and an extensive formation of gases at temperatures higher than 280 °C.

The liquid fraction increased with the increase of temperature giving the best yield at 280 °C (20%), from which it started to decline. Biochar yield was a function of pyrolysis temperature, this product represented as much as 72% at 200 °C, decreasing thereafter until it reached a minimum of ca. 14% at 300 °C. This decrease was accompanied by a concomitant formation of non-condensable gaseous products, which peaked at 300 °C to 70%. Since the bio-oil fraction decreased at 300 °C, showing a negative trend, higher temperatures were not further tested. Typically, fast pyrolysis experiments, and particularly those run in vacuum conditions have been performed at higher temperatures (≥400 °C); usually higher temperatures maximize bio-oil production around 50–70% depending on feedstock characteristics and other operational parameters [56,57].

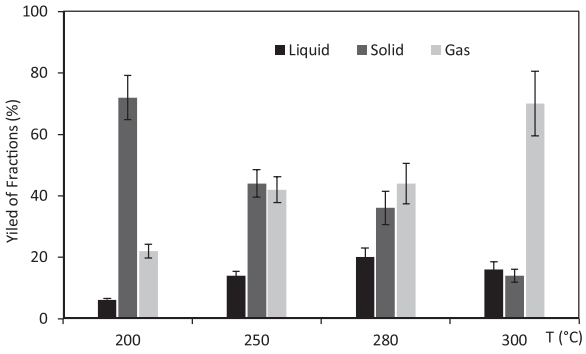

Fig. 1. Liquid, solid and gas fractions at different temperatures for pyrolysis of FO leaves. Data are expressed as means of three independent experiments ± SE.

The fact that in our experiments the maximum attainable liquid yield was obtained at a relatively low temperature was an unexpected result, and may suggest an interaction or synergism between FO biopolymers and the high percentage of resins present in these biomasses. The results of experiments in which switch grass was subjected to fast pyrolysis with a fixed bed reactor, and N_2 or CO_2 as carrier, showed a similar trend in the yield of the different fractions, although at higher temperatures [58]. These authors found that increasing temperature from 300 to 400 °C favored liquids (ca. 35% at 400 °C) as well as non-condensable gases, although the yields were lower for biochar (from 55% at 300 °C to 35% at 400 °C). Low oil yields have been documented in the pyrolysis experiments of *Euphorbia rigida* (ca. 22%) which has a comparable chemical composition with FO; in this work the application of catalysts increased bio-oil yields decreasing the formation of gaseous products [59].

The bio-oil was extensively analyzed by GC–MS technique, where the peak area percentage of the detected products depends on the response factor of the mass spectrometer detector, making it difficult to provide an accurate quantification of products. Bearing this in mind, the peak area of an individual compound was considered to be directly proportional to the concentration of such compound in the liquid pyrolysate. The peak area percentage of any compound was used for comparison purposes to assess its variability among the experimental conditions. According to the ion chromatograms of the bio-oils at different temperatures, three main families of compounds can be distinguished: long chain hydrocarbons (LCH), cyclic hydrocarbons (CyH) and aromatic hydrocarbons (ArH) (Fig. 2). Although LCH and CyH were present at all temperatures tested, their relative contribution varied in quasi an inverse mode as temperature increases. LCH represented ca. 40% of the total area at 200 and 250 °C, and dropped to 13% at the higher temperatures. This response could be explained by the conversion of longer chain carbohydrates into lower molecular weight

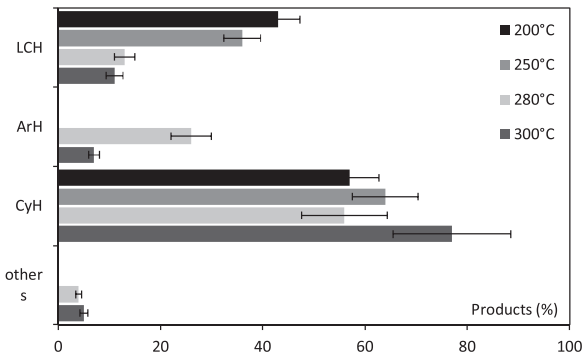

Fig. 2. Product distribution in the bio-oil from pyrolysis of FO leaves performed at different temperatures. Data are expressed as means of three independent experiments ± SE.

products through a combination of cleavage and cracking of these structures. Within the saturated and unsaturated hydrocarbons (straight and branched), tetracontane ($C_{40}H_{82}$), squalene ($C_{30}H_{50}$), octadecane ($C_{18}H_{38}$), and tetradecane ($C_{14}H_{30}$) were the main contributors. The high contribution of LCH is related to its presence in the original biomass, being major components of the resins found inside the resiniferous ducts which are present in high proportion in stems and leaves of FO and other *Flourensia* species [11,14]. Compounds such as n hexadecanoid acid (30%) and 1-octadecanol (15%) have been identified by GC–MS in leaves in which both epidermises have been peeled, whereas 1-heptatriacotanol may account for 14% of the total compounds identified in resins extracted directly from stem ducts. Other components deposited on the leaves epidermis (obtained by hexane partition) include ethyl butyrate and 2-butoxyethanol (ca. 17% and 14%, respectively); it may be possible that these smaller units may be conjugated during pyrolysis contributing to the higher molecular weight hyrocarbons found in this group (11,14).

Conversely, the amount of CyH compounds increased with temperature, being predominant at 300 °C. The mixture of cyclic hydrocarbons was mainly composed by terpenes, such as limonene, bisabolene and spathulenol (see Fig. 3), and hydrogenated naphthalenes. However, species contribution was affected by temperature. For instance, while spathulenol and polyhydro-naphtalenes were obtained in all reactions, spathulenol was predominant at 250 °C, and limonene appeared to be the major contributor at temperatures higher than 280 °C. In previous and ongoing studies on *Flourensia campestris* and FO, our group has identified an extensive list of terpenoids, particularly mono and sesquiterpenes, as majoritarian compounds of the volatile and non-volatile resins of leaves and stems. For instance, essential oils of leaves are exclusively composed by monoterpenes (up to 45%, e.g, pinenes, ocimenes) and sequiterpenes (up to 76%, e.g., bisabolene derivatives and their precursors as major components). Significant amounts of spathulenol have been also found in resins exracted from leaves and stems. Limonene and bisabolene are recognized as precursors to a range of commercially valuable products used in food, home, biomedical, cosmetic and personal care industries [60–62]. More recently, Bokinsky et al. [63] and Peralta-Yahya [64] have identified bisabolane (the reduced form of bisabolene) as an excellent alternative to D2 diesel fuel. Bisabolane was obtained through hydrogenation of its immediate precursor bisabolene, which in turn is produced through microbial synthesis. The physicochemical properties of bisabolane show that the cetane number (ca. 42) fall within the range of D2 diesel (40–45), and has an excellent performance at low temperatures, with a cloud point of −78 °C (cf. to −35 °C in D2 diesel and − 3 °C in biodiesel). Spathulenol, on the other hand, has shown the capacity to inhibit proliferation in the lymphocytes and can be regarded as an anti-inflammatory agent [65]. In addition, derivatives of this terpene have significant cytotoxic activity against a variety of cultured human cancer cells [66] being an important target for anticancer studies.

Identification of compounds that may represent high-valued products derived from pyrolyzed lignocellulosic biomasses and other feedstocks are subject of intense research [67–69]. In the case of *Nicotiana tabaccum*, nicotine may still be present as a majoritarian compound in the bio-oil after fast pyrolysis temperatures up to 450 °C [69]. *Taxus canadiensis* has been extensively investigated for the presence of taxol, a well-.known anticancer compound. Different authors were able to identify taxanes, taxol derivatives or precursors in the pyrolytic bio-oil of leaves and twigs [67–69].

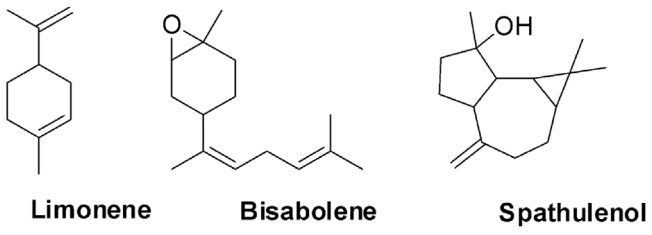

Fig. 3. Main terpenes detected in the pyrolysis of FO leaves.

The group of aromatics (ArH) was set up by monocyclic (substituted benzoic acids), polycyclic hydrocarbons (substituted naphthalenes) and substituted phenols (hydroquinone, guaiacols, syringols, among others). These compounds were formed at temperatures higher than 250 °C.

^{1}H NMR spectroscopy was used to characterize the fast-pyrolysis oils from FO leaves at different temperatures (Fig. 4). From the spectra it is evident that there were no major differences in the overall chemical makeup of the bio-oils obtained at different temperatures. The spectra were integrated over six different regions to quantify classes of hydrogen atoms in each bio-oil sample. The most upfield region of the spectra, from 0.5 to 1.8 ppm, representing aliphatic protons from methyl and methylene groups in hydrocarbon structures was more populated (~36–42% of all protons) indicating a significant aliphatic content in the mixture. The highest amount of this type of protons (42%) was observed in the bio-oil obtained at 250 °C (Fig. 4b), which is in concordance with the presence of a high content of natural hydrocarbon compounds. The next integrated region was from 1.8 to 3.1 ppm where the protons of acetone solvent were not considered in the quantification. This region represents protons on aliphatic carbon atoms that may be bonded to a C=C (aromatic or olefinic) and C=O double bonds, or are two bonds away from a heteroatom. High proton levels were observed in this region, ranging from 27 to 34%, where the highest value corresponded to the bio-oil generated at 300 °C (Fig. 4d). This is consistent with previous characterizations of this bio-oil that show high levels of natural hydrocarbons. The next section of the spectrum, from 3.1 to 4.5 ppm, represents protons on carbon atoms next to an aliphatic alcohol or ether. These protons appeared in significant amounts in the bio-oil obtained at 200 °C (21%) but their presence decreased as temperature increases (13% at 300 °C). Probably at high temperatures, dehydration processes are favored and alcohols are converted to olefin or aromatic compounds. The area between 4.5 and 6.0 ppm represents olefinic protons, which were characterized by their complex multiplicity. Low amounts of this type of protons were observed (5–7%) meaning that most of the alkene compounds presented highly substituted double bonds. The region of the spectrum corresponding to 6.0–9.0 ppm contains between 8 and 11% of the protons in the bio-oils, represented by hydrogen atoms in aromatic compounds and those deshielded olefinic hydrogens. The percentage of protons in this region was prevalent at temperatures higher than 250 °C. Trace amounts of acids and aldehydes were also detected in the downfield regions of NMR spectra (> 9 ppm) of bio-oils, although they are not observable at the scale presented in Fig. 4.

When vacuum pyrolysis of FO stems was carried out at 280 °C, a reduction in bio-oil was observed in comparison with leaves, generating significant amounts of biochar and non-condensable gas products (Fig. 5).

In order to calculate a real yield based on feedstock, a separation by chromatographic column of the pyrolytic bio-oil obtained from leaves at 280 °C was performed. The fractions were grouped according to its composition as determined by GC–MS. Results show that the mixture of phenol and terpene derivatives-which could not be separated into individual groups- represented 25% (w/w), while the yield of long chain hydrocarbons only reached 4% (w/w). The preponderance of terpenes and phenols was in agreement with the GC–MS analysis of the whole crude.

The composition of pyrolysate coming from untreated stems was remarkably different from that obtained in the experiments using untreated leaves. Key pyrolysis decomposition products were quantified and are shown in Fig. 5. In this case, the aromatic compounds accounted for 70% of the bio-oil composition, represented mainly by phenol derivatives (ArOH). Along the main compounds, typical lignin markers such as phenol, methoxy-phenols isomers and hydroquinone

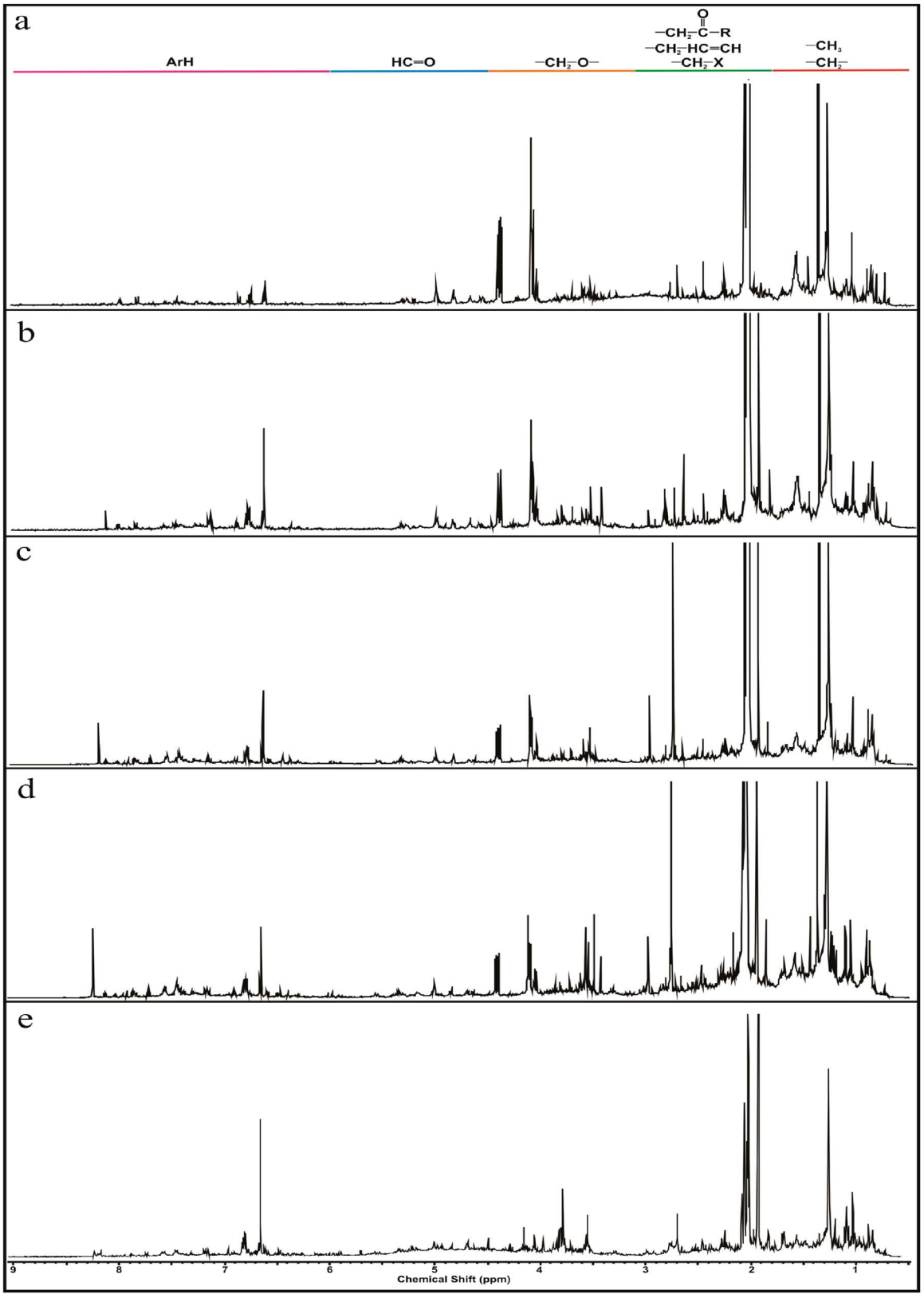

Fig. 4. ^{1}H NMR spectra of bio-oils from fast pyrolysis of FO leaves at: (a) 200 °C (b) 250 °C, (c) 280 °C and (d) 300 °C and (e) pyrolysis of FO stems at 280 °C.

were detected. Other aromatic compounds like furans, and other heterocyclic compounds were also distinguished. Cyclic hydrocarbons represented only 12% (quite different from 60% between 200 and 280 °C in leaves) of the total area, of which spathulenol derivatives could be detected in small amounts. Long chain hydrocarbons were also generated in low proportions at this temperature.

The higher percentage of phenol derivatives compared to the bio-oil derived from leaves was consistent with the larger content of lignin in stems (> 80%). These results would also agree with the range of temperatures at which lignin and hemicellulose are known to decompose

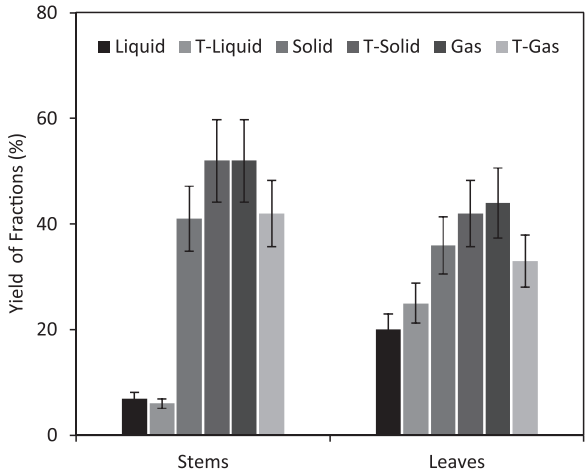

Fig. 5. Liquid, solid and gas yields for the pyrolysis (T = 280 °C) of leaves and stems of FO, untreated and acid treated (T) samples. Data are expressed as means of three independent experiments ± SE.

[16,58,70–72]. As described by Mohan et al. [73], results may also reflect the combined thermal responses of holocellulose and lignin, where holocellulose leads to the formation of furans and carbohydrates, and lignin generates phenolics (syringols and guaiacols). The complex molecule of lignin includes three main phenylpropane units namely para coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Phenolic compounds are formed by cracking these phenylpropane units in the macromolecular structure of lignocellulosic biomass. In vacuum pyrolysis experiments aimed at monitoring the evolution of phenols, the authors observed that for birch bark and sapwood, the maximum rate of transformations for lignin were also observed between 275–350 °C, with guaiacol derivatives resulting at lower temperatures, followed by syringol derivatives. The presence of these oxygenated compounds was higher than the values observed in fast pyrolysis of other lignocellulosic feedstock which ranged between 20–30% [45].

On the other hand, the high amount of cellulose in stems did not result in the formation of anhydrosugars as could be expected from results of previous studies of lignocellulosic biomasses. This result could be explained by the presence of inorganic salts, metal ions and ionic species that degrade anhydrosugars to low molecular weight compounds under pyrolysis conditions. This catalytic effect has been reported in the primary cellulose pyrolysis reactions giving a drastic reduction of levoglucosan yields in the resulting bio-oil [74].

Acid pretreatment of lignocellulosic materials enhance the anaerobic digestibility, the hemicellulose polymer can be solubilized, and cellulose become more accessible [75]. The acid catalyzed pyrolysis of FO leaves seems to produce a slightly increase in the formation of liquid products; while no changes are observable in the case of stems (see Fig. 5, T-Liquid values). These results may be at least partly explained by the differences in biopolymers composition of leaves and stems; stems show three times more cellulose and hemicellulose than leaves (Table 1). In this sense, compared to stems, acid solubilization of hemicellulose in leaves may have been more effective, and hence, may have promoted a more complete degradation of cellulose with the concomitant production of bio-oil. Furthermore, previous pyrolysis studies demonstrated that acid impregnation of cellulose-rich materials sometimes lead to low bio-oil yields in comparison with fresh raw materials, which was observed in our case for pyrolysis of treated stems [22,76,77]. On the other hand, the acid treatment of raw materials promoted the formation of the solid fraction at expenses of gaseous products (Fig. 5).

A remarkable effect of the phosphoric impregnation could be visualized on the composition of bio-oils from leaves and stems (Fig. 6a,b). In the case of pyrolyzed leaves, the formation of long chain hydrocarbons was favored at the expense of terpene-like compounds, while the contribution of the remaining products was very similar to that obtained for untreated leaves. Conversely, in phosphoric impregnated stems, terpenes production was enhanced relative to untreated material, while phenol content exhibited a substantial decrease compared to the pyrolysis of the raw stems.

Overall, the acid impregnation favored the degradation of leaves into bioliquid with similar level of selectivity towards terpenes and long chain hydrocarbons. For stems, the acid treatment increased the complexity of the liquid pyrolysate associated with a poor yield of the oil.

In summary, pyrolysis of untreated FO leaves generated a bio-oil enriched in sesquiterpenes at all reaction temperatures (max. 80% at 300 °C). Among these terpenes, spathulenol was the main representative compound. Taking into account the potential applications of bisabolene and of spathulenol derivatives the alternative generation of these types of bioactive nucleus from pyrolysis of FO deserves further investigation. The important contribution of long chain hydrocarbons at low pyrolysis temperatures also highlights the relevance of improving the yields of the bio-oil product. There is vast evidence that documents that product yields for a particular feedstock and conversion process, can vary widely depending on experimental setup conditions [16]. The phosphoric acid treatment of leaves enhanced bio-oil yields favoring the contribution of long chain hydrocarbons at relatively low temperature. Possible avenues to further improve bio-oil yields as well as selectivity of the process could include the use of other catalysts (in the pre-treatment or during the pyrolysis), and/or the increase of residence time of vapors during pyrolysis modifying the vacuum system to improve the degradation of the herbaceous material at low temperatures [73].

As demonstrated, the vacuum pyrolysis of untreated stems of FO could represent a promissory source of phenolic compounds with a wide range of applications. Phenol enriched mixtures can act as feedstock for further upgrading n the phenolic resins manufacturing [78], or used directly in boilers, engines and furnaces for electricity generation [21]. In this sense, additional investigations should also be undertaken to improve liquid yields while maintaining this selectivity.

3.3. Biochar characterization

New technologies, focused on the efficient transformation of biomass to charcoal [79] have increased the feasibility of utilizing biochar as solid fuel, to produce activated carbon, or as a sustainable soil amendment to mitigate global warming and improve soil properties and crop yields [29,33,80,81].

As shown in Fig. 1, biochar was the major product of pyrolysis of leaves at 200 °C and the second in importance at all other temperatures tested. At 280 °C, biochar yields remained high, averaging 40% for leaves and stems (Fig. 5). The physicochemical properties of this carbonaceous solid were investigated and the results are depicted in Table 2. The performance of the char formed from leaves in stimulating or inhibiting seed germination and seedling growth was also studied as a proxy to evaluate its possible use as soil amendment.

The composition of the carbonaceous materials showed differences, carbon and hydrogen content was higher for biochar formed from stems while oxygen and nitrogen were higher for biochar derived from leaves. Vacuum pyrolysis process reduced the O:C molar atomic ratio of initial stem feedstock from 0.5 to 0.3 (c.f. Tables 1 and 2), thus producing carbon-enriched solid products. The H:C ratio was also decreased in biochar from leaves compared to the initial biomass, which can be attributed to the partially or totally unsaturated compounds that were retained in the solid product during the pyrolysis process. For stems, the H:C molar ratio did not change after pyrolysis compared to the starting material, but oxygen content significantly decreased indicating a clear de-oxygenation of the biomass. These factors, among others, contribute to an improvement of HHV for biochars which reached 19.9 (leaves) and 23.6 MJ/kg (stems), compared to original feedstock (~18 MJ/kg). The energy density of these biochars is comparable to that of solid fossil

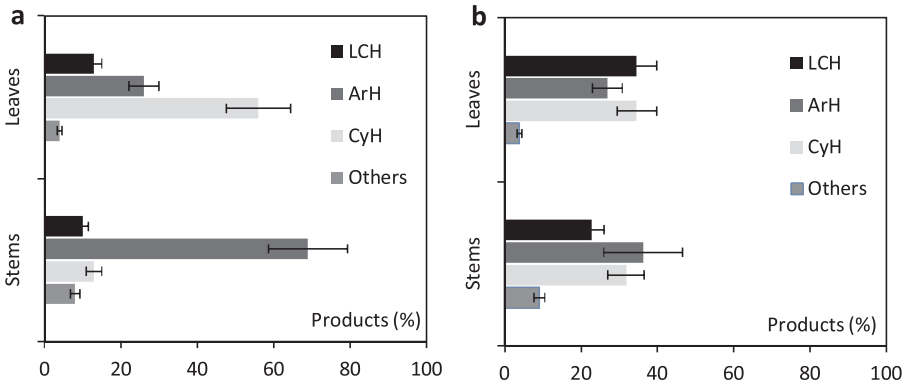

Fig. 6. Product distribution in the oil from FO leaves and stems pyrolysis at 280 °C: (a) without acid treatment, and (b) with phosphoric acid treatment. Data are expressed as means of three independent experiments ± SE.

Table 2
Characterization of biochar derived from pyrolysis of leaves and stems of FO at 280 °C.

	Biochar	
	From leaves	From stems
S_{BET} ($m^2\ g^{-1}$)	2.93	5.05
C (wt.%)a[a]	52.14 ± 0.05	60.78 ± 0.05
H (wt.%) [a]	3.03 ± 0.01	4.29 ± 0.01
N (wt.%) [a]	2.74 ± 0.03	1.78 ± 0.02
O (wt.%)[b]	36.1 ± 0.1	25.1 ± 0.01
H/C[c]	0.7	0.8
O/C[c]	0.5	0.3
Ash (wt.%)	5.95 ± 0.02	8.08 ± 0.03
Moisture (wt.%)	5.7 ± 0.01	10.9 ± 0.02
Volatile (wt.%)	20.9 ± 0.02	13.9 ± 0.02
Fixed carbon (wt.%)[b]	79.1 ± 0.02	86.1 ± 0.02
HHV (MJ Kg^{-1})[d]	19.9	23.6

[a] On dry basis.
[b] Calculated by difference.
[c] Atomic ratio.
[d] Calculated values as in Friedl et al. [39].

fuels, and as such they could be used to be co-fired as a solid fuel. With ca. 61% of carbon and a relatively low O/C ratio, stem-derived biochar could be potentially used for carbon storage, although its long-term soil stability should be further investigated. Indeed, Spokas et al. showed that the O/C ratio, which differs depending on the pyrolysis temperature, is linked to biochar stability [82]. This author explained that an O/C molar ratio lower than 0.2 provides a 1000-year biochar half-life. Analysis of BET area showed that both carbonaceous materials displayed a very small surface area with a negligible presence of pores, which is comparable to other biochars derived from woody and herbaceous biomass processed at similar temperatures [83,84]. Several authors have shown that pyrolysis temperature may influence the specific surface area of biochars. At higher temperatures (> 600 °C) a larger proportion of volatiles are removed and the formation of channel structures is promoted [25]. Conversely, biochars produced at lower temperatures generally exhibit lower specific surface area and a larger amount of functional groups adsorbed on the surface. As will be pointed out below the analysis of ethanolic extract of biochars indicated the presence of organic compounds adsorbed to the carbon surface, which certainly prevented the development of a porous structure of the solid material.

3.4. Bioassay of biochar water-extractable substances or water leachates

The biochar water extracts from the leaves of FO only reduced seed germination of *L. sativa* at the higher concentrations tested. At 7.5% (w/v) germination was inhibited by 20%, and at 10% (w/v) germination was nil (Fig. 7). However, further tests performed on non-germinating seeds showed that the inhibitory effect was transient, lasting only as long as the seeds were in contact with the biochar extract. All the seeds

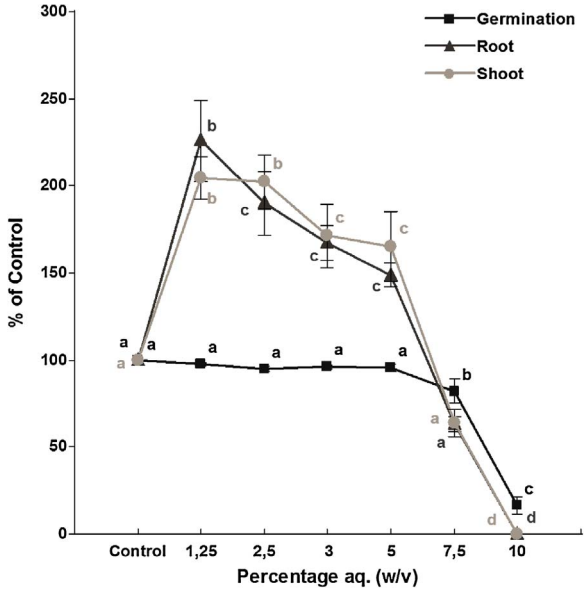

Fig. 7. Effects of bio-char water extractable substances from the pyrolyzed leaves of *F. oolepis* (at 280 °C) on germination and root and shoot growth of *L. sativa*, computed at the end of the experiment (day 3). Data are expressed as means of three independent bioassays (three replicates for each concentration (aqueous extracts) per bioassay) ± SE. Different letters (a–c) indicate significant differences between treatment effects when compared to the control (ANOVA, REML and DGC test, $p < 0.01$).

transferred to petri dishes with distilled water germinated producing seedlings with normal morphology. This response was consistent with the results of the Tetrazolium test, in which all seeds stained pink (which denotes that are viable) and exhibited no signs of damage in the tissues. These results clearly show that the arresting effect is not caused by toxic effects of the compounds dissolved in water, but could be at least partly due to an osmotic effect of the biochar extracts at the higher concentrations, since measured conductivity values for the 10% solution exceeded 3999 μS^{-1}.

In contrast, the biochar water extracts at the lower range of concentrations not only did not affect germination, but exhibited a drastic stimulatory effect on root and shoot growth (Fig. 7). Roots and shoots were 225 to ca. 160% longer than the control at concentrations between 1.5–5%. Growth inhibition was only visible at 7.5%. The mean effective concentrations of the biochar water extract that inhibited germination (Ecg50), root (Ecr50) and shoot (Ecs50) growth were 9.41, 8.25 and 8.25%, respectively. These results show that the biochar water extracts exhibit a hormetic type of response, enhancing growth at lower concentrations and causing inhibition at higher doses [85,86].

The few reports in which bioassays have been performed with biochar water extracts have yielded variable results, depending on feedstocks and the thermo-conversion processes involved [80,87–89]. For instance, germination of radish and corn seeds was reduced by

extracts from high volatile matter charcoal of macadamia nut shell (430 °C) [88]. Buss and Mašek found that leachates of bio-char produced from softwood pellets (550 °C) contaminated with high levels of volatile organic compounds (VOC) induced heavy toxicity to germination of *Lepidium sativum*, while low-VOC bio-chars did not show phytotoxicity [38]. Rogovska et al. [90] have reported no effect on corn seed germination but a decrease in seedling growth; particularly shoot length, in three out of six biochar extracts derived from different feedstocks at the highest temperature treatments. Toxic effects on blue-green and green algae have been found using pine biochars extracts at 450 °C [91] and Oleszczuk [92] also reported variable negative impacts on aquatic species of several organisms when biochars from different feedstocks were tested. Polycyclic aromatic hydrocarbons (PAHs) have been typically investigated in biochars for its possible toxic effects on a wide range of organisms, and several studies [90,92] support this contention. However, other results demonstrating the rather semi-volatile or non-volatile nature of PAHs [93], and their very low bioavailability and water solubility [94], suggest that these compounds would not necessarily be involved in the phytotoxic effects of biochar water extracts on seed germination. Alternatively, phenol derivatives present in the biochars may also exert toxic effects -through growth reduction-, as has been shown for green and blue-algae [91].

However, in the present study, the biochar extracts of FO showed a hormetic type of response, and only a transient inhibitory effect at higher doses. To our knowledge, this is the first study to report these type of responses in germination assays with biochar water extracts. The fact that inhibition was only transient highlights the importance of including this type of test in future experiments aimed at evaluating the effects of biochars extracts on seed germination and seedling growth. This should be also advisable in the experimental setting that has previously been proposed to test the effects of VOCs [95] and the direct contact of seeds with biochar and biochar leachates [38].

In order to investigate the possible compounds involved in the observed response, the ethanolic extract of the FO biochar was subjected to GC–MS yielding a main chlorinated compound with MW equal to 265, which could not be unequivocally identified by comparison with the NIST library. A vast array of chlorinated compounds naturally occurs in plants [96]. These include compounds with auxinic effects that could be considered plausible candidates of the hormetic type of response we found [96]. Furthermore, Engvild working with global gene expression arrays [97], found that the promoting growth effect of biochar in *Arabidopsis* and lettuce was strongly related to changes in auxin and brassinosteroids signaling. This provides additional evidence for the contention that a chlorinated, possibly auxin-type compound may be responsible for these unpredicted results, and highlights the need to confirm the identity of the compound/s.

In the case of stems (Fig. 8), biochar water extracts did not affect seed germination of *L. sativa* at any of the concentrations tested; all the seeds germinated produced seedlings with normal morphology. The growth promoting effect of these extracts was even more dramatic than those found in the biochar water extracts derived from leaves. Shoot and root growth was stimulated at all concentrations tested; values ranged from 150% to as much as 330% promotion of growth at concentrations between 5 and 7.5%, relative to the controls (Fig. 8).

The ethanolic extract of stems biochar was also subjected to GC–MS, but the chlorinated compound (MW 265) found in the ethanol extract of leaves biochar was not detected, and no other compound could be identified based on the NIST library available. The identification of the compound/s responsible for this extremely high growth stimulatory effect clearly deserves further investigation, not intended in the present study.

Although water lixiviates from biochars have not been particularly investigated as growth promoters, an increasing number of papers are demonstrating that biochar applied to the soil may increase plant growth, especially under low-fertility soil conditions [28,98–101].

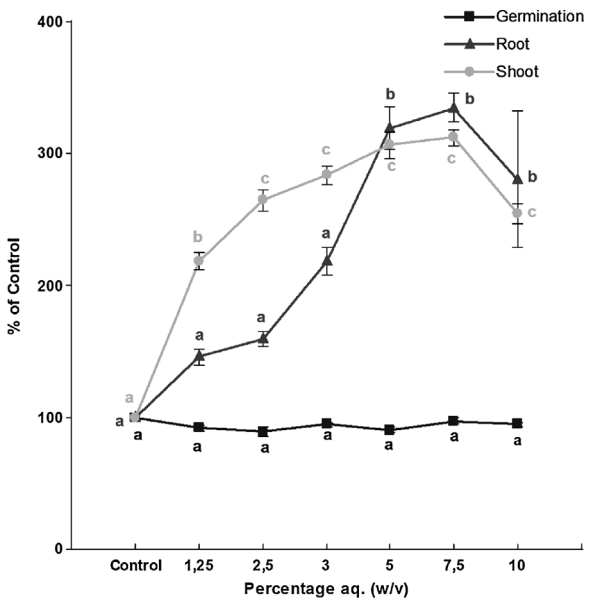

Fig. 8. Effects of bio-char water extractable substances from the pyrolyzed stems of *F. oolepis* (at 280 °C) on germination and root and shoot growth of *L. sativa*, computed at the end of the experiment (day 3). Data are expressed as means of three independent bioassays (three replicates for each concentration (aqueous extracts) per bioassay) ± SE. Different letters (a–c) indicate significant differences between treatment effects when compared to the control (ANOVA, REML and DGC test, $p < 0.01$).

4. Conclusion

In summary, the vacuum pyrolysis of leaves and stems in FO showed a compound predominance of long chain, cyclic and aromatic hydrocarbons. In leaves, the sesquiterpene spathulenol which is a high value-added product was the major compound in these reactions. The acid treatment of both types of biomass prior to pyrolysis led to the improvement of bio-oil yield in the case of leaves, increasing the amount of long chain hydrocarbons, which is a good prospect for future applications of this liquid.

Biochars showed improved HHV compared to the original feedstocks, with an energy density comparable to solid fossil fuels. The biochar water extracts from leaves exhibited a hormetic type of response, with a promoting growth effect (up to 250%), and only a transient inhibition of germination at higher concentrations (> 7.5%). The water extracts from stems did not affect seed germination and showed a remarkable promotion of growth (up to 330%). Thus, *F. oolepis* could be considered as a promissory species for the production of bio-oil and biochar; the latter could be an extremely useful by-product with potential application as plant growth regulator.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jaap.2017.06.026.

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