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Euphorbia characias as Bioenergy Crop: a Study of Variations in Energy Value Components According to Phenology and Water Status

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ABSTRACT: *Euphorbia characias* has drawn much attention as a potential bioenergy crop given its considerable amount of latex, rich in hydrocarbon-like compounds, and its ability to grow in large areas of semiarid lands. Compositions of major constituents with an energy value have been determined for the three phenological stages of this plant (preflowering, flowering, and postflowering) and different irrigation treatments. Metabolites from both nonpolar and polar extracts have been identified and quantified by GC−MS, GC-FID, HPLC-ELSD, and UPLC-PDA-MS. The results highlight that the end of the flowering period is the optimal harvesting time to maximize the yields of *E. characias* as a potential energy crop. The total water requirements to obtain the maximum yields of hexane- and methanol-extractables were determined for its annual development cycle.

KEYWORDS: *Euphorbia characias, phytochemical analysis, triterpenes, carbohydrates, flavonoids, GC−MS, UPLC-PDA-MS, HPLC-ELS, bioenergy crop, feedstock*

INTRODUCTION

Green plants are able to take up atmospheric CO_2, thus reducing carbon to carbohydrates with the help of sunlight via the photosynthetic process. Furthermore, some plants are able to reduce carbohydrates all the way to hydrocarbons (petroleum-like compounds). In 1967, Calvin spearheaded the idea that hydrocarbon-producing plants could be used as future oil and other chemical sources.[1] Thereafter, several *Euphorbia* spp., particularly *E. lathyris* L., and other latex-bearing plants, have been extensively explored as possible alternative fuel sources to crude oil products and fine chemicals given their high contents of hydrocarbon-like compounds.[2] The genus *Euphorbia* is one of the largest and most widely distributed in the spurge family (Euphorbiaceae), in which most species are found in temperate regions. These plants are herbs, shrubs, and often cactus-like, growing in nearly all climates types, and are characterized by the presence of milky-latex sap. *Euphorbia* latex consists of lipids, rubbers, resins, and sugars as well as several proteins and many different enzymes such as peroxidases, proteases, esterases, phosphatases, and lipases.[3] Within this genera, the perennial shrub *E. characias* commonly grows on rocky slopes, along road shoulders, and in open grounds in Mediterranean regions and reaches a height of more than 1 m. Phytochemical studies into the latex and shoots of *E. characias* have shown the presence of varied metabolites, such as jatrophane,[4] *ent*-atisane, *ent*-abietane, *ent*-pimarane, and kaurane[5] type diterpenoids, besides esterified or free fatty acids, sterols, tetracyclic and pentacyclic triterpenols, and two quercetin glycosides.[5−9] The chemical diversity of isoprenoid constituents in this genus has been considered to be of biological and chemotaxonomic interest.[10] In addition, and similarly to other *Euphorbia* spp., *E. characias* has drawn much attention as a potential bioenergy crop due to its substantial amount of latex, rich in hydrocarbon-like compounds, and its ability to grow in large areas of semiarid lands.[9,11−13]

From the bioenergy point of view, both dry matter yields and metabolite compositions are important aspects to be considered in any plant that is investigated as a potential energy crop. In this context, both the developmental stage of the plant and its water availability[14−16] are essential factors that affect plant physiology and, consequently, biomass yields and metabolite composition. Therefore, it is expected that experimental assays considering both factors might improve the production of high-energy value constituents of *E. characias*. Indeed, despite several studies having been conducted to identify all components of the *E. characias* nonpolar extract, no study to date has focused on the metabolic changes occurring from different plant developmental stages and irrigation doses.

The goal of the present research was to establish the irrigation water requirements and the adequate harvesting time to optimize the *E. characias* crop as a sustainable energy source. For this purpose, variations in composition and amount of the major constituents with an energy value have been determined from its three phenological stages (preflowering, flowering, and postflowering) and different irrigation treatments. We recently reported the application of quantitative ^{13}C NMR spectroscopy for metabolite profiling purposes to nonpolar extracts of wild *E.*

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characias.[17] In the current work, major metabolites from both nonpolar and polar extracts have been identified and quantified. Due to the complexity of the samples, effective gas chromatography (GC) and liquid chromatography (HPLC or UPLC) technologies coupled to a mass spectrometry (MS) detector for metabolite identification, and flame ionization detector (FID), evaporative light scattering detector (ELSD), and photodiode array (PDA) detector have been used for metabolite quantification according to the extract.

MATERIALS AND METHODS

Reagents. Internal standards (IS) (tetracosane, D-(+)-trehalose and flavanone) were purchased from Sigma-Aldrich Co. (St Quentin Fallavier, France) as well as reference compounds (nonpolar: palmitic acid, linoleic acid, linolenic acid, phytol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, squalene, heptacosane, nonacosane, hentriacontane, tritriacontane, β-sitosterol, lanosterol, β-amyrin, lupen-3-one, lupeol, and cycloartenol; polar: D-(+)-glucose, D-(−)-fructose, sucrose, myo-inositol, citric acid, D-(+)-malic acid, L-pyroglutamic acid, L-aspartic acid, L-glutamine, quercetin-3-β-glucoside, quercitrin and quercetin). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and methoxyamine hydrochloride were purchased from Acros Chemical Co. (New Jersey, USA) and Sigma Chemical Co. (St. Louis, MO), respectively. Hexane, dichloromethane, and pyridine were analytical grade, while acetonitrile and methanol were UPLC grade. All of these solvents were purchased from Scharlab SL (Barcelona, Spain).

Plant Material and Field Experiments. Seeds of *E. characias* L. subsp. *characias* were obtained from CEQA (Mediterranean Agroforestry Institute), at the Polytechnic University of Valencia (UPV). Plants were grown in a greenhouse in UPV facilities from June 2010 to March 2011. Then, 384 plants were transplanted to 24 hexagonal containers with a 3.8 m^2 area and 0.9 m in depth, in an open field in the IVIA facilities in Moncada (Valencia, Spain). Sixteen plants were grown in each container with a planting pattern of 0.4 × 0.4 m, which implies a planting density of 62500 plants per hectare. Irrigation water (80 mm) was applied immediately after transplanting to ensure plant establishment before the irrigation treatments. Soil was analyzed and classified as sandy loam with pH 8.4 and contained 18% carbonates, 5% lime, and 0.86% of organic matter.

Water Stress Experiments. Twenty-four hexagonal containers were divided into four groups in which different irrigation treatments (T1 to T3) and a control (T0) were randomly arranged (six replicates per treatment). Soil moisture was monitored using a frequency domain reflectometry (FDR) technology (capacitance sensors), provided with a capacitance probe with multiple sensors (Diviner 2000, Sentek Sensor Technologies, Stepney, Australia). The probe is swiped in and out of the access plastic tubes (3 for each treatment) and inserted into different soil depths, allowing the sensors to take measurements in a few seconds and detecting changes in dielectric constant of surrounding media by changes in the operating frequency in 10 cm intervals. Hexagonal containers were irrigated the first time when the moisture content was 13.7% (w/v, 60% field capacity) at 30 cm below soil. The volume irrigations were 30 mm (T3), 15 mm (T2), 7.5 mm (T1), and 0 mm (control only received rainwater, T0) per treatment, that corresponded to 100, 50, 25, and 0% of the total water evaporated, respectively. The following supplementations were identically performed when soil moisture in T3 was 13.7% (w/v, 60% field capacity) at 30 cm below soil. At the end of the study six treatments were applied, and the total amounts of water supplied accounted for 180 mm (T3), 90 mm (T2), and 45 mm (T1). The occasional rainfall was also monitored through the course of the experiment, being responsible for an increase of 370 mm in the total amount of water added to the treatments. Variations of leaf water potential were determined according to Turner and Hsiao.[18,19]

Preparation of Extracts. Between 3 and 6 representative plants per treatment (T1−T3 and control T0) were harvested from each container and dried in an oven at 65 °C to constant mass, approximately 72 h. Leaves and stems were separated from each plant and powdered. The powdered material (5 g) was extracted with hexane (50 mL) in a Soxhlet apparatus for 4 h, followed by MeOH extraction for an additional 4 h. Solvents were evaporated under reduced pressure to obtain the hexanic and methanolic extracts.[20]

Fiber Composition. The neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined according to Van Soest et al.[21] using a thermo-stable amylase (Thermamyl L120, Novo Nordisk, Gentofte, Denmark). Each analysis was performed in triplicate over both leaf and stem bagasses from plants in each developmental stage and different irrigation treatments T1−T3 and control T0.

Estimation of Rubber and High Molecular Weight Esters. *Rubber Precipitation.* Samples of hexanic extracts (30 mg) were saponified in a closed vial by treatment with 2 N KOH in EtOH (2 mL), heating at 90 °C and stirring for 2 h. Then, the unsaponifiable material was extracted from the alkaline solution (pH ∼10) with CH_2Cl_2. Next, the aqueous layer was acidified with 2 N HCl aqueous solution (pH ∼1) and the fatty acids were obtained by extraction with CH_2Cl_2.[13] Unsaponifiable samples (10 mg) were weighted into an Eppendorf, dissolved in MeOH (1 mL), and centrifuged at 9000 rpm for 5 min at room temperature. The supernatant was separated and the pellet was dissolved in MeOH (1 mL) and then centrifuged again. This MeOH-centrifugation process was performed at least four times for each sample. The pellet was dissolved in hexane, dried by nitrogen stream, and weighed. Pellet samples (5 mg) were dissolved in 1 mL of IS solution (0.2 mg/mL of tetracosane in CH_2Cl_2) and injected into the GC-FID system. Because the GC-FID chromatogram showed the presence of hentriacontane and hexacosanol in this nonpolar fraction, the content of rubber was considered as the difference between the total weight of the pellet and the calculated amounts of hentriacontane and 1-hexacosanol by employing the corresponding linear regression equations. The rubber precipitation process was performed in triplicate.

High Molecular Weight Esters (Nonvolatiles). The same procedure based on MeOH-centrifugation (4 times) was applied to samples of hexanic extracts (30 mg) without previous saponification reaction. The high molecular weight esters were estimated as the difference between the weights of nonsaponified pellets and the rubber, hentriacontane, and hexacosanol contents calculated after saponification following the same procedure described above.

Sample Clean-up of Methanolic Extract. Samples of methanolic extract (50−80 mg) were passed through a solid phase extraction (SPE) C_{18} cartridge (1 g, 6 mL) model ExtraBond (Scharlab, Barcelona, Spain), previously preconditioned with methanol (18 mL) followed by water (18 mL). First, the sample was eluted with water to collect the more polar compounds (SPE1), especially sugars. Next, the column was eluted with methanol to collect the retained polar residues fraction (SPE2), especially flavonoid glycosides.[22] Both fractions SPE1 and SPE2 were dried in a rotatory evaporator under reduced pressure at 40 °C maximum.

HPLC-Semipreparative of Nonpolar Metabolites. Commercial standards of butyrospermol, 24-methylenecycloartenol and octacosanal are not available and were purified using HPLC-semipreparative for their unambiguous identification by NMR spectroscopy. Samples of leaf hexanic extracts were injected onto a semipreparative RP-HPLC instrument (Waters 600E system, Milford, MA, USA) equipped with a solvent delivery pump unit (Waters 600E), and coupled to a photodiode array detector (Waters 2996 PDA) and an evaporative light-scattering detector (Waters 2420 ELSD). The separation of metabolites was carried out using a Kromasil 100 (10 × 250 mm, 5 μm particle size) C18 column (Teknokroma, Barcelona, Spain) operating at 22 °C and using 100% MeOH as mobile phase in a flow rate of 3 mL/min.

Spectroscopic Analysis. ^1H and ^{13}C NMR experiments of the rubber (*cis*-polyisoprene) enriched fraction from the pellet as well as butyrospermol, 24-methylenecycloartenol, and octacosanal from leaf hexanic extracts were recorded on a Bruker AVIII-500 (500 MHz) instrument (Bruker, Karlsruhe, Germany).[17]

Metabolite Identification. *GC–MS Analysis.* Major secondary metabolites of both hexanic and methanolic extracts were identified from the samples, after being previously trimethylsilyl (TMS) derivatized. Compounds were mostly identified by comparing retention times (t_R) and mass spectral data with TMS derivatized analytical standards which were commercially purchased or previously purified using HPLC. Only a few compounds not commercially available were tentatively identified on the basis of their MS fragmentation patterns and high probability matches according to NIST database information (Version 2, Gaithersburg, MD).

Nonpolar compounds from hexanic extracts (hydrocarbons-like) were identified by GC coupled to a mass spectrometer (PerkinElmer Clarus 500 gas chromatograph, Wellesley, MA) operating in the electron impact mode and equipped with a ZB-5 MS (30 m × 0.25 mm × 0.25 μm particle size) capillary column (Phenomex Inc., Torrance, CA). The oven temperature was programmed at 90 °C for 1 min, then ramped at 20 °C/min to 210 °C, increased at 10 °C/min to 260 °C, and then ramped at 4 °C/min to 340 °C for 10 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min. An autosampler injected 1 μL samples into a split/splitless injector at 250 °C, which operated in split mode (split ratio, 9:1; split flow, 10 mL/min). The GC interface temperature was set at 250 °C. The electron energy was 70 eV and the ion source was at 180 °C. Mass spectra were acquired using full scan monitoring with a scan range of 55–600 mass-to-charge ratio (m/z) at 0.2 scans/s. Data were recorded and processed with TurboMassVer5.2.0 software (PerkinElmer Inc.). The trimethylsilyl (TMS) ethers of fatty acids and alcohols displayed a major peak $[M\text{-}15]^+$ due to methyl loss, whereas the most abundant metabolites with triterpene skeletons exhibited the target $[M]^+$ and a major ion peak $[M\text{-}15\text{-}SiCH_3\text{–}H_2O]^+$.

Polar compounds (carbohydrates) from methanolic extracts were identified by using the same GC–MS instrument and conditions described above. The oven temperature was programmed at 110 °C for 1 min, then ramped at 10 °C/min to 210 °C, increased at 5 °C/min to 260 °C, and then ramped at 4 °C/min to 340 °C for 5 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The split/splitless injector operated in split mode (split ratio, 29:1; split flow, 30 mL/min).

Derivatization of Nonpolar Extracts. TMS derivatives of the hexanic extracts (5 mg) were prepared by the addition of 100 μL of anhydrous CH_2Cl_2 and 100 μL of BSTFA with 1% TMCS to a 1.5 mL amber GC autosampler vial.[23] The mixture was stirred overnight at room temperature. After derivatization, the sample was reconstituted in 1 mL of CH_2Cl_2 and introduced into GC–MS for analysis.

Derivatization of Polar Extracts (Carbohydrates). TMS derivatives of the methanolic extracts (5 mg) were prepared by a two-step procedure involving a methoximation-trimethylsilylation process to a 1.5 mL amber GC autosampler vial. For methoximation, a dried sample (5 mg) of methanolic extract was treated with methoxyamine hydrochloride solution (20 mg/mL in pyridine) (100 μL) and stirred overnight at room temperature. Next, BSTFA with 1% TMCS (100 μL) was added as silylation reagent and stirred for 3 h at room temperature.[24] After derivatization, the sample was reconstituted in 1 mL of CH_2Cl_2 and introduced into GC–MS for analysis.

UPLC-PDA-Q-TOF Analysis. High resolution ESI-MS data of the less polar residues fraction (SPE2), containing mainly flavonoid glycosides, were recorded on a Micromass quadrupole time-of-flight (Q-TOF) spectrometer coupled to an Acquity UPLC-PDA system (Waters, Milford, MA, USA) via an electrospray ionization (ESI) interface. Separation was performed on a Waters Acquity BEH C18 column (150 × 2.1 mm i.d., 1.7 μm). The solvent system consisted of 0.1% formic acid in acetonitrile (phase A) and 0.1% formic acid in ultrapure water (phase B). Gradient conditions were as follows: 100% B at 0 min for 1 min to 55% A in 30 min, held for 2 min, returned to 100% B in 1 min, and equilibrated for 2 min before the next injection; the flow rate was 0.4 mL/min; the column and sample temperatures were kept at 40 and 20 °C, respectively; the sample injection volume was 2 μL. UV spectra were acquired between 210 and 800 nm with a 1.2-nm resolution and 20 points/s sampling rate. The ESI source was operated in negative ionization mode with the capillary and cone voltages at 3.0 kV and 45 kV, respectively. The temperature of the source and desolvation was set at 120 and 300 °C, respectively. The cone and desolvation gas (nitrogen) flows were 500 L/h and 50 L/h. The collision energy was set at 5 eV. ESI data acquisition was collected in Centroid mode in a full scan range from m/z 50–1500 at 0.2 s per scan. The mass spectrometer was calibrated using a polyethylene glycol (PEG) mixture from 200 to 1500 MW (resolution specification 5000 fwhm, deviation <5 ppm RMS in the presence of a known lock mass). All data were acquired using Masslynk NT4.1 software (Waters Corp., Milford, MA, USA).

Metabolites Quantification. *GC-FID Analysis for Nonpolar Metabolites.* The content of each metabolite from hexanic extracts was quantified using a PerkinElmer Clarus 500 gas chromatograph with a flame ionization detector (GC-FID; Wellesley, MA) equipped with a column ZB-5 MS (30 m × 0.25 mm × 0.25 μm; Phenomex Inc., Torrance, CA). The temperature of FID was set at 300 °C, and the oven temperature program and helium flow rate used were the same as for GC–MS analysis. An autosampler injected 1 μL samples into a split/splitless injector at 250 °C, operating in split mode (split ratio, 19:1; split flow, 20 mL/min). Samples (10 mg) of nonderivatized hexanic extracts were dissolved in 1 mL of IS solution (0.2 mg/mL of tetracosane in CH_2Cl_2) to be injected into the GC system. Concentrations of compounds in the hexanic extracts (g of compound per 100 g of hexanic extract) were quantified by previous calibration. Thus, calibration curves were constructed by GC-FID with palmitic acid, linoleic acid, linolenic acid, heptacosane, nonacosane, 1-hexacosanol, triacontane, hentriacontane, 1-octacosanol, tritriacontane, β-sitosterol, lanosterol, β-amyrin, lupen-3-one, cycloartenol, and lupeol. Because the low amounts of butyrospermol and 24-methylenecycloartenol after purification and their commercial unavailability, concentrations of these metabolites were calculated using calibration curves from lanosterol and cycloartenol, respectively. Data were recorded and processed with TotalChrom Workstation software (PerkinElmer Inc.).

HPLC-ELSD Analysis for Carbohydrates. A sample (5 mg) of the more polar fraction (SPE1) obtained by C_{18} SPE was reconstituted in 1 mL of IS (trehalose at 5 mg/mL in H_2O) and injected (5 μL) onto a HPLC instrument (Waters 600E system, Milford, MA, USA) that was equipped with a solvent delivery pump unit (Waters 600E) and coupled to a Waters 2420 ELSD detector. The separation of carbohydrates was carried out using a ProntoSIL 120-3-amino column (125 × 4.6 mm, 3 μm) from Bischoff Chromatography (Leonberg, Germany) and using CH_3CN/H_2O (90:10) as mobile phase with a flow rate of 1 mL/min.

UPLC-PDA Analysis for Flavonoids. A sample (5 mg) of the less polar residues fraction (SPE2, mainly flavonoid glycosides) was reconstituted in 1 mL of IS (flavanone at 1 mg/mL in MeOH), injected onto a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager, sample manager, column compartment, and 2996 PDA detector, connected to Waters Masslynx 4.1 software. The separation was carried out using a Waters BEH C18 column (2.1 × 100 mm, 1.7 μm) at 37 °C. The optimal chromatographic conditions were established: solvent system, phase A, 1% formic acid in acetonitrile, and phase B, 1% formic acid in water; gradient separation system: 100% B at 0 min for 1 min, 55% A at 30 min; flow rate of 0.4 mL/min and injection volume of 5 μL.

External Calibration Curves. Calibration curves were obtained for different nonpolar and polar analytes in different concentrations (25, 100, 200, 500, 1000, and 1500 μg/mL) by injecting in triplicate. The nonpolar, less polar (SPE2), and more polar (SPE1) analytes were dissolved in the following IS solutions: tetracosane in CH_2Cl_2 (0.2 mg/mL), flavanone in MeOH (1 mg/mL), and trehalose in H_2O (5 mg/mL), respectively. For each analysis, the responses taken into account were the peak area ratio, that is the ratio of the analyte peak area over the IS peak area. Linear regression equations were obtained ($Y = a + bX$) by plotting the peak area ratio (Y) versus the ratio of the analyte concentration over the IS concentration (X).

Data Analysis. Contents of metabolites of samples from leaves and stems were determined from each period (phases I, II, and III) and irrigation treatment (T1–T3 and control T0). These contents were

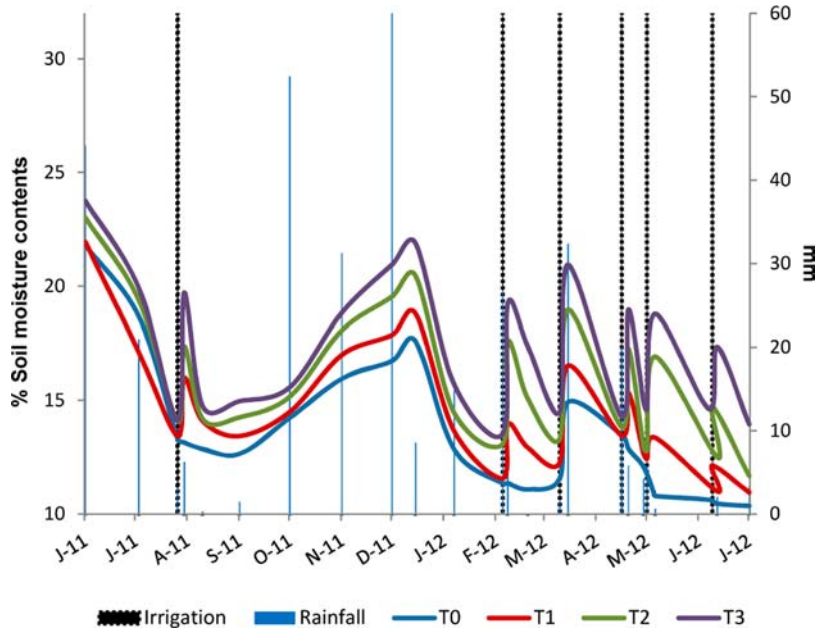

Figure 1. Variations of soil moisture content for control T0 (rainfall) and each treatment (T1-T3) from June 2011 to July 2012 of *E. characias* growing-season. The vertical scale on the right indicates the amount of rainfall (mm).

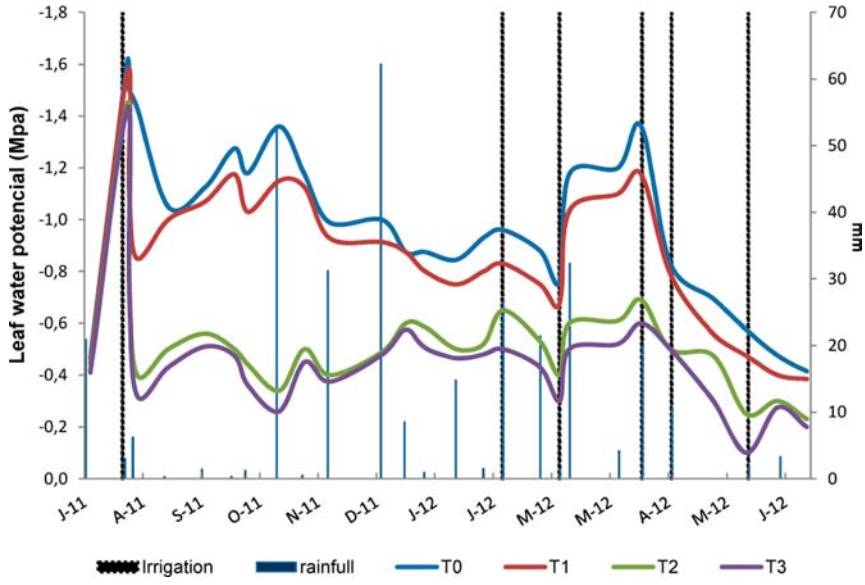

Figure 2. Variations of leaf water potential for control T0 and each irrigation treatment (T1−T3) from July 2011 to June 2012 of *E. characias* growing-season.

expressed as percentage of each compound per 100 g of dry weight and were calculated by triplicate (n = 3) and arranged in a matrix suitable for analysis of variance. A three-way ANOVA was carried out for each metabolite with the software Statgraphics Centurion XVI (Statpoint Int., Warranton) considering three factors: period, irrigation treatment, and aerial part (leaf or stem). For clarity purposes, two-way ANOVAs considering only two of the three factors were also considered. The interactions were also studied. Additional ANOVAs were conducted to study the content of fibers and total metabolites in both hexanic and methanolic extracts. Means were compared using the LSD test (Least Significant Differences) considering 95% confidence intervals. By plotting residuals of each ANOVA model on a normal probability plot, it was found that residuals followed an approximately normal distribution. Furthermore, one or two outliers were identified in a few cases, and the ANOVA was repeated after removing such abnormal values.

RESULTS AND DISCUSSION

Water Stress Experiments. Besides irrigation after planting (80 mm) in April 2011, six other irrigations were applied per container until July 2012, which was the experimental period for the three treatments performed: 30 mm (T3), 15 mm (T2), and 7.5 mm (T1). For all of them, the first irrigation was applied in July 2011 and next irrigations as follows: one in January, one in February, two in April, and one in June 2012, when field capacity decreased by 40% at 30 cm below soil. As expected, different water doses supplied resulted in different patterns of soil moisture content, monitored by a FDR probe (Figure 1). The corresponding leaf water potential variations registered through the course of the experiment are illustrated in Figure 2. Evolution of shoot dry weights per plant

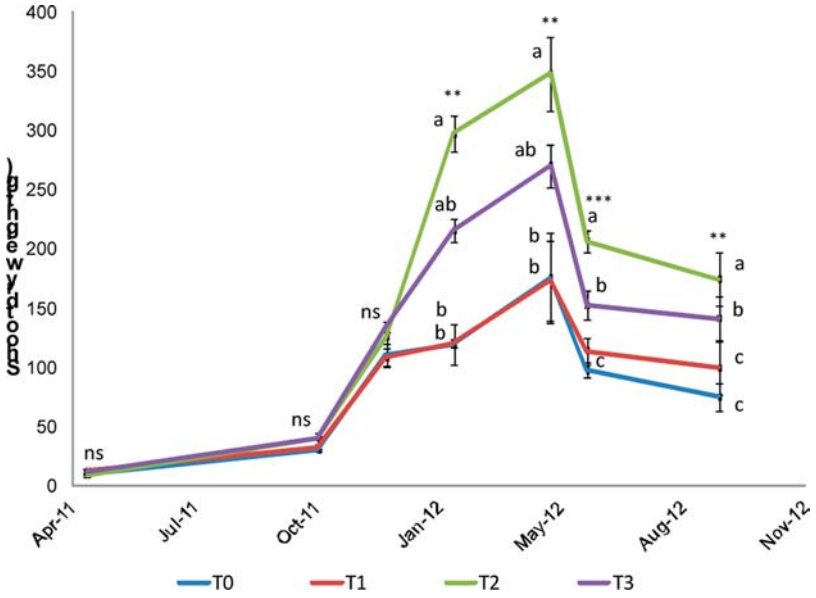

Figure 3. Variations of shoot dry weight for T0 and each treatment (T−T3) during the growing season of *E. characias* from April 2011 to September 2012. Equal letters indicate that differences are not statistically significant (ns).

Table 1. Percentages (g of Dry Extract or Fiber per 100 g of Aerial Part Dry wt) of High Energetic Components of *E. characias* Shoots from the Three Phenological Stages (Phases I, II, and III) and Different Irrigation Treatments (T1−T3 and Control T0)

		shoot aerial part (%)[a]		shoot extracts[a,c] (%)		shoot fibers[a,c] (%)		
	irrigation treatment[b]	L	S	hexanic	methanolic	cellulose	hemicellulose	lignin
phase I	T0	71.80 ± 2.77 a	28.07 ± 2.77 d	8.17 ± 0.22 a	21.57 ± 0.29 a	15.28 ± 0.38 b	10.77 ± 1.54 b	8.23 ± 0.47 a
	T1	64.25 ± 3.08 a	35.75 ± 3.08 d	7.38 ± 0.37 a	21.41 ± 0.62 a	14.68 ± 0.07 b	11.02 ± 0.07 b	7.95 ± 0.17 a
	T2	64.67 ± 2.01 a	35.33 ± 2.01 d	7.72 ± 0.04 a	19.91 ± 0.73 a	15.11 ± 0.04 b	10.21 ± 0.19 b	7.79 ± 1.97 a
	T3	64.53 ± 2.00 a	35.47 ± 2.00 d	7.85 ± 0.31 a	16.39 ± 0.84 b	15.24 ± 1.18 b	10.94 ± 0.37 b	6.95 ± 0.83 a
phase II	T0	50.11 ± 2.06 b	49.89 ± 2.06 c	6.56 ± 0.22 b	15.82 ± 3.18 b	22.53 ± 4.08 a	15.68 ± 1.07 a	5.44 ± 0.76 b
	T1	48.06 ± 2.78 b	51.94 ± 2.78 c	6.30 ± 0.11 b	19.09 ± 0.73 a	22.96 ± 2.92 a	15.83 ± 2.24 a	5.17 ± 0.78 b
	T2	52.93 ± 0.97 b	47.07 ± 0.97 c	5.82 ± 0.33 b	19.28 ± 0.59 a	23.98 ± 1.60 a	14.27 ± 1.77 a	4.24 ± 0.09 b
	T3	54.91 ± 4.09 b	45.09 ± 4.09 c	5.59 ± 0.23 b	18.69 ± 0.18 ab	24.06 ± 2.14 a	16.01 ± 2.18 a	4.82 ± 0.87 b
phase III	T0	26.24 ± 3.72 d	73.76 ± 3.72 a	8.14 ± 0.82 a	10.70 ± 0.68 c	24.86 ± 1.42 a	11.22 ± 0.02 ab	6.97 ± 0.97 a
	T1	37.00 ± 5.56 c	63.00 ± 5.56 b	8.02 ± 1.07 a	15.96 ± 0.12 b	24.33 ± 1.42 a	12.88 ± 0.33 ab	7.39 ± 0.02 a
	T2	30.14 ± 4.21 cd	69.86 ± 4.21 ab	7.61 ± 0.16 a	18.88 ± 0.38 ab	23.10 ± 0.48 a	13.40 ± 0.50 ab	7.89 ± 0.97 a
	T3	31.07 ± 4.00 cd	68.93 ± 4.00 ab	7.40 ± 0.71 a	10.36 ± 1.85 c	20.74 ± 0.20 a	12.15 ± 0.52 ab	5.94 ± 1.30 a
	period	***	***	***	**	***	***	***
	treatment	ns	ns	ns	**	ns	ns	ns
	interactions	*	*	ns	**	ns	ns	ns

[a]Means ± SEM. L, leaves; S, stems. [b]Irrigation treatment applied: T0 (rainwater), T1 (7.5 mm), T2 (15 mm), and T3 (30 mm). [c]Calculated according to the formula: yield = [(L·E) + (S·E)]/100; where L is leaf dry weight (%), S is stem dry wt (%), and E is hexanic or methanolic extract or cellulose, hemicellulose, or lignin (%); Different letters between samples in the same column represent statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001), according to a two-ANOVA with factors: period (phases I, II, and III) and irrigation treatment (T1−T3 and control T0). ns: not statistically significant (p > 0.05).

during the experimental period is graphically represented in Figure 3, giving a sigmoid curve[25] that shows three well-defined growth stages (phases I−III) for *E. characias*. First, phase I (preflowering), which extended from planting in April 2011 to December 2011 and comprised the early vegetative stage with slow shoot growth; During phase II (flowering) from December 2011 to May 2012, plants underwent a rapid vegetative growth by changing to the reproductive stage, in which significant shoot growth occurred with the subsequent development of flowering shoots and inflorescences; finally, phase III (postflowering) covered the period from May to September 2012, when plants returned to early vegetative growth and during which leaf loss became significant (Figure 3). Under the experimental conditions used to study the irrigation requirements besides rainfall (370 mm), the total amount of irrigation water supplied to the experiments was 180 mm (T3), 90 mm (T2), and 45 mm (T1), distributed in six irrigations. Figure 3 shows that plant growth and, accordingly, shoot dry weights and biomass productions differed among the irrigation treatments applied. The highest water dose T3 (30 mm) produced significantly lower shoot dry weight, suggesting that this semiarid adapted plant becomes susceptible to excess water when overirrigated. The shoot dry weights were also much lower in the control plants (T0) with only rainfall and those irrigated with 7.5 mm (T1), particularly in phases II and III if compared with the plants subjected to treatments T2 and

Table 2. Percentages (g of Dry Extract or Fiber per 100 g of Aerial Part Dry wt) of High Energetic Components of *E. characias* Aerial Parts (Leaves and Stems) from the Three Phenological Stages (Phases I, II, and III) and Different Irrigation Treatments (T1−T3 and Control T0)

		extracts (%)[a]				fibers (%)[a]					
		hexanic		methanolic		cellulose		hemicellulose		lignin	
		L	S	L	S	L	S	L	S	L	S[b]
phase I[b]	T0	8.80 ± 0.03 c	6.55 ± 0.18 c	22.53 ± 0.21 a	19.11 ± 0.74 ab	12.03 ± 0.23 b	23.57 ± 0.74 b	11.77 ± 1.28 b	8.23 ± 2.23 b	8.08 ± 0.04 a	8.62 ± 1.58 a
	T1	7.96 ± 0.35 c	6.35 ± 0.31 c	22.23 ± 0.17 a	19.95 ± 1.59 a	10.58 ± 0.08 b	22.05 ± 0.05 b	11.18 ± 0.07 b	10.73 ± 0.07 b	8.37 ± 0.08 a	7.20 ± 0.33 a
	T2	8.54 ± 0.13 c	6.22 ± 0.26 c	22.35 ± 0.16 a	15.44 ± 0.24 cd	11.92 ± 0.56 b	20.96 ± 1.13 b	8.80 ± 0.22 b	12.80 ± 0.94 b	6.95 ± 1.37 a	9.33 ± 3.07 a
	T3	8.60 ± 0.60 c	6.48 ± 0.16 c	17.70 ± 1.36 a	14.02 ± 0.47 d	11.96 ± 1.02 b	21.21 ± 1.46 b	11.35 ± 0.99 b	10.20 ± 0.75 b	6.10 ± 0.72 a	8.51 ± 1.04 a
phase II[b]	T0	8.27 ± 0.12 b	4.85 ± 0.32 b	16.55 ± 4.48 a	15.10 ± 1.06 cd	21.33 ± 5.63 a	23.74 ± 2.52 a	13.18 ± 1.92 a	18.19 ± 0.22 a	6.55 ± 0.88 b	4.33 ± 0.63 b
	T1	7.96 ± 0.54 b	4.76 ± 0.56 b	21.39 ± 0.82 a	16.96 ± 0.96 bc	22.88 ± 2.58 a	23.04 ± 3.24 a	14.20 ± 1.98 a	17.33 ± 2.48 a	5.32 ± 0.80 b	5.04 ± 0.76 b
	T2	7.00 ± 0.36 b	4.49 ± 0.24 b	20.38 ± 1.52 a	18.05 ± 0.67 ab	19.26 ± 1.83 a	29.28 ± 1.35 a	12.49 ± 0.05 a	16.28 ± 3.83 a	4.45 ± 0.35 b	4.00 ± 0.20 b
	T3	6.70 ± 0.30 b	4.23 ± 0.31 b	19.18 ± 0.73 a	18.10 ± 0.49 ab	23.21 ± 2.41 a	25.11 ± 1.83 a	14.67 ± 1.84 a	14.64 ± 2.59 a	4.98 ± 1.16 b	4.64 ± 0.52 b
phase III[b]	T0	10.23 ± 0.86 a	7.39 ± 0.74 a	15.98 ± 5.71 a	8.83 ± 0.45 e	15.64 ± 0.49 b	28.14 ± 2.10 a	6.40 ± 0.95 c	12.94 ± 0.34 a	6.51 ± 1.30 b	7.13 ± 0.86 a
	T1	8.73 ± 0.43 a	7.60 ± 0.49 a	20.48 ± 1.47 a	13.30 ± 0.57 d	14.08 ± 1.06 b	31.94 ± 2.88 a	7.29 ± 1.01 c	16.17 ± 1.11 a	6.93 ± 0.76 b	7.66 ± 0.48 a
	T2	9.95 ± 0.85 a	6.60 ± 0.17 a	20.70 ± 0.78 a	18.09 ± 0.67 ab	14.64 ± 0.61 b	26.75 ± 0.42 a	6.23 ± 0.93 c	16.50 ± 0.32 a	6.15 ± 0.95 b	8.65 ± 1.81 a
	T3	10.03 ± 0.93 a	6.22 ± 0.61 a	15.44 ± 1.44 a	8.07 ± 1.82 e	13.20 ± 0.29 b	24.14 ± 0.15 a	5.50 ± 0.85 c	15.15 ± 0.37 a	4.78 ± 1.12 b	6.47 ± 1.39 a
	period	***	***	ns	***	***	**	***	***	*	**
	treatment	ns	ns	ns	***	ns	ns	ns	ns	ns	ns
	interactions	ns	ns	ns	***	ns	ns	ns	ns	ns	ns

[a]Means ± SEM. L, leaves; S, stems. [b]Phase I is preflowering, phase II is flowering, and phase III is postflowering period. Different letters between samples in the same column represent statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), according to a two-ANOVA with factors: period (phases I, II, and III) and irrigation treatment (T1-T3 and control T0); ns: not statistically significant ($p > 0.05$).

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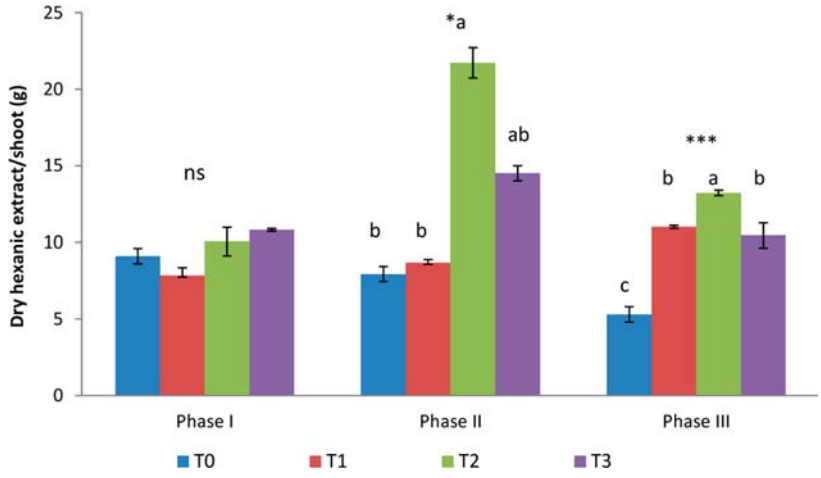

Figure 4. Hexanic extract yields per shoot for *E. characias* harvested from the three phenological stages (phases I–III) and irrigation treatments (T1–T3) and control T0.

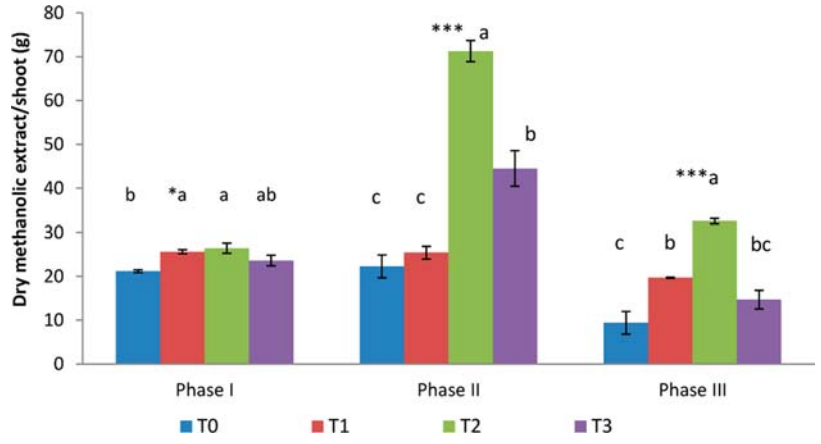

Figure 5. Methanolic extract yields per shoot for *E. characias* harvested from the three phenological stages (phases I–III) and irrigation treatments (T1–T3) and control T0.

T3. This is probably due to the higher temperatures during these periods which caused a higher evaporation rate, and influenced leaf stomata transpiration and concomitant decreases in the leaf water potential (Figure 2).[26,27] As other authors have reported,[28,29] the water deficit under the T0 and T1 conditions, together with the higher temperatures in phases II and III, could constrain photosynthesis by stomatal closure affecting biomass growth, leaf expansion, and, consequently, shoot dry weights (Figure 3). Indeed, this experiment revealed that maximum plant growth was obtained at phase II by applying 15 mm of irrigation dose (T2), in which the shoot dry weights per plant was about 2-fold higher than those of control (Figure 3). Accordingly, we established that the irrigation requirements for *E. characias* were about 460 mm during the experimental period (April 2011 to July 2012) to obtain the maximum dry weight per plant. Furthermore, the highest shoot dry weights per plant were obtained 1 year after planting (from April 2011 to May 2012). This means that only five irrigations of 15 mm (T2) of water were applied until this time, besides rainfall (370 mm), which implies a total of 445 mm (4450 m^3/ha).

Extracts and Biomass Yields. The percentage of yields of hexanic and methanolic extracts, as well as of lignocellulose biomass (cellulose, hemicellulose and lignin), were determined for each phenological stage (phases I–III) and irrigation treatment. These results are reported in Table 1 for shoots (leaves and stems altogether) and in Table 2 for the separated aerial parts (leaves and stems). Significant variations were found in the chemical composition of *E. characias* among the different phenological stages. As mentioned above, the highest plant dry weights of about 348 g of dry matter per plant were reached at the end of phase II under T2 irrigation conditions (Figure 3). In this phase, a fast growth and development occurs followed by flowering (Table 1), which is in accordance with Coppola and Brunori.[30] Our results revealed a slight decrease in relative yields (% of dry wt) of the hexanic extract from phase II, principally in stems (Table 2). This decrease could be due to the energy needs required to complete fast growth and development, which may be achieved from carbohydrate reserves in detriment of hydrocarbons biosynthesis. On the other hand, methanolic extract yields (% dry wt) fluctuated in accordance with the phenological stages and irrigation treatments, especially in phases II and III. It is hardly surprising that overirrigated plants showed diminished methanolic extract yields in phase III, which was observed for plants grown under T3 irrigation conditions (Table 1). Nonetheless, observing the same phenomenon in nonirrigated plants (T0; in phase II and III; Table 1) was less expected. In fact, this can be explained by the stomatal closure result obtained under nonirrigation (T0) conditions, which are close to drought stress and cause low

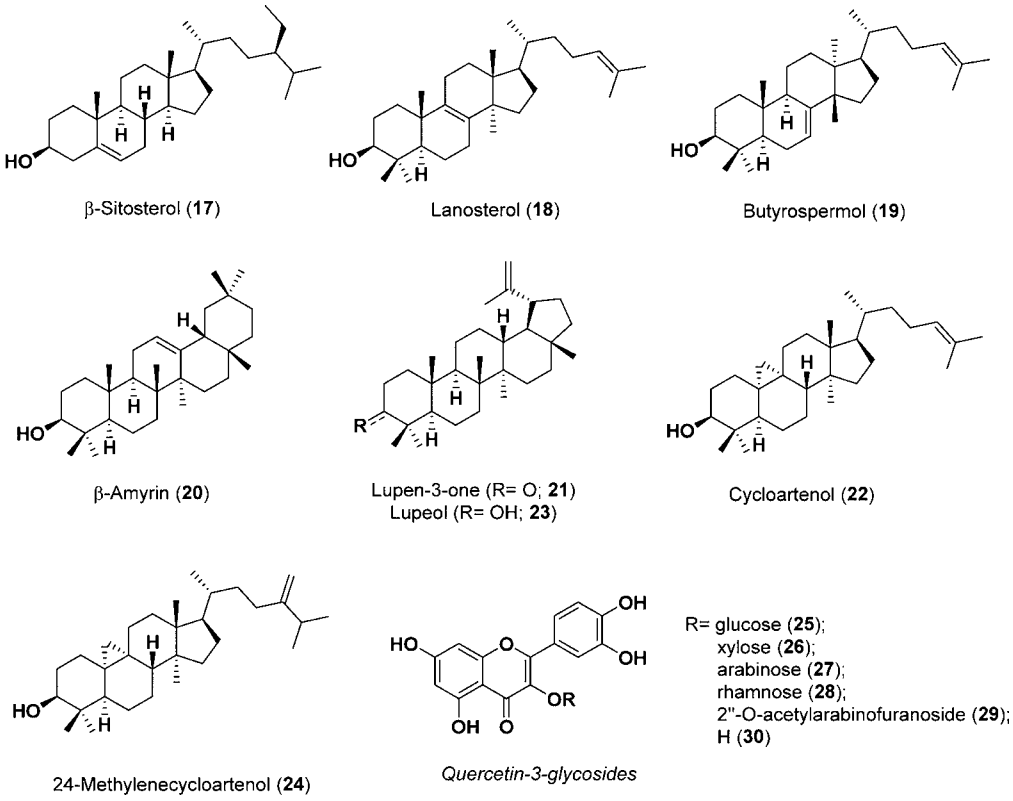

Figure 6. Secondary metabolites identified from hexanic (triterpene and sterol hydrocarbons) and methanolic (flavonoids) extracts of *E. characias* shoots.

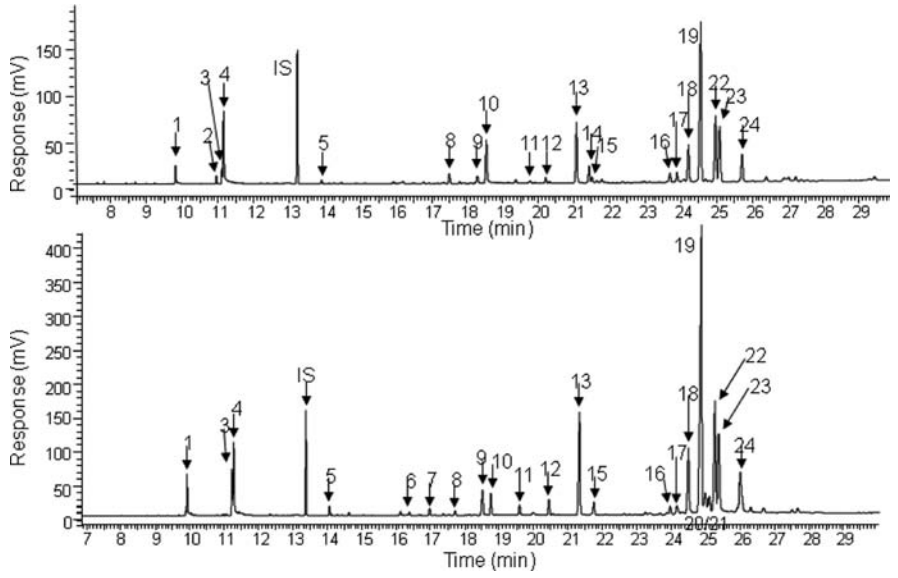

Figure 7. GC-FID chromatograms of leaf (A) and stem (B) of hexanic extracts from plants harvested at phase II (May 2012) and under T2 irrigation treatment. Assignments: **1**, palmitic acid; **2**, phytol; **3**, linoleic acid; **4**, linolenic acid; **5**, pentacosene; **6**, heptacosane; **7**, tetracosanol; **8**, squalene; **9**, nonacosane; **10**, hexacosanol; **11**, triacontane; **12**, octacosanal; **13**, hentriacontane; **14**, α-tocopherol; **15**, octacosanol; **16**, tritriacontane; **17**, β-sitosterol; **18**, lanosterol; **19**, butyrospermol; **20**, β-amyrin; **21**, lupen-3-one; **22**, cycloartenol; **23**, lupeol; **24**, 24-methylenecycloartenol; **IS**, tetracosane.

intercellular CO_2 levels to limit the photosynthetic process. Regarding lignocellulose contents, cellulose and hemicellulose yields (% dry wt) rose during fast vegetative growth (phase II), whereas lignin contents lowered during this period (Table 1). It seems that an increase in shoot dry weights at phase II enhanced the cellulose and hemicellulose contents by reducing the lignin and hexane-extractable yields. From an energy point of view, high-cellulose content biomass rather than lignin is preferred because the conversion of cellulose into glucose via acid/enzymatic hydrolysis, along with the subsequent fermentation of glucose into ethanol, is easier than with the lignin-rich biomass.

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Taking into account that the shoot dry weights per plant change with the different plant phenological stages and irrigation treatments, the total yields expressed as hexane-extractables (mainly hydrocarbons-like) or methanol-extractables (mainly carbohydrates) in grams per unit of plant were calculated and are graphically represented in Figures 4 and 5. The results showed that the highest yields per plant from both hexanic and methanolic extracts, containing metabolites with high energy values were found in phase II and under irrigation treatment T2 (15 mm).

Phytochemical Composition. The major metabolites of *E. characias* shoots were identified and quantified from the leaf and stem extracts in the three plant phenological stages (phases I–III). The metabolite concentrations, given as percentages of hexanic and methanolic extracts (g of metabolite per 100 g of hexanic or methanolic extract), were calculated for all the irrigation treatments (T1–T3). By means of ANOVA, it was found that the differences with respect to the control were not statistically significant ($p > 0.05$) for any metabolite. However, when bearing in mind the presence of variations of shoot dry weights between different irrigation conditions (T0–T3), the largest metabolite amounts in hydrocarbon-like compounds, carbohydrates, and flavonoids corresponded undoubtedly to phase II and applying T2 irrigation dose.

Hexanic Extracts. Twenty-four metabolites were identified in the hexanic extracts by GC–MS after TMS-derivatization. Identified triterpene and sterol hydrocarbons are drawn in Figure 6, and their GC-FID chromatograms are shown in Figure 7 for the optimal T2 irrigation treatment in phase II (flowering). The metabolite concentrations in the hexanic extracts and their distributions in leaves and stems are reported in Tables 3 and Table 4. The most significant changes in the phytochemical compositions were found between the three phenological stages and between leaves and stems depending on the metabolites. Fatty acids, particularly palmitic and linolenic acid, reached their maximum concentration during the flowering period and decreased afterward. The major alkane in the extracts was hentriacontane, reaching the highest concentrations in phase III (Table 3). This result was not unlikely because environmental factors, such as light and humidity, can affect wax production to provide the hydrophobic barrier of the plant surface aimed at preventing nonstomatal water loss.[31] Among the two long-chain alcohols encountered in *E. characias*, hexacosanol was the predominant one, mainly in leaves, with almost 2-fold higher concentrations than stems (Table 4). The most abundant compounds of hexanic extracts were triterpen-3-ols, especially from stem extracts (25–27% and 34–40% for leaves and stems, respectively, Table 4). It is well-known that the *Euphorbia* genus accumulates high concentrations of triterpenoids in latex.[32] Indeed, triterpenoids are synthetized from sugars in laticifers during a process regulated through an osmotically sensitive organelle.[33] These secondary metabolites play an important ecological role in the interaction of plants with the environment. Among triterpen-3-ols, a clear predominance of butyrospermol (euphane-type skeleton) was detected in all the plant phenological stages (9.26–11.01% and 13.76–15.91%, for leaves and stems, respectively), followed by cycloartenol (lanostane-type skeleton), lupeol (lupane-type skeleton), and 24-methylenecycloartenol. In addition, lupeol concentrations remained almost unchanged for both aerial parts during the three phenological stages. β-Amyrin (oleanane-type skeleton) and lupenone (lupane-type skeleton) were detected almost exclusively in

Table 3. Metabolite Composition (g of Metabolite/100 Hexanic Extract) from Hexanic Extract of *E. characias* Grown under T2 Irrigation Treatment

			percentage composition of hexanic extracts (%)[a]								
			phase I		phase II		phase III				
structure	compd	t_R (min)[b]	L	S	L	S	L	S	part	period	interactions
fatty acids	**1**	9.7	1.05 ± 0.27 c	1.74 ± 0.63 b	1.91 ± 0.51 b	2.65 ± 0.33 a	0.00 ± 0.00 d	0.00 ± 0.00 d	**	***	*
	3	11.2	3.23 ± 0.23 b	3.96 ± 0.61 a	3.26 ± 0.45 b	3.69 ± 0.41 ab	2.57 ± 0.21 c	2.15 ± 0.18 c	ns	***	**
	4	11.3	7.54 ± 1.02 a	5.81 ± 1.02 b	7.14 ± 0.88 a	4.54 ± 0.41 c	3.26 ± 0.26 d	3.06 ± 0.24 d	***	***	***
alkanes (C_{27}–C_{33})	**6**	16.4	<0.1 c	<0.1 c	0.25 ± 0.10 a	0.24 ± 0.11 ab	0.12 ± 0.05 bc	0.20 ± 0.12 ab	ns	***	ns
	9	18.4	0.62 ± 0.15 c	1.60 ± 0.37 a	0.53 ± 0.20 c	1.14 ± 0.25 b	0.74 ± 0.24 c	1.07 ± 0.15 b	***	*	ns
	11	19.6	0.10 ± 0.05 c	0.24 ± 0.09 a	0.10 ± 0.04 c	0.18 ± 0.07 b	0.18 ± 0.03 b	0.21 ± 0.05 ab	***	*	*
	13	21.2	4.12 ± 0.69 c	8.56 ± 1.33 b	4.51 ± 0.87 c	5.40 ± 1.22 c	10.71 ± 1.53 a	10.97 ± 1.33 a	***	***	***
	16	23.7	0.51 ± 0.12 c	0.47 ± 0.11 cd	0.35 ± 0.11 cd	0.29 ± 0.13 d	1.34 ± 0.26 a	0.76 ± 0.18 b	***	***	***
cis-polyisoprene			15.70 ± 0.51 a	7.29 ± 1.32 b	13.57 ± 0.45 a	10.87 ± 1.58 ab	14.52 ± 2.41 a	12.80 ± 2.41 a	*	ns	ns
high MW compds			22. 79 ± 1.12 a	11.72 ± 1.50 d	21.37 ± 0.90 ab	14.40 ± 0.79 cd	15.37 ± 1.47 cd	16.85 ± 2.52 bc	**	ns	***

[a]Means ± SEM. L, leaves; S, stems. [b]t_R by GC-FID; Compounds: **1**, palmitic acid; **3**, linoleic acid; **4**, linolenic acid; **6**, heptacosane; **9**, nonacosane; **11**, triacontane; **13**, hentriacontane; **16**, tritriacontane. Different letters between samples at each period and aerial part in the same row represent statistically significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) according to a two-ANOVA with factors: period (phases I, II ,and III) and aerial part (leaves and stems); ns: not statistically significant ($p > 0.05$).

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Table 4. Metabolite Composition (g of Metabolite/100 Hexanic Extract) from Hexanic Extract of *E. characias* Grown under T2 Irrigation Treatment

			percentage composition of hexanic extracts (%)[a]								
			phase I		phase II		phase III				
structure	compd	t_R (min)[b]	L	S	L	S	L	S	part	period	interactions
alcohols (C_{26}, C_{28})	**10**	18.6	6.20 ± 0.71 b	4.22 ± 0.53 c	8.37 ± 1.10 a	4.31 ± 0.37 c	7.79 ± 1.23 a	4.65 ± 0.20 c	***	**	ns
	15	21.6	2.02 ± 0.14 b	2.31 ± 0.20 a	2.02 ± 0.15 b	1.85 ± 0.18 bc	1.86 ± 0.12 bc	1.79 ± 0.10 c	ns	***	**
triterpenes and sterols	**17**	24.0	0.78 ± 0.04 d	0.94 ± 0.16 bc	1.0 ± 0.10 ab	1.07 ± 0.11 a	0.87 ± 0.06 cd	1.03 ± 0.11 ab	***	***	ns
	18	24.4	1.67 ± 0.31 bc	2.69 ± 0.49 a	1.40 ± 0.31 c	1.96 ± 0.57 b	1.78 ± 0.45 bc	2.67 ± 0.54 a	***	**	ns
	19	24.7	9.26 ± 1.26 b	14.57 ± 2.07 a	9.41 ± 1.27 b	13.76 ± 2.25 a	11.01 ± 1.52 b	15.91 ± 2.25 a	***	ns	ns
	20	24.9	0.10 ± 0.02 b	0.82 ± 0.24 a	0.16 ± 0.07 b	0.91 ± 0.23 a	<0.1 b	0.23 ± 0.09 b	***	***	ns
	21	25.1	<0.1 c	1.27 ± 0.36 a	<0.1 c	0.88 ± 0.25 b	<0.1 c	0.95 ± 0.19 b	***	ns	ns
	22	25.3	6.40 ± 1.18 bc	9.15 ± 1.71 a	5.76 ± 0.83 c	7.90 ± 1.18 ab	6.41 ± 0.96 bc	8.63 ± 1.39 a	***	ns	ns
	23	25.4	5.15 ± 0.47 bc	6.59 ± 1.25 a	5.58 ± 0.65 b	4.57 ± 0.45 c	5.48 ± 0.60 b	5.24 ± 0.73 bc	ns	*	***
	24	26.0	2.29 ± 0.30 c	4.62 ± 0.83 a	1.70 ± 0.27 d	3.21 ± 0.46 b	2.27 ± 0.41 c	3.07 ± 0.19 b	***	***	ns

[a]Means ± SEM. L, leaves; S, stems. [b]t_R by GC-FID. Compounds: **10**, hexacosanol; **15**, octacosanol; **17**, *β*-sitosterol; **18**, lanosterol; **19**, butyrospermol; **20**, *β*-amyrin; **21**, lupen-3-one; **22**, cycloartenol; **23**, lupeol; **24**, 24-methylenecycloartenol. Compounds **19** and **14** quantified by calibration curves of **18** and **22**, respectively. Different letters between samples at each period and aerial part in the same row represent statistically significant differences ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) according to a two-ANOVA with factors: period (phases I, II, and III) and aerial part (leaves and stems); ns: not statistically significant ($p > 0.05$).

stems (0.23−1.27%) for all of the plant developmental stages, whereas their amounts were less than with 0.16% in leaves (Table 4). Although cycloartenol is the biosynthetic precursor of sterols, only small amounts of *β*-sitosterol were detected in leaves and stems (0.78−1.03%; Table 4). Rubber (ca. 7−15%) and high-molecular-weight esters (HMWE), probably including esterified triterpenes with long-chain fatty acids (ca. 12−23%), were estimated by gravimetric analysis after saponification and the precipitation process (Table 3). Rubber, *cis*-1,4-polyisoprene, which has been previously identified and characterized in *E.* characias,[34] was identified from the pellet by ^{1}H NMR spectrometry given the presence of three major signals at 5.12, 2.04, and 1.68 ppm, corresponding to the olefinic proton (*H*C═C), the methylene proton (CH_2-C═C), and the methyl proton (*cis*-CH_3-C═C), respectively. As regards distribution, leaf hexanic extracts contained a higher amount of both rubber and HMWE in phases I and II (the preflowering and flowering periods), but they occurred almost in equal amounts at both aerial parts in phase III (postflowering).

Methanolic Extracts. Six metabolites were identified from the SPE1 (100% H_2O) methanolic extracts by GC−MS after TMS-derivatization: pyroglutamic acid, D-glucose, D-fructose, inositol, *myo*-inositol, and sucrose. In addition, D-fructose, D-glucose, and sucrose were further quantified by the HPLC-ELSD analysis from the three developmental stages of the plants, which were grown under the optimal T2 irrigation conditions (Table 5). A section of the HPLC-ELSD chromatogram from leaf extract is depicted in Figure 8. Sucrose is the major product of photosynthesis that acts as a transport molecule in growth, development, storage, signal transduction, and acclimation to environmental stress.[35] The concentration of this nonreducing disaccharide was almost 2-fold higher than those of D-fructose and D-glucose in phase I (preflowering), but its concentration decreased progressively by half in phase III (postflowering) at the expense of increasing yields of D-fructose and D-glucose, probably due to the enhanced activity of the invertases to provide cells with energy for respiration and with carbon for the synthesis of many different compounds.[36]

Furthermore, six flavonoids and flavonoid glycosides were identified by the UPLC-PDA-MS-QTOF analysis in SPE2 fraction (100% MeOH) from the methanolic extracts of *E. characias* in the three phenological stages. These secondary metabolites were quercetin glycosides: quercetin-3-arabinoside and quercetin-3-rhamnoside (quercitrin), which are two flavonoid glycosides previously reported[5] of *E. characias* and quercetin-3-*β*-glucoside, quercetin-3-xyloside, quercetin-3-(2″-*O*-acetyl)arabino-furanoside, and their precursor quercetin (flavon-3-ol skeleton). Their structures and their LC-MS/MS data are provided in Figure 9 and Table 6, respectively. Only three flavonoid glycosides were tentatively identified on the basis of their MS fragmentation patterns.[37] In addition, the major flavonoids of *E. characias* shoots were further quantified from the optimal T2 irrigation treatment using the UPLC-PDA detector (Table 5). Quantifications of unavailable metabolites were determined by the calibration curve of quercetin-3-*β*-glucoside. The metabolite concentrations from the leaf and stem extracts are summarized in Table 5, and a section of their UPLC-PDA chromatograms is provided in Figure 9. Flavonoid glycosides are usually produced in the late flavonoid biosynthesis stage and they accumulate in the vacuoles of epidermal cells, or occasionally in epicuticular waxes.[37] Among their physiological roles, flavonoids possess photoprotective properties and UV−B radiation modifies their production, which is

Table 5. Metabolite Composition (g of Metabolite/100 g Methanolic Extract) from Methanolic Extract of *E. characias* Grown under T2 Irrigation Treatment

			percentage composition of methanolic extracts (%)[a]								
			phase I		phase II		phase III				
structure	compd	t_R (min)[b]	L	S	L	S	L	S	part	period	interactions
carbohydrates	**25**	13.0	14.90 ± 0.11 c	20.26 ± 1.37 b	17.41 ± 1.95 bc	26.03 ± 1.11 a	17.26 ± 1.88 bc	19.61 ± 1.76 b	***	**	*
	26	18.0	15.34 ± 0.34 c	19.17 ± 1.24 bc	18.73 ± 2.03 bc	25.97 ± 2.44 a	21.0 ± 2.88 b	28.44 ± 2.55 a	***	***	ns
	27	50.0	28.90 ± 2.25 a	23.23 ± 2.56 b	15.67 ± 0.26 c	14.68 ± 0.46 cd	12.15 ± 1.26 de	11.65 ± 0.24 e	*	***	*
flavonoids	**28**	10.5	0.66 ± 0.13 b	0.19 ± 0.05 c	2.23 ± 0.26 a	0.14 ± 0.04 c	0.90 ± 0.32 b	0.77 ± 0.24 b	***	***	***
	29	11.3	0.40 ± 0.09 bc	0.30 ± 0.11 c	1.05 ± 0.04 a	0.49 ± 0.19 bc	0.58 ± 0.20 b	0.44 ± 0.13 bc	***	***	**
	30	11.6	0.95 ± 0.35 a	0.25 ± 0.03 b	1.25 ± 0.20 a	0.27 ± 0.09 b	1.03 ± 0.35 a	0.27 ± 0.05 b	***	ns	ns
	31	11.9	10.70 ± 2.19 b	4.15 ± 0.99 c	16.57 ± 1.92 a	4.06 ± 1.38 c	14.30 ± 1.91 a	5.46 ± 1.04 c	***	**	**
	32	13.3	0.07 ± 0.03 b	0.02 ± 0.00 b	0.18 ± 0.05 a	0.03 ± 0.01 b	0.12 ± 0.05 ab	0.03 ± 0.01 b	**	ns	ns
	33	14.7	0.30 ± 0.09 c	0.83 ± 0.19 ab	0.78 ± 0.21 a	0.76 ± 0.33 ab	0.68 ± 0.30 abc	0.42 ± 0.17 bc	ns	ns	*

[a]Means ± SEM. L, leaves; S, stems. [b]t_R (min) by HPLC-ELSD detector for **25**–**27** and UPLC-PDA for **28**–**33**. Compounds: **25**, D-fructose, **26**, D-glucose; **27**, sucrose; **28**, quercetin-3-*β*-glucoside; **29**, quercetin-3-xyloside; **30**, quercetin-3-arabinoside; **31**, quercitrin; **32**, quercetin-3-(2″-*O*-acetyl)arabinofuranoside; **33**, quercetin; Flavonoids **29**, **30**, **32** quantified by calibration curve of **28**. Different letters between samples at each period and aerial part in the same row represent statistically significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) according to a two-ANOVA with factors: period (phases I, II, and III) and aerial part (leaves and stems); ns: not statistically significant ($p > 0.05$).

Figure 8. HPLC-ELSD chromatograms of samples taken in (A) December 2011, (B) April 2012, and (C) September 2012 of leaf SPE1 methanolic extracts of plants harvested under T2 irrigation treatment. Assignments: **25**, D-fructose; **26**, D-glucose; **27**, sucrose; **IS**, trehalose.

probably the reason why they appear in the leaves of *E. characias* in considerable amounts, mainly in phases II and III.[38] The most abundant flavonoid glycoside was quercitrin, being 4-fold more concentrated in leaves than in stems (16.57% versus 4.06%) in the flowering period (phase II).

A two-way ANOVA was carried out with two factors (irrigation dose, developmental stage or aerial part) for the major constituents: hydrocarbon-like metabolites (fatty acids, *n*-alkanes, alcohols, and triterpen-3-ols), carbohydrates (fructose, glucose, and sucrose), flavonoid glycosides (mostly quercitrin), and lignocellulose-biomass (cellulose, hemicellulose, and lignin). Figure 10 shows the interaction plots for each major family of metabolites (g per unit of plant). This plots graphically highlight the highest amounts of rich energy constituents of *E. characias* from the different growing stages. Although data from the four irrigation doses are only available for hydrocarbon-like compounds, Figure 10 suggests that the highest amounts of metabolites were obtained from phase II (coinciding with the end of flowering) and under T2 irrigation conditions.

Moreover, this experimental study allowed us to obtain further results from an energy point of view. Indeed, under the T2 irrigation and the experimental field conditions (planting density of 60 000 plants/ha), the hexanic and methanolic extract yields may be around 1287 kg/ha and 4284 kg/ha, respectively. Consequently, we were able to reach an amount of at least 5500 kg/ha of chemicals with a high-energy value per

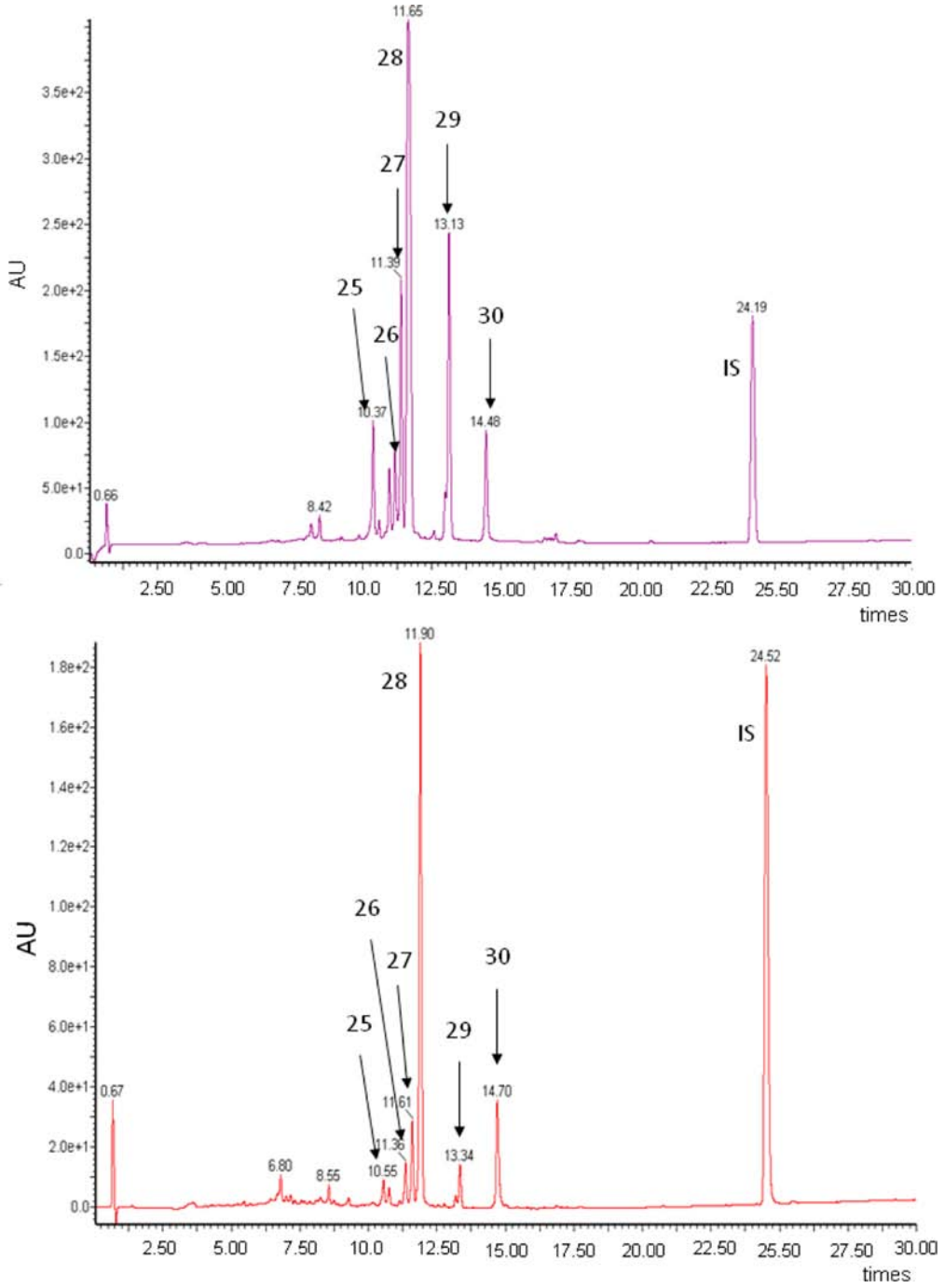

Figure 9. UPLC-PDA chromatograms of samples taken in April 2012 (phase II) of leaf (A) and (B) stem of SPE2 methanolic extracts from plants harvested under T2 irrigation treatment. Assignments: **28**, quercetin-3-*β*-glucoside; **29**, quercetin-3-xyloside; **30**, quercetin-3-arabinoside; **31**, quercitrin; **32**, quercetin-3-(2″-O-acetyl)arabinofuranoside; **33**, quercetin; **IS**, flavanone.

year, although these amounts may be substantially greater by augmenting planting density in the field (by at least 2- or 3-fold). After also, taking into account Demirbas'[39,40] empirical equations, we were able to predict high heating values based on either the lignocellulose-biomass chemical composition (17 MJ/kg, with the equation HHV = 0.088*L* + 16.8218, where *L* is the weight percentage of lignin) or the elemental analysis of hexane-methanol extractives (40 MJ/kg and 18 MJ/kg for the hexanic and methanolic extracts, respectively, with the equation HHV = 0.335*C* + 1.423*H* − 0.154*O* − 0.145*N*, where *C*, *H*, *O*, and *N* are the weight percentages of dry extracts). Therefore, the energy content of lignocellulose-biomass is similar to other energy plant species, and falls within the 17−21 MJ/kg range, such as *Miscanthus* and switchgrass;[41] the energy content from the hexanic extract is only slightly lower than diesel (45 MJ/kg) and the methanolic extract was somewhat higher than wood (15 MJ/kg).

To summarize, we have established that the end of phase II (particularly, the end of the flowering period) is the optimal harvesting time to maximize *E. characias* yields as a potential energy crop. The total water requirements to obtain the maximum yields of hexane- and methanol-extractables are

Table 6. Compounds Identified in Fraction SPE2 from Methanolic Extract of *E. characias* and Their LC-MS/MS Data[a]

analyte	MW (Da)	t_R (min)	λ (nm)	MS [M-H] (m/z)	MS/MS [M-H] (m/z)
28	464	10.34	256, 355	463.09	301.03
29	434	11.15	256, 355	433.08	300.04
30	434	11.38	256, 353	433.08	301.04
31	448	11.65	256, 350	447.09	301.04
32	476	12.99	256, 354	475.09	300.03
33	302	14.47	255, 371	301.04	151.01

[a]Analytes: **28**, quercetin-3-β-glucoside; **29**, quercetin-3-xyloside; **30**, quercetin-3-arabinoside; **31**, quercitrin; **32**, quercetin-3-(2″-*O*-acetyl)-arabinofuranoside; **33**, quercetin.

approximately 445 mm of water (4450 m^3/ha) for their annual development cycle. Accordingly, plants produced during the flowering period the largest amounts of the hexane-extractables that contain high energy hydrocarbons-like compounds, which can be converted directly into biofuels by established catalytic hydrocracking.[42] The methanol-extractables provide vast contents of easily fermentable sugars, while a lower content of flavonoid glycosides can be hydrolyzed to obtain aglycones (with antioxidant properties that are useful as nutraceutical components) and free fermentable sugars. In addition, the lignocellulose-biomass of the plant under study, which is a cellulose-rich biomass, can also produce fermentable sugars by previous enzymatic or chemical hydrolysis.[42]

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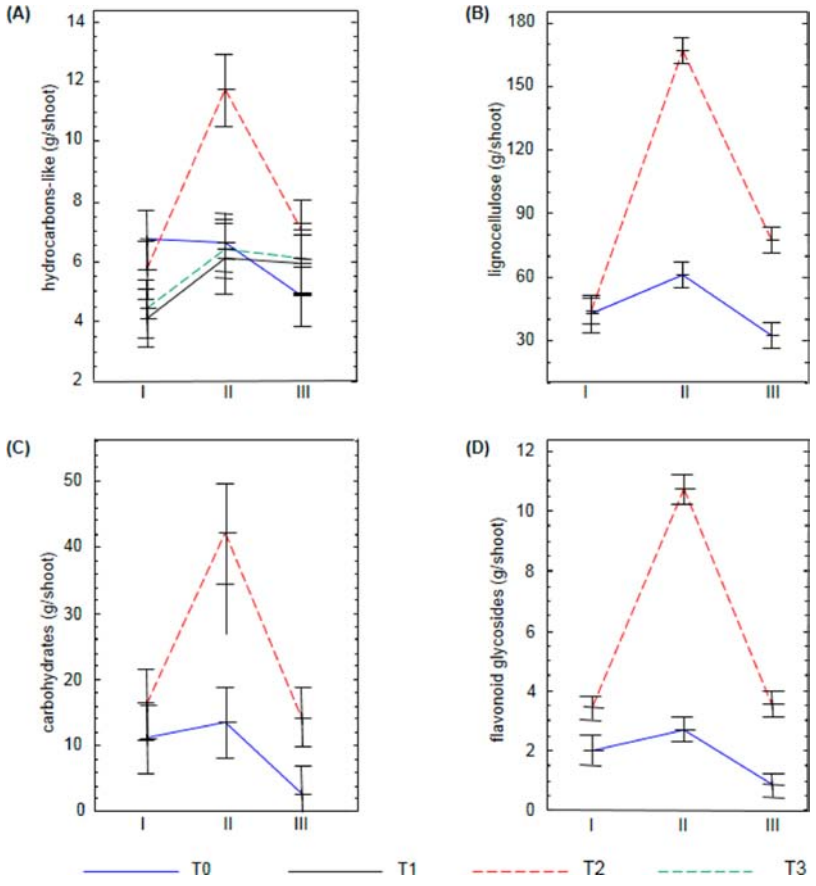

Figure 10. ANOVA interaction plot showing the effect of irrigation dose and developmental stage of the plant on the amount (g/dry shoot weight) of major constituents: (A) hydrocarbon-like metabolites; (B) lignocellulose-biomass; (C) carbohydrates; (D) flavonoid glycosides. Intervals of least significant differences (LSD) are calculated with a confidence level of 95%.

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