Extracellular lipids of *Camelina sativa*: Characterization of chloroform-extractable waxes from aerial and subterranean surfaces

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Abstract

Camelina sativa (L.) Crantz is an emerging low input, stress tolerant crop with seed oil composition suitable for biofuel and bioproduct production. The chemical compositions and ultrastructural features of surface waxes from *C. sativa* aerial cuticles, seeds, and roots were analyzed using gas chromatography and microscopy. Alkanes, primary fatty alcohols, and free fatty acids were common components of all analyzed organs. A particular feature of leaf waxes was the presence of alkyl esters of long-chain fatty acids and very long-chain fatty alcohols, ranging from C₃₈ to C₅₀ and dominated by C₄₂, C₄₄ and C₄₆ homologues. Stem waxes were mainly composed of non-sterol pentacyclic triterpenes. Flowers accumulated significant amounts of methyl-branched *iso*-alkanes (C₂₉ and C₃₁ total carbon number) in addition to straight-chain alkanes. Seed waxes were mostly primary fatty alcohols of up to 32 carbons in length and unbranched C₂₉ and C₃₁ alkanes. The total amount of identified wax components extracted by rapid chloroform dipping of roots was 280 µg g⁻¹ (fresh weight), and included alkyl hydroxycinnammates, predominantly alkyl coumarates and alkyl caffeates. This study provides qualitative and quantitative information on the waxes of *C. sativa* root, shoot, and seed boundary tissues, allowing the relative activities of wax biosynthetic pathways in each respective plant organ to be assessed. This detailed description of the protective surface waxes of *C. sativa* may provide insights into its drought-tolerant and pathogen-resistant properties, and also identifies *C. sativa* as a potential source of renewable high-value natural products.

Keywords

Camelina sativa, cuticle, seed, root, waxes, triterpenoids, alkyl hydroxycinnamates, gas chromatography, mass spectrometry, scanning electron microscopy

1. Introduction

Camelina sativa (L.) Crantz, also known as ‘false flax’ or ‘gold of pleasure’, is an oilseed crop of the Brassicaceae family. It is native to south-eastern Europe and south-western Asia and has been cultivated for its oil and fibre since ancient times (Bouby, 1998). It is cultivated today on a small scale for the health food market as its oil is rich in ω -3 fatty acid (α -linolenic acid) and its protein-rich seed meal can be used for animal feed (Colombini et al., 2013; Putnam et al., 1993). There is considerable recent interest in developing *C. sativa* as a biofuel and high-value chemical production crop, especially on the Great Plains of North America (Iskandarov et al., 2014). *C. sativa* has a relatively short growth cycle (85-100 days to maturity) and is easily transformed by *Agrobacterium tumefaciens* (Lu and Kang, 2008). There have also been recent advances in the genomics of *C. sativa*, including large-scale gene sequencing and transcriptomic studies (Liang et al., 2013; Mudalkar et al., 2014; Nguyen et al., 2013). *C. sativa* is thus a good plant for basic research studies as well as for translation to field applications using genetically engineered varieties.

The advantages of *C. sativa* as a biofuel and bioproduct crop plant are its low input costs and ability to grow on marginal agricultural lands (Iskandarov et al., 2014; Putnam et al., 1993). *C. sativa* is frost-tolerant and requires less water and fertilizer than other oilseed crops such as Canola (*Brassica napus*) (Iskandarov et al., 2014). It also has a relatively high level of resistance to insect pests and plant pathogens that negatively affect other Brassicaceae crops (Francis and Warwick, 2009). These traits, as well as its short growing cycle, allow *C. sativa* to be grown on lands that are not currently used for food crops or in double cropping systems with *C. sativa* sown in late fall or early spring followed by another crop such as wheat or soybean. The mechanism(s) behind the

stress-tolerant attributes of *C. sativa*, and whether they can be further improved, are currently unclear.

All land plants have aliphatic and phenolic-based barriers present at environmental interfaces that serve to protect them against water loss and various stresses. Their aerial surfaces are coated with a cuticle, which is comprised of cutin and waxes (Samuels et al., 2008). Cutin is a polymer mostly made up of glycerol and long-chain (C16 and C18) fatty acid derivatives overlaying the cell wall and serves as the structural backbone of the cuticle. Cuticular waxes are mixtures of low polarity compounds, including very-long-chain ($\geq C_{20}$) straight-chain aliphatics (e.g. fatty acids, fatty aldehydes, alkanes, primary and secondary fatty alcohols, wax esters, and β -diketones), triterpenoids, and phenolic-lipids (e.g. alkylrescorcinols) (von Wettstein-Knowles, 2012). Intracuticular waxes are embedded in the cutin matrix and epicuticular waxes, often in the form of crystals, cover the outer surface. Cuticular waxes serve the essential function of limiting non-stomatal water loss (Riederer and Schreiber, 2001; Vogg et al., 2004). Epicuticular waxes being in direct contact with the environment play key roles in plant-insect and plant-pathogen interactions as well as in the shedding of dust, spores, and water droplets (Kerstiens, 1996). Cuticular wax composition varies substantially between species, organs of the same plant, developmental stages and environmental conditions (Jetter et al., 2006). Another hydrophobic surface barrier is suberin. Similar to cutin, suberin is a polyester of glycerol, phenolics and fatty acid derivatives (long-chain and very-long-chain) (Franke and Schreiber, 2007). Suberin is found on the inner face of cell walls of certain tissue layers, for example the root endodermis and peridermis. There are also waxes associated with suberin, and when in roots termed root waxes (Espelie et al., 1980; Kosma et al., 2012; Li et al., 2007). Suberin-associated or root wax compounds variably include long-chain and very-long-chain fatty acids, primary fatty alcohols, alkanes,

monoacylglycerols, sterols, and alkyl hydroxycinnamates (fatty alcohols esterified with coumaric, caffeic, or ferulic acids) (Kosma et al., 2012).

The surface waxes of *C. sativa* may confer, in part, its relative tolerance to various abiotic and biotic stresses. These extracellular lipids may also represent an additional source of high-value components that can be extracted from the plant. We present here a detailed chemical description of the extracellular waxes of *C. sativa* aerial and root organs.

2. Results and Discussion

Wax mixtures from stems, leaves, flowers, seeds and roots of *C. sativa* were extracted by rapid immersion in chloroform, silylated, and analyzed by gas chromatography (GC). Molecular components were identified from both their GC-retention behaviour and characteristic mass spectra (MS), and quantified by flame ionization detection (FID).

2.1. Leaf cuticular wax

Leaf samples were analyzed at two different growth stages along the vegetative or primary stem, identified as “top” (youngest) and “bottom” (oldest) (Fig. 1A). Total leaf wax loads were not significantly different between the top and bottom portions, which accumulated $6.3 \pm 1.1 \mu\text{g cm}^{-2}$ and $5.8 \pm 0.8 \mu\text{g cm}^{-2}$ (mean $\pm$ SD., $n = 4$; Fig. 2, inset), respectively. However, they differed in their relative compositions of wax esters and alkanes (T-test, two-sided $P < 0.05$). Wax esters, were the dominant compounds in both top and bottom leaves (64 and 69 mol %, respectively), followed by very-long-chain primary fatty alcohols, ranging from C_{22} to C_{34} (26-27 mol %), fatty acids (4-5 mol %) and

odd carbon number alkanes (C₂₉ - C₃₃) (3 and 1 mol % in top and bottom leaves, respectively) (Fig. 2).

Scanning electron microscopy studies of leaf surfaces revealed an absence of epicuticular wax crystal structures (Fig. 1H). Similarly, smooth surfaces without crystals were observed on leaves of *A. thaliana* ecotypes Wassilewskija (WS), Landsberg *erecta* (Ler) (Jenks et al., 1995), and C24 (Teusink et al., 2002). Wax loads in these *A. thaliana* ecotypes (1.6, 1.0, and 1.1 $\mu\text{g cm}^{-2}$, respectively) were comparable to total waxes accumulated on *C. sativa* leaves. It is possible that the leaves on these species are covered solely by an amorphous wax layer because a critical amount of lipids is necessary to form crystals, although it is well documented that crystal formation is also influenced by wax chemical composition (Baker, 1982; Rowland et al., 2006). The wax loads on leaves of *Brassica oleracea* and *B. napus* are much higher (25-50 $\mu\text{g cm}^{-2}$) than *C. sativa* leaves and their leaf surfaces are characterized by densely packed dendritic tubes of wax crystals (Baker, 1974; Holloway et al., 1977). The leaf wax compositions of *B. oleracea* and *B. napus* are also very different, being dominated by alkanes, ketones, and secondary alcohols.

2.1.1. Composition of wax esters found in leaf cuticular wax

Alkyl esters of long-chain fatty acids and long-chain fatty alcohols (wax esters), with carbon chain-lengths ranging from C₃₈ to C₅₀, were present in the waxes extracted from leaves but not in those extracted from other organs. Wax esters were quantified by GC-FID, and equaled $4.7 \pm 0.6 \mu\text{g cm}^{-2}$ in top leaves and $4.6 \pm 0.5 \mu\text{g cm}^{-2}$ in bottom leaves (Fig. 2, inset). By comparison, wax ester content in Ler and WS ecotypes of *A.*

thaliana stem cuticular wax is 0.17 and 0.2 $\mu\text{g cm}^{-2}$, respectively, with only traces present in the rosette leaf cuticular wax fractions (0.002 $\mu\text{g cm}^{-2}$) (Jenks et al., 1995).

Saturated wax ester homologues were identified according to their molecular ions (M^{+}). Like wax esters found in cuticular waxes of other species, each *C. sativa* wax ester peak was composed of a mixture of constitutional isomers. To characterize these isomeric mixtures, we compared the relative amounts of the acyl moieties within each ester group present in the mass spectrum. The alcohol ($R'OH$) and acid ($RCOOH$) moieties of the isomeric mixtures were identified according to their most abundant diagnostic fragments, $[R'-H]^{+}$ and $[RCOOH_2]^{+}$, respectively, which are products of cleavage near the ester bond (Table 1) (Aasen et al., 1971; Lai et al., 2007; Sümmchen et al., 1995; Urvanova et al., 2012). For each even-numbered wax ester (C_{38} - C_{48}), the protonated acyl fragments $[RCOOH_2]^{+}$ had higher intensities and were used to calculate the abundance of each wax ester isomer ($n = 4$). The most abundant ions representing the alcohol moiety of the esters were radical cations $[R'-H]^{+}$, produced by elimination of the acyl moiety; these were identified to confirm isomer structure.

Table 1 details the relative amounts of different isomers in the major wax ester homologues (C_{38} , C_{40} , C_{42} , C_{44} , C_{46} , C_{48} and C_{50}). Small amounts of odd-carbon number ester homologues (0.06-0.16 $\mu\text{g cm}^{-2}$ each) with carbon chain lengths C_{41} , C_{43} , C_{45} , C_{47} and C_{49} , were also identified (data not shown); their isomer compositions are not reported here because the method is reliable only when substantial concentrations of components are present in the mixture (Aasen et al., 1971). The most abundant wax esters were C_{42} , C_{44} and C_{46} , which comprised 18, 26 and 13 mol % of the total wax esters of bottom leaves, respectively, and 19, 25 and 18 mol % of the total wax esters in top leaves, respectively (Fig. 2). Substantial amounts of C_{40} (10-11 mol %), and C_{48} (8-11 mol %) were also found in both young and old leaves. The predominant wax ester

isomers were composed of eicosanoic acid (20:0) and 1-tetracosanol (24:0-OH), constituting 68% of the isomeric molecular species in the C₄₄ wax esters, eicosanoic acid (20:0) and 1-docosanol (22:0-OH), constituting 52% of the C₄₂ wax esters, and eicosanoic acid (20:0) and 1-hexacosanol (26:0-OH), constituting 63% of the C₄₆ wax esters (Table 1).

2.1.2. Acyl chain compositions in wax esters

The predominant fatty acids in the wax esters were hexadecanoic acid (16:0) (23 mol %), eicosanoic acid (20:0) (46 mol %), and docosanoic acid (22:0) (18 mol %) (Fig. 3A). Approximately 6 mol % of the wax esters contained the 24:0 acyl moiety. Only a small proportion of wax esters (4 mol %) contained the 18:0 acyl moiety (Fig. 3A) even though roughly equal amounts of free hexadecanoic (16:0) and octadecanoic (18:0) acids were present in leaf cuticular waxes (Fig. 2 and 3A). This observation may imply that at least two wax synthases are involved, one more specific for 16:0 with little activity for 18:0, and one for 20:0 and 22:0 acyl substrates. By contrast, 16:0 is the predominant acyl moiety (~75%) of the wax esters found in *A. thaliana* stems (Lai et al., 2007; Li et al., 2008). Our results also show that there is no obvious relationship between the percentages of free and esterified fatty acid (Fig. 3A). For example, even though 20:0 and 22:0 were the most abundant acyl chains in esters the corresponding free fatty acids were not found in top or bottom leaf waxes.

2.1.3. Alkyl chain compositions in wax esters

Free and esterified alcohols were compared to infer possible substrates for wax ester synthesis in *C. sativa*. Free alcohols were quantified by GC-FID (Fig. 2) and ester-bound

alkyl moieties were deduced from the MS-calculated acyl percentages and the total wax ester chain lengths (Fig. 3B). The most abundant esterified fatty alcohol, 1-tetracosanol (24:0-OH), constituted about 39% of the ester-bound fraction, whereas 1-hexacosanol (26:0-OH) was the most abundant free alcohol (41 mol %). The 26:0 and 22:0 homologues were the next abundant alkyl moieties in wax esters, representing 24 and 27 mol % of the bound alcohols. Longer chain ($\geq 28:0$ -OH) fatty alcohols were either present in small amounts in the wax esters compared to the corresponding free species (1-octacosanol – 28:0-OH, 1-triacontanol – 30:0-OH), or only found in the free forms (1-dotriacontanol – 32:0-OH, 1-tetratriacontanol – 34:0-OH). These observations suggest a preference of the wax synthase enzyme(s) for $C_{22} - C_{26}$ species; however, it is also possible that the longer chain alcohols are not readily available at the sites of wax ester synthesis.

2.2. Stem cuticular wax

Stem samples representing four different growth stages along the bolt were analyzed for their wax compositions (Fig. 1A). “Top” and “side” (secondary stem/shoot) samples represented young stem, whereas “middle” and “bottom” constituted intermediate and older primary stem portions, respectively. All these yielded mixtures of very-long-chain aliphatics, dominated by alkanes, and pentacyclic triterpenes with small amounts of primary fatty alcohols and free fatty acids (Fig. 4). Surprisingly, secondary alcohols and ketones, which are abundant components of the cuticular waxes of *A. thaliana* stems (Li-Beisson et al, 2013) and *B. napus* organs (Molina et al., 2008; Pu et al., 2013), were not found in the analyzed *C. sativa* organs.

Chromatographic analysis of wax components also revealed a major difference in the total wax coverage between the top and bottom of the shoot (Fig. 4, inset). Wax loads

correlated with the presence and density of crystals. The highest wax amount and crystal density was found in top stems ($15.5 \pm 6.5 \mu\text{g cm}^{-2}$; mean $\pm$ SD, $n = 4$), followed by side shoots ($11.0 \pm 5.8 \mu\text{g cm}^{-2}$). Side shoots presented a smaller number of crystals per area unit than top stems (Fig. 1B-E). In middle and bottom stem samples, the extracted waxes dropped to $5.3 \pm 2.7 \mu\text{g cm}^{-2}$ and $1.6 \pm 0.9 \mu\text{g cm}^{-2}$, respectively. The fact that no crystals were observed on these lower surfaces (Fig. 1F-G) suggests that a critical wax concentration for crystallization was not reached. Because the compound class distribution remains similar along the stem (Fig. 4), it is unlikely that the lack of crystals is attributed to differences in chemical composition. Differences in surface microstructure may reflect different eco-physiological roles of cuticular wax in top versus bottom stem. For example, epidermal surface features like wax crystals create a micro-scale surface roughness that is too small for insect claws to grip, effectively reducing the contact area between the plant surface and insect footpads thereby reducing insect traction (Whitney and Federle, 2013). The differential presence of wax crystals on the top portion of the stem versus the bottom portion of the stem could be indicative of mechanisms to deter herbivorous insects from the reproductive organs (flowers) of *C. sativa*.

Pentacyclic triterpenoids of the ursane, oleanane and lupane groups, which constitute widespread plant natural products with nutraceutical properties (Jäger et al., 2009), were abundant constituents of *C. sativa* cuticular stem waxes. Trimethylsilyl (TMSi) ether and/or ester derivatives of erythrodiol, betulin, oleanolic acid, betulinic acid and ursolic acid were identified by direct comparison with mass spectra and chromatographic retention behavior of authentic standards (Fig. S1). Three compounds, namely α -amyrin, β -amyrin and uvaol, were identified by comparison with published spectra (Heinzen et al., 1996; Wang et al., 2011; Wang et al., 2010) (Fig. S2 A, E, F). In rapidly expanding shoots, namely top and side samples, the relative abundances of each of these

secondary metabolites were comparable. Ursane-type triterpenoids, corresponding to α -amyrin and its metabolites ursolic acid and uvaol, were predominant (71%; Fig. 4).

Oleanane-derived triterpenoids, represented by β -amyrin and two of its oxidized derivatives, oleanolic acid and erythrodiol, constituted 24% of the total triterpenoid fraction. Betulin and betulinic acid, but not their precursor, lupeol, constituted 5% of the total cuticular waxes in young stems. In more mature stems (middle stem), the relative content of α -amyrin and β -amyrin decreased but was compensated by a relative increase in the proportions of their metabolites ursolic acid, uvaol, and erythrodiol. Likewise, an increase in the proportion of betulin was also observed in this fraction. Nevertheless, the total content of pentacyclic triterpenoid components, as well as the total wax coverage, decreased substantially in the bottom (older) portions of stems, indicating that increasing stem girth and wax production are not necessarily synchronized processes in *C. sativa* stems.

2.3. Flower wax

Identified wax components found in wax extracts from whole open flowers (Fig. 5) totaled 264 ± 48 $\mu\text{g/g}$ flower sample, and corresponded to 40% of the total chromatographic area. Most of the unidentified peaks had spectra characteristic of disaccharides. Alkanes were the most abundant aliphatic wax components (68% of the identified wax load) with the most abundant alkane being unbranched nonacosane (C_{29}). Branched alkanes, namely *iso*-nonacosane (C_{29} *iso*) and *iso*-hentriacontane (C_{31} *iso*), were identified from diagnostic ions $[\text{M} - 43]^+$ ($[\text{M} - \text{C}_3\text{H}_7]^+$) and $[\text{M} - 15]^+$ ($[\text{M}^+ - \text{CH}_3]^+$) which were much more abundant than in their respective *n*-alkanes (Bauer, 2002). These *iso*-alkanes were present in relatively large amounts. Free fatty acids and fatty

alcohols (i.e. octadecanol) constituted 16% and 1% of the flower wax mass, respectively (Fig. 5). The triterpenoid fraction (15% of the identified aliphatics) included α - and β -amyrin (Fig. S2), and a mixture of co-eluting sterols, namely campesterol-TMSi ether (diagnostic ions: m/z 129, 343, 367, 382, 472 $[M]^+$), and two unidentified sterols with $[M]^+$ m/z 470 and 481.

2.4. Seed wax

Mature *C. sativa* seeds have a size intermediate to the size of *A. thaliana* and *B. napus* seeds (Li et al., 2006) (Fig. S3). If modeled as an ellipsoid, *C. sativa* seed has a surface area of 0.062 cm², which is 2 times smaller than that of *B. napus* and 17 times larger than *A. thaliana* seeds (Li et al., 2006). Although we cannot assume that the extracted waxes are exclusively deposited at the seed surface because both cuticle(s) and suberized cells may be present in more internal seed coat layers (Molina et al., 2008), it is useful to normalize wax mass to unit of seed area for the purpose of comparison with other *C. sativa* organs and also with published data.

Figure 6 shows the composition of waxes readily extracted from mature seeds of *C. sativa* via rapid immersion in chloroform. The identified components (70% of the total chromatographic area) gave a total of 242 ± 56 ng cm⁻², which was the lowest wax coverage of the analyzed *C. sativa* surfaces. This load was in the same range as *A. thaliana* seed surface wax content (370 ng cm⁻²) (Li-Beisson et al., 2013), and more than 3×10^3 times lower than *B. napus* seed wax coverage, which contains 800 ± 0.2 μ g wax cm⁻² (Molina et al., 2008). Alkanes, mainly nonacosane (C₂₉) and hentriacontane (C₃₁), and very-long-chain primary fatty alcohols ranging from C₂₄ to C₃₂, including odd-numbered carbon chain lengths, were predominant (Fig. 6). Seed waxes of other

species of Brassicaceae, namely *B. napus* and *A. thaliana* (Beisson et al., 2007; Molina et al., 2008), are instead dominated by nonacosane (C₂₉ alkane) and its 15-hydroxy and oxo derivatives, resembling the composition of *A. thaliana* leaf waxes (Rashotte et al., 2001). Most of the primary fatty alcohols in *C. sativa* seed waxes were C₂₈-C₃₂ in length (including odd-chain homologues), much longer than the predominant fatty alcohols found in the waxes of other organs analyzed. This indicates that further elongation of very-long-chain fatty acyl-CoAs occurs in seeds, perhaps by a seed coat-specific fatty acid elongase. A fatty acyl reductase capable of reducing the C₂₈-C₃₂ fatty acyl-CoAs to fatty alcohols must also be present in seeds.

2.5. Root wax

Eight-week-old roots of *C. sativa* plants contain a well-developed suberized periderm, as indicated by the characteristic autofluorescence of cell wall structures observed by fluorescence microscopy (Fig. 1I). Quick solvent immersion allows extraction of periderm-associated waxes (Espelie et al., 1980; Li et al., 2007). Entire *C. sativa* roots were dipped in chloroform for 1 min and previous studies have shown that most of the waxes extracted by this process derive from the taproot and are likely associated with the suberized periderm (Kosma et al., 2012).

Gas chromatographic analyses of taproot chloroform extracts revealed the presence of a complex mixture of compounds (Fig. S4). To better identify and quantify *C. sativa* root wax constituents, root wax extracts were subjected to solid-phase extraction (SPE) and then fractionation following a previously described procedure (Gutiérrez et al., 2008) with some modifications (see Experimental). Use of this fractionation technique allowed the isolation and identification of approximately 25% of root wax compounds representing 276 ± 58 µg per gram of root fresh weight (Fig. 7). Identified root wax

constituents presented a composition similar to *A. thaliana* root waxes (Li et al., 2007), including a profile dominated by alkyl caffeates (62 mol % of total identified compounds) and alkyl coumarates (27 mol % of total identified compounds) with traces of alkyl ferulates. The presence of alkyl caffeates is indicative of an acyltransferase activity orthologous to that described in *A. thaliana* (Kosma et al., 2012) for the synthesis of alkyl caffeates in *C. sativa* taproots. Previously unreported in *A. thaliana*, C₁₆ alkyl coumarates and caffeates and trace amounts of C₁₇ and C₂₁ alkyl caffeates were found in the root waxes of *C. sativa*. Other identified root wax constituents included the sterols campesterol, ergosterol, and β -sitosterol (C₂₈: 1, C₂₉: 2, and C₂₉: 1 sterols, respectively), C₁₈-C₂₈ primary fatty alcohols, and C₂₀-C₂₄ monoacylglycerols (both α and β stereoisomers). Lanosterol, taraxerol, β -amyrin, glutinol, α -amyrin, and cycloartenol peaks represented 4%, 6%, 2%, 6%, 3%, and 2%, respectively, of the total chromatogram area of fraction B. An additional non-sterol triterpenoid representing <1% of the total chromatogram area of fraction B was tentatively identified as taraxasterol but could not be distinguished from its geometric isomer Ψ -taraxasterol based on the mass spectrum (Fig. S2). The presence of a different suite of triterpenoids in root versus stem waxes suggests the presence of triterpene synthases with different specificities in these respective organs.

3. Conclusions

This study provides qualitative and quantitative information on the surface waxes of *C. sativa* aerial and underground organs. Substantial chemical diversity was observed in the wax mixtures extracted from the various organs. Despite the short phylogenetic distance from *A. thaliana*, *C. sativa* presented organ-specific wax compositions that were in most instances substantially different from those of *A. thaliana*. The information

presented herein will serve as a basis for future genetic and biochemical studies on plant wax and natural product biosynthesis, and to evaluate the impact of such waxes on *Camelina sativa*'s resistance to stresses. For example, our results indicate that the stem cuticle of this species is a non-sterol, triterpene-rich lipid layer, thus representing an attractive system to study the metabolism of such bioactive compounds. Modification of surface wax loads or compositions of *C. sativa* may further improve the protective properties of these hydrophobic barriers and thereby further increase the stress-resistant attributes of this oilseed crop. Further, it will help to establish *C. sativa* as a platform for the production of value-added natural products of industrial utility in addition to its use as an oil seed crop.

4. Experimental

4.1. Plant material and growth conditions

Camelina sativa (L.) Crantz cv. Celine seeds were obtained from Dr. Chaofu Lu (Montana State University, USA). Surface sterilized seeds were planted on autoclaved PRO-MIX MPV potting mixture (Premier Tech Horticulture) containing 20-20-20 fertilizer and grown in an environmental chamber at 22 °C in a 16-h-light/8-h-dark photoperiod with 100–120 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity and ambient humidity. Seeds were harvested from fully mature, dry plants by manually opening seed pods and then carefully sieving away any non-seed material.

4.2. Wax extractions

The surface waxes of aerial organs were all extracted by immersion of organs in the low polarity solvent chloroform. Leaf, stem, and flower organs from 7-8-week-old plants were dipped for 30 s in enough chloroform (8-24 mL) to cover the organ completely. The solution also contained 1 µg each of *n*-tetracosane (24:0 alkane), 1-pentadecanol (15:0-OH) and heptadecanoic acid (17:0), which served as internal quantification standards. The *n*-tetracosane, 1-pentadecanol and heptadecanoic acid standards were obtained from Sigma-Aldrich and were at least 98% purity. For the flowers, 15-20 partially or fully opened flowers were used. The fresh weight of the flowers was measured prior to dipping. For the leaves, two leaves were taken just below the first silicle ('top' leaves) and two leaves were taken about 5-9 cm above the soil ('bottom' leaves). The leaves were scanned to determine the total surface area. For the stems, 7 to 8 cm sections were taken from different parts of the stem: (1) starting just below the first fully formed secondary inflorescence ('top stem'), (2) centered around the 15th and 16th leaves counting from the bottom ('middle' stem), (3) starting about 5 cm above the soil ('bottom' stem), and (4) from a mature secondary inflorescence starting immediately next to the point of attachment with the primary inflorescence ('side' shoot) (see Fig. 1A for visual depiction). The diameters and lengths of the stem sections were measured using a digital caliper to determine the surface area. For seed waxes, 100 mg of dry mature seeds were dipped for 2 min in 8 ml of chloroform containing the internal standards described above. The above chloroform extracts were then evaporated under a gentle stream of nitrogen.

For root wax extractions, the roots from 8-week-old plants were dug out of soil, washed with tap water, air dried at room temperature, and then dipped for 1 min in chloroform. The solution also contained 5 µg each of *n*-tetracosane (24:0 alkane), 1-pentadecanol (15:0-OH), heptadecanoic acid (17:0), tridecyl (13:0)-ferulate, heptadecyl (17:0)-coumarate, and nonadecyl (19:0)-caffeate, which served as internal quantification

standards. The synthesis and purification of tridecyl (13:0)-ferulate and heptadecyl (17:0)-coumarate is described in Kosma et al., 2012; these standards were >95% pure. The synthesis and purification of nonadecyl (19:0)-caffeate is described below; this standard was >95% pure. The extracts were filtered through glass wool and then evaporated under a gentle stream of nitrogen gas. The extracts were then subjected to fractionation by solid-phase extraction (SPE) using aminopropyl-phase cartridges (Sep-Pak™ Vac 3cc, 500 mg, Waters Ltd.). The fractionation procedure was similar to that described by (Gutiérrez et al., 2008) with some modifications. The above dried chloroform extracts were redissolved in 0.5 mL of hexane:chloroform (4:1) and loaded onto separate SPE cartridges, which had been conditioned with 4 mL of hexane. The columns were then eluted with a sequential series of solvents of increasing polarity: 8 mL of hexane (fraction A), 6 mL of hexane: chloroform (5:1) (fraction B), 8 mL of dichloromethane (fraction C), 8 mL of chloroform (fraction D), 10 mL of diethyl ether: acetic acid (98:2) (fraction E), and 6 mL of methanol (fraction F). Fraction A contained alkanes, fraction B contained triterpenoids, fraction C contained fatty alcohols, alkyl ferulates, sterols, and a series of unidentified compounds; fraction D contained a series of unidentified compounds, fraction E contained alkyl coumarates, alkyl caffeates, and monoacylglycerols. Root wax fractions were evaporated under a gentle stream of nitrogen and analyzed as silylated derivatives by GC-MS as described below (per temperature program used for all waxes except leaf waxes). Root wax constituents from each fraction were quantified using uncorrected peak areas from total ion chromatograms (TIC), unless otherwise noted, relative to internal standards that co-eluted with each fraction. Sterols and fatty alcohols were quantified relative to the 1-pentadecanol (15:0-OH) internal standard found in fraction C. Alkyl coumarates and monoacylglycerols were quantified relative to the heptadecyl coumarate (17:0-coumarate) internal standard found in fraction E. Alkyl caffeates were quantified relative

to the nonadecyl caffeate (19:0-caffeate) internal standard found in fraction E. Alkyl ferulates were quantified relative to the tridecyl ferulate (13:0-ferulate) internal standard found in fraction C. Several constituents were quantified using single ions diagnostic of a given class of constituents: the peak areas of the $m/z = 249$ fragments were used for the alkyl ferulates relative to the tridecyl ferulate internal standard ion fragment and the peak areas of the $m/z = 219$ fragments were used for the 16:0-, 17:0-, and 21:0-caffeates relative to the 19:0-caffeate internal standard ion fragment.

4.3. GC-FID and GC-MS analysis

Wax extracts were derivatized by dissolving the samples in 100 μ L of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) plus 100 μ L of pyridine. The samples were sealed under nitrogen gas, briefly vortexed, and then incubated for 10 min at 110 °C. The derivatized samples were allowed to cool down to room temperature and then evaporated under a gentle stream of nitrogen gas. The samples were re-suspended in heptane:toluene (1:1 v/v) for analysis by gas chromatography. For chemical identifications, the samples were analyzed on an Agilent 6850 gas chromatograph equipped with an Agilent 5975 mass spectrometer. Splitless injection was used with a HP5-MS column (30 m length, 0.25 mm inner diameter, 0.25 μ m film thickness). Temperature settings were as follows: inlet 350 °C, detector 320 °C, oven temperature program was set to 130 °C for 2 min and increased to 325 °C at a rate of 5 °C per minute, oven temperature was then held at 325 °C for 10 min. The helium flow rate was set at 1.5 mL per minute. For most quantifications, the samples were analyzed on an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID). Splitless injection was used with a HP5-MS column (30 m length, 0.25 mm inner diameter, 0.25 μ m film thickness). Temperature settings were as follows: inlet 350 °C,

detector 320 °C, oven temperature program was set to 130 °C for 2 min and increased to 325 °C at a rate of 5 °C per minute, oven temperature was then held at 325 °C for 10 min. The helium flow rate was set at 1.5 mL per minute. For leaf waxes, which contained very-long-chain wax esters, temperature settings were as follows: inlet 350 °C, detector 345 °C, oven temperature program was set to 130 °C for 2 min and increased to 345 °C at a rate of 5 °C per minute, oven temperature then stayed at 345 °C for 10 min. The helium flow rate was set at 1.5 mL per minute. Compounds were identified by their relative retention times and characteristic mass spectra by comparison to authentic standards (Fig. S1), or to published MS data (Bauer, 2002; Christie, 2013; Heinzen et al., 1996; Wang et al., 2011; Wang et al., 2010).

4.4. Determination of wax ester calibration response factors

Because split / splitless injection is known to bias against high molecular weight compounds, calibration curves for wax esters (WEs) with overall carbons C₃₈, C₄₀, C₄₂, C₄₄ and C₄₆ were generated using commercially available standards (Nu-Check Prep Inc., Elysian, MN). Standard curves were generated as follows (modified from Iven et al., 2013): 1) three independent replicate mixtures of WE standards were prepared and used to generate a dilution series of analytes containing 0.002-0.160 mg of each WE and analyzed by GC-FID under the same experimental conditions as the *C. sativa* wax samples; 2) each individual dilution was spiked with a constant amount (1 µg) of internal standard (*n*-tetracosane), which was the same amount of IS used for the analysis of *C. sativa* waxes; 3) calculation of normalized peak areas (peak area/standard area) versus WE amount (mg) generated non-linear (third order polynomial) regression curves ($R^2 > 0.98$) in the range of the standard amounts (Fig. S5 A-E). These calibration curves were generated using Prism 6 software package (GraphPad Software, Inc., La Jolla, CA,

USA). Significant differences in detector response were found with increasing analyte molecular weight. As a result, each wax ester amount was interpolated from their respective calibration curves, with the exception of C₄₈ and C₅₀ for which the C₄₆ calibration curve was used to estimate their loads in samples. Calibration curve coefficients are shown in Fig. S5 F.

4.5. Calculation of seed wax surface area

A. thaliana and *B. napus* seeds have been modelled as ellipsoids and spheres, respectively, to calculate surface areas (Li et al., 2006). *C. sativa* seeds have a shape similar to that of *A. thaliana* seeds and thus were modeled as ellipsoids. We examined 30 seeds using a stereomicroscope (Leica M205C) and calculated seed dimensions (i.e. long and short axis lengths) using Leica LAS EZ software, which allows measuring and annotation (see example in Supplemental Figure 3). Averages of such dimensions were used to calculate the ellipsoid surface area with an online calculator (<http://keisan.casio.com/exec/system/1223392149>).

4.6. Nonadecyl caffeate synthesis and purification

Nonadecyl caffeate (19:0-caffeate) was synthesized via modification of methods described by Spener and Mangold, 1972. Potassium salts of caffeic acid were first prepared by reacting equimolar amounts of caffeic acid (Sigma-Aldrich) with potassium hydroxide in an alcoholic solution (ethanol) at room temperature. Ethanol was removed by evaporation under nitrogen gas at 50 °C leaving a dry residue of potassium caffeate. Equimolar amounts of potassium caffeate and commercially available nonadecyl methanesulfonate (Nu-Chek Prep, Inc.) were mixed in *N,N*-dimethylformamide under

nitrogen gas with constant stirring at 120 °C for 4 h in a silicon oil bath. The reaction mixture was allowed to cool to room temperature and washed with 1 volume of water three times. The organic phase of the washed reaction product was chromatographed on silica thin-layer chromatography plates (PK6F; Whatman) using multiple developments with 3% ethanol in chloroform. The nonadecyl caffeate band was identified by fluorescence quenching under UV light. Nonadecyl caffeate was eluted from the silica with chloroform:methanol:water (5:5:1, v/v/v) and recovered in the chloroform phase after phase partitioning by addition of chloroform and 0.88% KCl (w/v), followed by washing the chloroform phase twice with methanol:water (1:1, v/v) and drying of the organic phase over sodium sulfate. Extracts were evaporated to dryness under nitrogen gas, recovered as waxy solids, desiccated for 2 d, and dry weights collected. Purity was >95% as determined by GC-MS of the silylated product and TLC analysis of the underivatized products.

4.7. Microscopy

4.7.1. Scanning electron microscopy

Stems from 7-8-week-old *C. sativa* plants were air dried on stubs and coated with gold-palladium particles using a Hummer VII sputter coater (Anatech Ltd.). Leaves from these plants were placed directly onto a pre-cooled cryo stage for imaging. The samples were examined in a Vega\\XMU variable pressure scanning electron microscope (Tescan) at an accelerating voltage of 15 kV.

4.7.2. Fluorescence microscopy

The roots from 8-week-old plants were dug out of the soil and carefully brushed clean. Thin sections were made by hand using a razor blade. The sections were mounted in 50 % glycerol and observed under UV light using a using an Axio Imager M2 compound fluorescent microscope (Zeiss).

Acknowledgments

We thank Micaëla Chacón and Tanya Hiebert (Dept. of Biology, Carleton University) for assistance with the lipid extractions, Dr. Jianqun Wang (Carleton University Nano Imaging Facility) for assistance with the SEM imaging, and Debora França (Algoma University, funded by the Brazilian Science Without Borders Program) for preparing publication-quality mas spectral figures. We thank Prof. John Ohlrogge (Michigan State University) for use of gas chromatographs and general support. This work was funded by grants from the Natural Sciences and Engineering Research Council of Canada (O.R. and I. M.), Algoma University (I.M.), and the National Science Foundation of the United States (grant no. MCB–0615563) and the Great Lakes Bioenergy Research Center (under Department of Energy contract no. DE–AC02–05CH11231) (D.K.K.).

Figure Legends

Fig. 1. Images of *Camelina sativa* organs analyzed for surface wax composition.

(A) Eight-week-old plant highlighting stem and leaf regions analyzed. Scale bar = 5 cm. (B) Scanning electron microscopy (SEM) image of top stem epidermis. Scale bar = 20 μm . (C) SEM close-up image of top stem epidermis. Scale bar = 5 μm . (D) SEM image of side shoot epidermis. Scale bar = 20 μm . (E) SEM close-up image of side shoot

epidermis. Scale bar = 5 μm . (F) SEM image of middle shoot epidermis. Scale bar = 20 μm . (G) SEM image of bottom shoot epidermis. Scale bar = 20 μm . (H) SEM image of adaxial surface of leaf taken from middle of the shoot. Scale bar = 20 μm . (I) Ultraviolet light-excited autofluorescence of tap root cross-section; arrow shows suberized periderm. Scale bar = 200 μm .

Fig. 2. Cuticular wax components extracted from *Camelina sativa* leaves by rapid chloroform-dipping. Wax composition from leaves harvested along two positions of the shoot are shown: “top” (younger) and “bottom” (older). * Amounts of C48 and C50 wax esters were calculated using the calibration curve for the C46 standard, therefore the values for these two esters may be less accurate. Inset: total leaf wax coverage; bottom leaf fraction includes odd-chain wax esters, which are not shown in the detailed graph. Mean and standard deviation bars are shown ($n = 4$). Unidentified components represented $4.5 \pm 2.7\%$ of the total peak area in the chromatogram.

Fig. 3. Chain-length distributions of free and esterified fatty acids (A) and fatty alcohols (B) in *Camelina sativa* leaf cuticular waxes. Error bars represent standard deviation of the mean ($n = 4$).

Fig. 4. Developmental variation in *Camelina sativa* stem cuticular wax load and composition. Waxes were isolated from four different sections of the stems (“top” and “side” samples, representing the youngest tissues), intermediate development stage (“mid”), and older stems (“bottom”). Inset: total stem wax coverage by wax class composition. Mean and standard deviation bars are shown ($n = 4$). Note: calibration curves for triterpenoids were not generated and it is possible that amounts are underestimated here.

Fig. 5. Profiling of *Camelina sativa* flower waxes. Mean and standard deviation bars are shown ($n = 4$). FW = fresh weight. *The sterol fraction was comprised of a mixture of co-eluting sterols, one of which was identified as the campesterol TMSi-ether.

Fig. 6. Profiling of *Camelina sativa* seed waxes. Mean and standard deviation bars are shown ($n = 4$).

Fig. 7. Characterization of *Camelina sativa* root waxes. Chain length distribution of individual wax components identified in root extracts per gram of fresh weight (FW). Mean and standard deviation bars are shown ($n = 4$). MAGs: monoacylglycerols (C20-C24 refer to the chain lengths of the acyl moieties esterified to glycerol). Coumarates, ferulates and caffeates represent alkyl esters of each hydroxycinnamic acid derivative (C16-C22 represent the chain lengths of the alcohol part of these esters). FW = fresh weight.

Table Legends

Table 1. Relative isomeric composition of saturated wax esters in *Camelina sativa* leaf cuticular wax and mass spectral data. Values were calculated from the intensities of characteristic ions containing the acyl chain in mass spectra of saturated wax esters. Mean values ($n = 4$) and SD are given for each alkyl ester.

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Table 1. Relative isomeric composition of saturated esters in *Camelina sativa* leaf cuticular wax and mass spectral data. Values were calculated from the intensities of characteristic ions containing the acyl chain in mass spectra of saturated wax esters. Mean values ($n = 4$) and SD are given for each alkyl ester.

Wax ester (WE) (CN:DB) ^a	Components (acid–alcohol)	% of isomer ^b	SD	[M] ⁺	[RCO ₂ H ₂] ⁺	[R' – 1] ⁺
WE 38:0	14:0–24:0	16.44	3.78	565	229	336
	16:0–22:0	79.79	3.21		257	308
	18:0–20:0	1.8	0.9		285	280
	20:0–18:0	1.2	0.7		313	252
	Minor isomers ^c	0.8	0.1			
WE 40:0	14:0–26:0	4.5	2.8	593	229	364
	16:0–24:0	78.4	1.5		257	336
	18:0–22:0	11.5	3.0		285	308
	20:0–20:0	3.5	1.2		313	280
	Minor isomers ^c	2.1	0.7			
WE 42:0	16:0–26:0	36.6	11.5	621	257	364
	18:0–24:0	8.3	1.6		285	336
	20:0–22:0	51.9	12.1		313	308
	22:0–20:0	1.1	0.5		341	280
	Minor isomers ^c	2.1	0.8			
WE 44:0	16:0–28:0	3.4	2.5	649	257	392
	18:0–26:0	3.9	1.0		285	364
	20:0–24:0	68.2	2.2		313	336
	22:0–22:0	21.9	5.5		341	308
	Minor isomers ^c	2.6	0.5			
WE 46:0	16:0–30:0	1.1	0.4	677	257	420
	18:0–28:0	1.1	0.3		285	392

	20:0–26:0	62.8	6.0		313	364
	22:0–24:0	27.2	4.7		341	336
	24:0–22:0	5.5	1.7		369	308
	Minor isomers ^c	2.3	0.4			
WE 48:0	20:0–28:0	34.2	5.3	705	313	392
	22:0–26:0	45.1	2.7		341	364
	24:0–24:0	15.9	2.9		369	336
	Minor isomers ^c	1.5	0.2			
WE 50:0	20:0–30:0	17.7	1.3	733	313	420
	22:0–28:0	30.8	7.1		341	392
	24:0–26:0	44.9	8.1		369	364
	26:0–24:0	3.3	0.9		397	336
	28:0–22:0	3.2	0.9		425	308

^a CN, carbon number of wax ester; DB, number of double bonds.

^b The percentage of a single isomer of a saturated wax ester was calculated based on intensities of the [RCO₂H₂]⁺ ion from 4 biological replicates.

^c Wax ester isomers constituting less than 1% of the mixture were pooled and included in the "minor isomers" category.

Graphical Abstract

Analyses of chloroform-extractable waxes of *Camelina sativa*'s aerial and subterranean surfaces show both overlapping and distinctive chemical compositions within the same species.