Research article

Leaf cuticular wax composition of a genetically diverse collection of lettuce (*Lactuca sativa* L.) cultivars evaluated under field conditions

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ARTICLE INFO

ABSTRACT

Cuticular waxes of plants impart tolerance to many forms of environmental stress and help shed dangerous human pathogens on edible plant parts. Although the chemical composition of waxes on a wide variety of important crops has been described, a detailed wax compositional analysis has yet to be reported for lettuce (*Lactuca sativa* L.), one of the most widely consumed vegetables. We present herein the leaf wax content and composition of 12 genetically diverse lettuce cultivars sampled across five time points during their vegetative growth phase in the field. Mean total leaf wax amounts across all cultivars varied little over 28 days of vegetative growth, except for a notable decrease in total waxes following a major precipitation event, presumably due to wax degradation from wind and rain. All lettuce cultivars were found to contain a unique wax composition highly enriched in 22- and 24-carbon length 1-alcohols (docosanol and tetracosanol, respectively). In our report, the dominance of these shorter chain length 1-alcohols as wax constituents represents a relatively rare phenotype in plants. The ecological significance of these dominant and relatively short 1-alcohols is still unknown. Although waxes have been a target for improvement of various crops, no such work has been reported for lettuce. This study lays the groundwork for future research that aims to integrate cuticular wax characteristics of field grown plants into the larger context of lettuce breeding and cultivar development.

1. Introduction

An edible member of the Compositae family, lettuce (*Lactuca sativa* L.) is one of the most popular leafy vegetables in the world and is an important part of the American diet [1,2]. In the US and Western Europe, lettuce is usually consumed in green salads and

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sandwiches, while in some eastern countries like China and Egypt the stems rather than leaves are the preferred edible portion [3]. Lettuce is cultivated in many countries around the world, with China, US, and India being the top producers [4]. In 2021, the production of head, romaine, and loose-leaf type lettuce was approximately 35.1 million hundredweight (cwt), 12.5 million cwt, and 27.2 million cwt, respectively [5]. Although raw lettuce is 94–95% water, the nutritional value of lettuce is still considerable, being a good
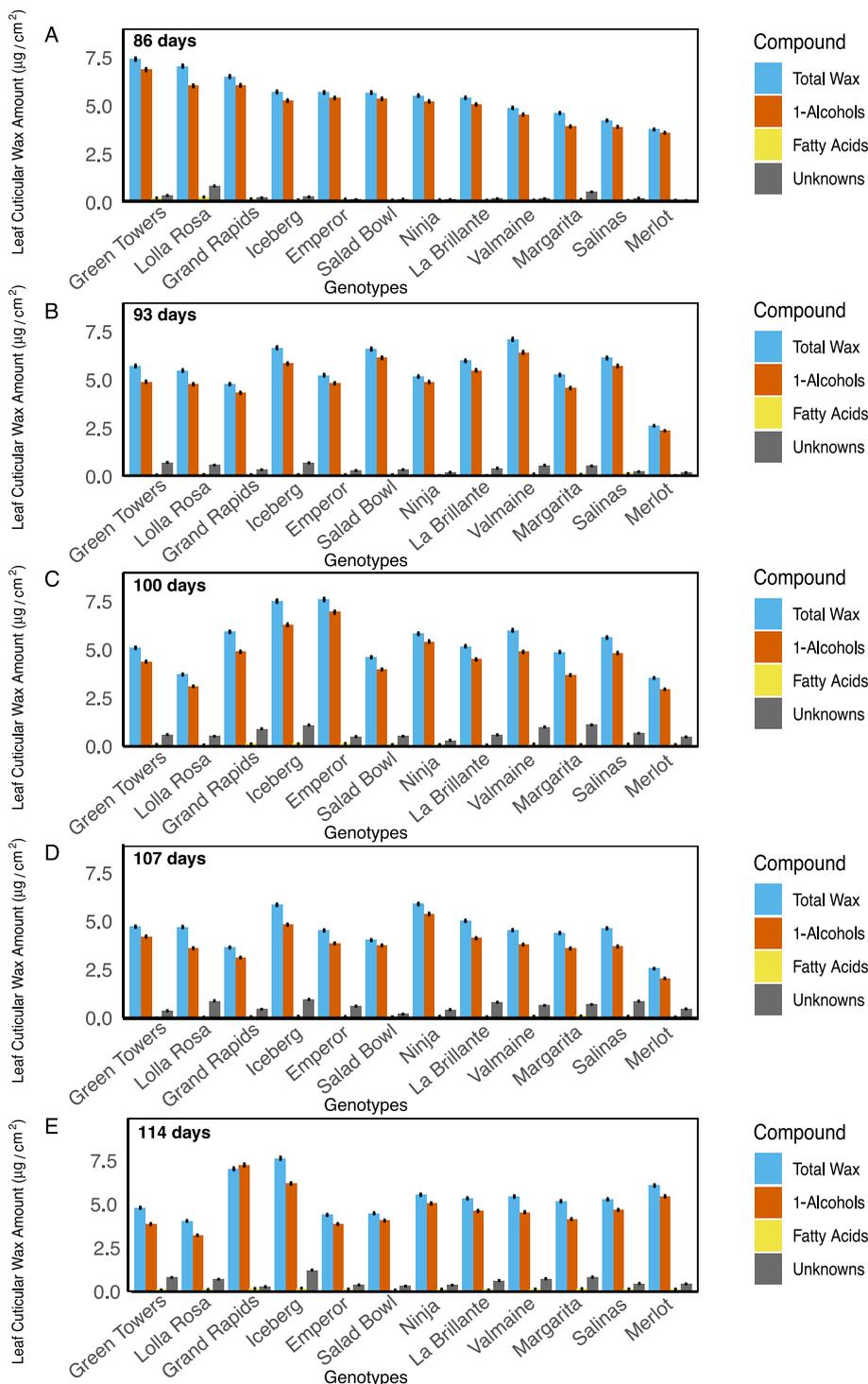

Fig. 1. The concentrations (μg/cm^2) of the total wax compound and the major wax constituents (1-Alcohols, Fatty Acids, and Unknowns) across cultivars on each sampling date (86, 93, 100, 107, and 114 days after planting) are depicted in panels A to E, respectively. Different cultivars are labeled on the horizontal axis for each sampling date, and concentrations of each wax compound are labeled on the vertical axis.

source of certain vitamins, minerals, fiber, and nutraceutical compounds, and low in calories, sodium, and fat [6,7]. Lettuce is a cool-season crop and its optimal temperatures for growth are 23 °C during the day and 7 °C during the night [8]. Lettuce is produced year-round in the US with production mainly concentrated in California and Arizona [5]. The cool coastal valleys of California grow the summer crops while the winter crops are cultivated in the desert valleys of Arizona and California [9].

The availability of irrigation water for lettuce production continues to be a challenge in arid and semiarid regions of the US Desert Southwest due to supra-optimal heat and high evaporative demand [10,11]. Exposure to supra-optimal heat and high vapor pressure deficits can negatively influence the performance and yield of lettuce [12]. Plants that experience heat and water deficits can induce a wide range of physiological responses that help balance evaporative cooling with conservation of tissue water content at levels high enough to protect cellular organelles and membranes from the damaging effects of heat and dehydration stress [13–16]. Studies have shown that an important adaptation conferring increased tolerance to heat and water deficit is a change in the content and composition of the plant cuticle [17].

The cuticle forms the outermost surface over nearly all aerial plant organs, forming a hydrophobic barrier that limits water movement out of the plant [18,19]. The cuticle is composed of a cutin polymer that is coated and embedded with epicuticular and intracuticular waxes, respectively [20,21]. Depending on the plant species and organ, plant waxes are chemically diverse, and often include a mixture of very long chain fatty acids, aldehydes, 1- and 2-alcohols, alkanes, various ketones, esters, and triterpenoids [21–23]. Recent studies in a variety of plant species have shown that water deficits can cause a significant change in the wax phenotype, with water deficits causing total wax amounts to be elevated significantly and wax chemical composition and ultra-structure to change dramatically [24–28]. Studies have linked these changes to reductions in cuticle permeability, reduced plant water loss, extended maintenance of higher relative water contents in tissues, and improved growth and overall tolerance to water deficit environments such as those created by climatic drought [16,29–31].

In addition to the cuticle being critical in mitigating water loss to the environment, the cuticle is also the first line of defense against pathogens and pests as well as microbes that can cause human illness [32,33]. Cases of human food-borne illnesses from contamination of leafy vegetables by *Escherichia coli* have increased in recent years [34–36]. Recent studies have shown that leaf cuticular waxes of lettuce can provide a protective role that prevents attachment of human pathogens such as *Salmonella* spp. [37,38], and this is due to its water shedding capacity (i.e. lotus effect).

This study provides a detailed examination of variation in leaf cuticular waxes on field-grown lettuce plants from six major market classes of lettuce. Our primary objective was to evaluate and characterize variations in cuticular wax compounds with a specific focus on tracking the dynamics of wax profile changes during the pre-flowering stage. In addition, this work investigates the hypothesis that statistically significant variations in wax compounds occur within and across lettuce market classes. We further hypothesized that wax composition changes significantly over time due to plant growth and its interaction with the environment. To address these questions, the amounts of total wax, wax compound classes, and total amounts for individual chemical constituents, were sampled at five different time points from the leaves of 12 diverse lettuce cultivars representing six lettuce market classes. Whereas a general description of waxes on greenhouse-grown lettuce has been reported previously [24,37,39], our study examines lettuce waxes for the first time under field-grown conditions and expands the analysis to provide a more detailed assessment of 12 additional cultivars. Our analyses and results provide insight into lettuce wax composition and properties that might be investigated further for developing new lettuce cultivars that are more climate resilient or can mitigate pathogen contamination.

2. Results and discussion

Previous greenhouse studies reported the total amounts of the major cuticular wax classes on lettuce leaves [24,37,39]; however, no studies have yet presented a more detailed assessment of the individual wax constituents by chain length distribution. Moreover, wax composition on lettuce grown under field conditions has yet to be reported, and this is a critical need before initiating breeding efforts to modify plant waxes for lettuce crop improvement. To date, only partial analysis of leaf cuticular wax chemical composition has been published, and this was limited to four cultivars (the Butterhead cultivar 'Meikoningin', the red Romaine cultivar 'Outred-geous', and the green loose-leaf cultivars 'Two Star' and 'Tropicana') evaluated from greenhouse-grown plants. Lettuce cultivars are classified into horticultural types based on morphology and can be broadly divided into head (round head with a compact heart), romaine (upright loose head lacking a significant heart), and loose-leaf type lettuce. In this study, we examined the leaf waxes on the head forming Butterhead types 'Ninja' and 'Margarita', the head forming crisphead types 'Emperor' and 'Salinas', and the head forming Batavia types 'Iceberg' and 'La Brillante'. This report also presents wax profiles for two cultivars of romaine lettuce 'Valmaine' and 'Greentowers', the green loose-leaf cultivars 'Grand Rapids' and 'Salad Bowl', and the red loose-leaf cultivars 'Lolla Rosa' and 'Merlot'.

Although our studies did not include any of the four cultivars examined previously for leaf waxes [37,39], the values reported here for total wax, ranging from 2.5 to 7.6 µg/cm², and the proportions of major wax constituents (Fig. 1) are comparable to those previously reported in Lu et al. (2015) [40] and Ku et al. (2020) [37], except total amounts were found to be slightly lower in our study. This difference might be explained by the weathering effects of sun, wind, and precipitation that field-grown plants encounter in comparison to greenhouse-grown plants; however, one cannot rule out that it is due to the use of different cultivars for the respective studies. We observed significant variation (P < 0.0001, F test) in the total cuticular wax amounts between lettuce cultivars over the 28-day time course during which samples were collected (Fig. 1). Among cultivars, we observed a nearly two-fold variation in the total wax load between the lowest and highest cultivars. During the time course, the ranking of the highest to lowest wax cultivar shifted considerably, with some of the highest wax containing cultivars on day 86 transitioning to become some of the lowest wax containing cultivars by day 114. In some cases, these changes were associated with variation in growth rates of specific cultivars (Fig. 2A & B) and

may have been due to relative changes in leaf area expansion for later fully expanded leaves. Salad Bowl, Green Towers, Iceberg, and Valmaine, with visibly larger leaves, tended to have less wax per leaf area. Hypothetically, yet not validated experimentally, within species variation leading to larger leaves may be associated with more elongated cells with larger vacuoles rather than an increased cells per area. This is not to contradict mitotic division as a driver of organ expansion, but instead to highlight that during cell maturation there could be a dilution effect caused by the produced wax being distributed over a larger cell surface area (as suggested in Jenks and Ashworth, 1999 [29]). We observed significant changes in wax composition for each of these lettuce cultivars during the developmental phase examined. As previously demonstrated Jenks et al., 1996 and Tipple et al., 2013 [41,42], wax production and distribution can be heterogenous with respect to the specific phenological stages of plant development. In the context of our work, we used cultivars from different market classes that have varied growth and development patterns that resulted in the sampled plants varying slightly within developmental phase. To what degree the variation we report for wax profiles among these cultivars is due to differences in development unique to the genotype, or to genotypic differences in response to precipitation, wind and blowing sand, and/or high solar radiation is a subject for future studies [43,44].

The primary wax constituents observed in our varieties were 1-alcohols, which accounted for 84–92% of total waxes (Fig. 1), and very long chain free fatty acids, which represented 2–3% of total waxes. We also report the presence of four unknown wax constituents that accounted for about 5–14% of total waxes. Mass spectral ion fragmentation patterns were suggestive of triterpenoids; however, further research is needed to verify the exact molecular identity of the unknowns. Notably, Bakker et al. (1998) [24] previously reported the presence of trace amounts of the triterpenoid squalene, but only in very old leaves. Finally, our analyses revealed trace amounts of alkanes in the wax samples, even though both Lu et al. (2015) [40] and Ku et al. (2020) [37] reported these at slightly higher levels in several lettuce samples ("Two Star" lettuce leaves), but still in very low abundance relative to the other lettuce wax constituents. It is possible that the field environment of our studies, which included higher solar radiation (Arizona deserts routinely have solar radiation levels surpassing 2000 μmol m^{-2} s^{-1}, far exceeding the supplemental light levels provided in the greenhouses used in previous studies), rain, wind, and freezing temperatures, conditions that are not present in greenhouse environments, accounts for the altered alkane deposition relative to other wax constituents. It is known that environmental conditions can alter plant wax compositional profiles [30,45].

Our detailed wax analyses showed that the main, very long chain free fatty acids on lettuce were the C24, C26, and C28 free fatty acid homologues (Supplemental Fig. 1). Fatty acids remained relatively low in abundance in comparison to the 1-alcohols, and their ratios remained stable throughout the time course for all cultivars with the C24 being most abundant, followed by the C26, and then the C28 fatty acid being least abundant (Supplemental Fig. 1). The amount of the four unknown wax compounds also maintained stable ratios relative to one another for all cultivars throughout the sampled time points, with unknown compound four being the most abundant compound (Supplemental Fig. 2). The unknowns observed in this study possess mass spectra ion fragmentation patterns consistent with triterpenoids. Terpenoids have been shown to play important roles in plants, especially in their ability to confer resistance to insects [46,47]. However, additional research is needed to identify the exact molecular structure of these unknown compounds as well as to investigate their potential ecological roles with respect to lettuce.

Although the total amounts of leaf wax classes on four lettuce cultivars were previously reported, the individual molecular
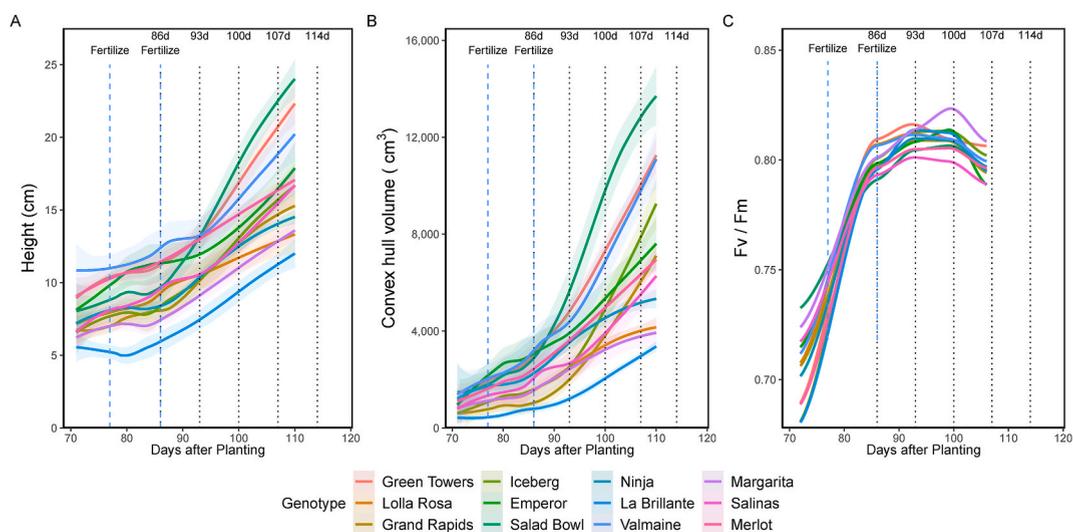

Fig. 2. Lettuce growth and photosynthetic capacity over the growing season. The change in plant height (cm, panel A), convex hull volume (cm^3, panel B), and photosynthetic efficiency (Fv/Fm; the ratio of variable fluorescence to maximum fluorescence after dark adaptation, unitless, panel C) for the 12 lettuce cultivars across the growing season. In each panel, the blue vertical dashed lines represent the days on which the plants were fertilized while the vertical black dashed lines represent the days on which lettuce leaf samples were collected for wax compositional analysis. The x-axis demarcates time as days after planting which occurred on November 13, 2023. It should be noted that phenotyping ceased prior to the last leaf sample collection date.

compounds within each class have not been documented for lettuce. Our analysis shows that lettuce leaves have a relatively unique wax constituent profile compared to other crop plants previously studied. Specifically, our results demonstrate that the 1-alcohols class is composed of relatively high amounts of the 22 carbon 1-alcohol (1-docosanol), and this represents a major flux in the metabolic pathway towards production of the C22 homologues in lettuce wax production, rather than the longer 26, 28, and 30 carbon compounds typically observed in other plants. The C22 1-alcohol is the most abundant wax constituent in most of the samples analyzed for these varieties across all five time points (Figs. 3 and 4). The C24 1-alcohol (1-tetracosanol) was also elevated, and its amounts tracked very closely with the C22 1-alcohol, at nearly all time points being slightly less abundant than the C22 1-alcohol (Figs. 3 and 4). Such high abundance of the C22 and C24 1-alcohols is rare among plant taxa, being previously reported for the C24 1-alcohol only in one Arabidopsis ecotype named CT-1 in a screen of 40 ecotypes [48]. Comparatively, the Yukon ecotype of a drought and salt tolerant extremophile *Eutrema salsugineum* (Pall.) Al- Shehbaz & Warwick (also in the *Brassicaceae* family, like Arabidopsis) produced much higher amounts of the 24 carbon 1-alcohols than all other 1-alcohol chain lengths [28]. Both the Yukon and Shandong ecotypes of *E. salsugineum* also produced especially high amounts of the C24 free fatty acid, revealing a unique high metabolic flux towards production of the C24 homologues in the wax metabolic pathway. The presence of elevated amounts of C22 and C24 1-alcohols in the lettuce wax profile might be explained by several biosynthetic routes including the presence of novel fatty acyl-CoA reductase (FAR) enzymes, whose role in the synthesis of 1-alcohols in plant waxes is well established [49–51]. There might also be unique regulation of the FAR genes or enzymes [52], or perhaps a unique biosynthetic pathway leading to production of shorter chain 1-alcohols. Notably, there are six FAR-like genes identified in the lettuce genome when using *Arabidopsis thaliana* FAR1 as a query (data not shown; Phytozome release V13 [53]), compared to the eight FAR genes present in *Arabidopsis thaliana*. Whether any of these FAR-like genes are involved in wax synthesis in lettuce remains to be determined.

To investigate the temporal relationship between plant growth and development with wax profiles across the growing season, we employed a high throughput phenotyping (HTP) platform to quantify and characterize phenotypic trait variability. As determined using the HTP system, the height of most lettuce plants doubled during the period from 71 to 110 days post-germination, with some
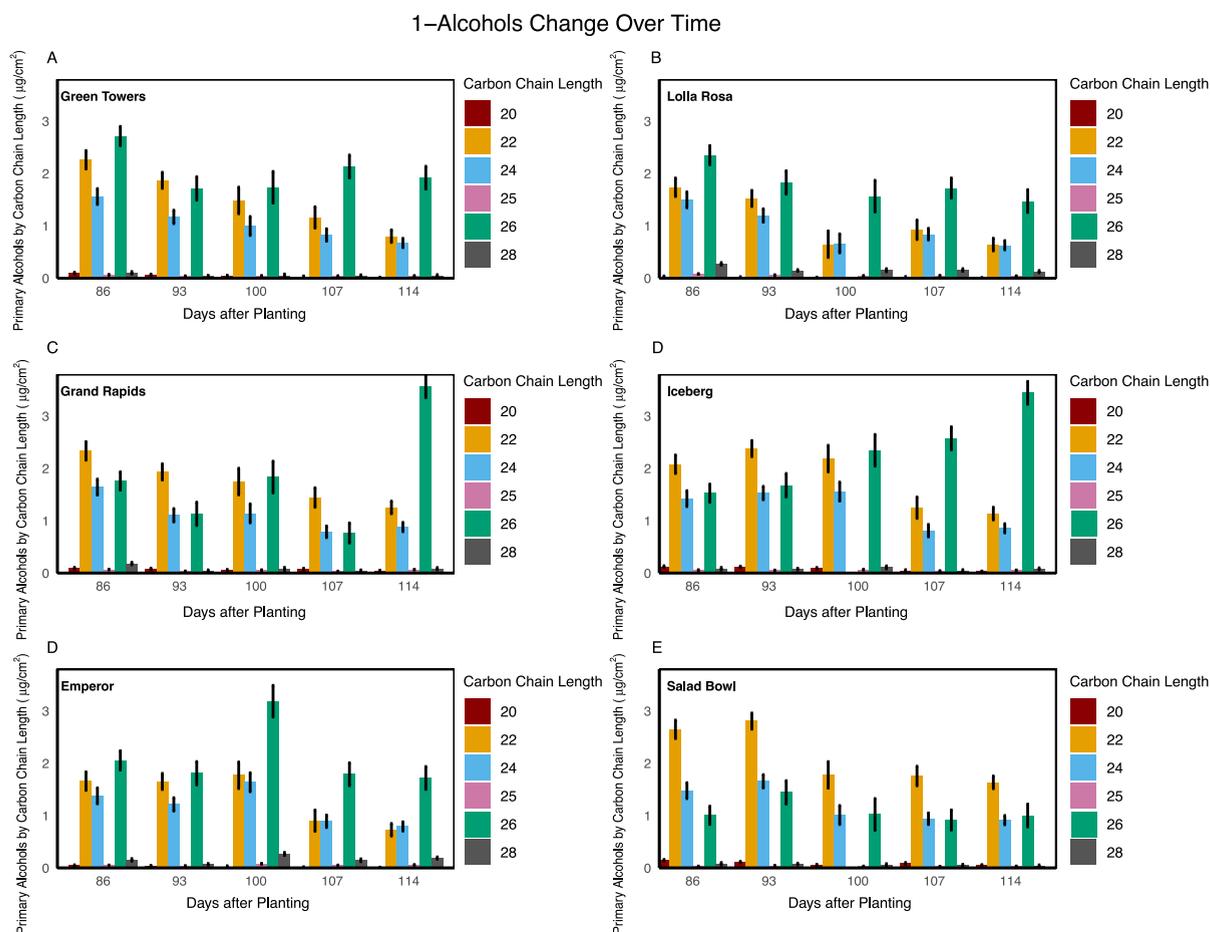

Fig. 3. The distribution of 1-Alcohol by carbon chain length (C20, C22, C24, C25, C26, and C28) for different lettuce cultivars (Green Towers, Lolla Rosa, Grand Rapids, Iceberg, Emperor, and Salad Bowl) is illustrated in panels A to E across five sampling time points. Chain-length distributions are labeled on the horizontal axis for each cultivar at different time points. The concentrations of the 1-alcohol by carbon chain length (ug/cm²) were labeled on the vertical axis.
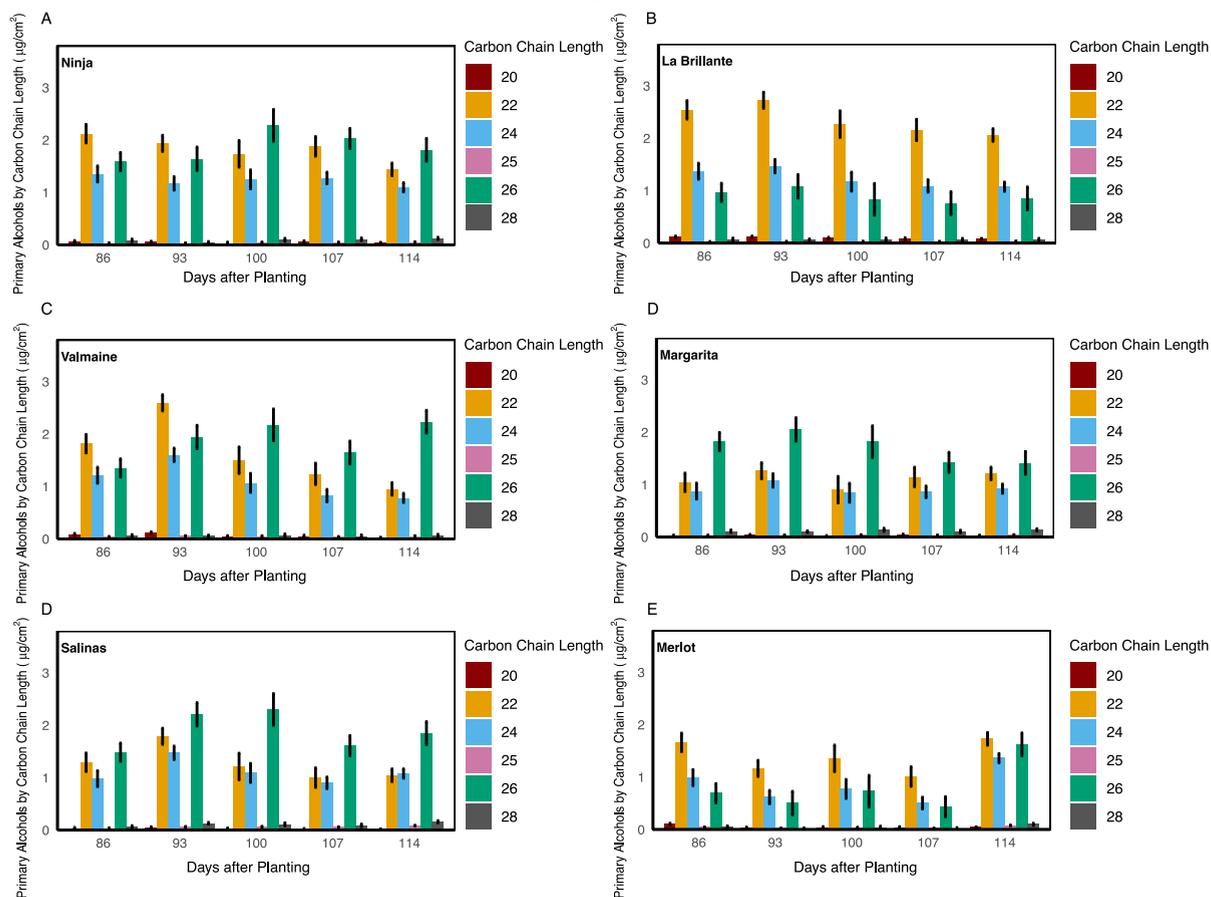
1–Alcohols Change Over Time

Fig. 4. The distribution of 1-Alcohol by carbon chain length (C20, C22, C24, C25, C26, and C28) for different lettuce varieties (Ninja, La Brillante, Valmaine, Margarita, Salinas, and Merlot) is depicted in panels A to E across five time points. Labeling is as in Fig. 3.

cultivars growing larger than others: Salad Bowl nearly tripled its height, from 8.20 to 23.7 cm while the cultivars Green Towers, Iceberg, and Grand Rapids also exhibited a pronounced increase in height compared to other cultivars (Fig. 2A). In terms of plant convex hull volume, a proxy trait for plant size and computed using point clouds, Salad Bowl, Green Towers, Iceberg, and Valmaine exhibited a relatively higher volume growth rate than other types during the 90 to 114-day period after planting (Fig. 2B). In contrast, La Brillante, Margarita, and Lolla Rosa showed a reduced rate of volume increase indicating a slower growth rate. On day 114, the average volume of Salad Bowl was five times that of La Brillante, approximately 13,321.6 cm^3 compared to 3278.2 cm^3, respectively (Fig. 2B).

Though the standard deviations of total wax content at each time point were high within this genetically diverse population, we did observe trends in mean leaf wax quantities over the collection period (Table 1). For the first three sampling time points the values remained consistent but then on day 107, there was a noticeable decrease in waxes; however, by day 114 they had partially recovered. The 1-alcohols, being the most abundant wax constituent class on all lettuce cultivars examined, followed this same trend where values

Table 1
Summary statistics of lettuce wax totals and principal constituents. Mean and standard deviation (SD) for total leaf waxes, 1-alcohols, fatty acids, and unknown compounds, in μg/cm^2, measured on 12 cultivars sampled on five different days within the growing season after planting (DAP).

DAP	Total Leaf Wax		1-Alcohols		Fatty Acids		Unknown Compounds	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
86	5.5	1.1	5.1	1.0	0.2	0.1	0.3	0.2
93	5.6	1.1	5.0	1.1	0.1	0.1	0.4	0.2
100	5.5	1.3	4.7	1.2	0.1	0.1	0.7	0.3
107	4.6	0.9	3.9	0.8	0.1	0.1	0.6	0.2
114	4.9	1.0	4.3	1.0	0.1	0.1	0.5	0.3

were highest on day 86, 5.1 μg/cm^2, and then declined to their lowest level on day 107, 3.9 μg/cm^2, with partial recovery by day 114. The measured fatty acids did not display any changes over the sampling time points, largely staying consistent at 0.1 μg/cm^2. Finally, the unknown compounds exhibited an increasing pattern from day 86–100, 0.3–0.7 μg/cm^2, respectively, before decreasing again to 0.5 μg/cm^2 by day 114. A likely explanation for the reduction of waxes was the major precipitation event in the field before the observed reduction in total wax amount and total 1-alcohols on day 107 (Supplemental Fig. 3). This suggests that the precipitation event likely promoted degradation of the wax on the leaf surface, even if only temporarily, as was described by Baker and Hunt (1985) [44]. Heavy winds with blowing sand and a decrease in daily temperature (Supplemental Fig. 3) were also associated with the precipitation event, and these environmental factors could also have played a role in degrading the wax amounts on these plants, either through abrasion and/or reduction in wax biosynthetic rates, respectively. The reason fatty acids and unknowns did not increase more during the precipitation event is uncertain but could be associated with their relative location within the wax layer and/or the fact they have higher chemical polarity. The impact of rain, variation in field temperature, wind, and wind-blown sand on wax deposition on plants has not been previously examined in detail. However, these environmental factors should be considered in future studies that aim to optimize wax amounts under diverse field environments and climates.

From day 86 to day 114, the amounts of the C22 and C24 1-alcohols on lettuce typically decreased relative to the amounts of the C26 1-alcohol (1-hexacosanol), which tended to increase, except for the one cultivar Margarita (Figs. 3 and 4). Margarita C22 and C24 to C26 1-alcohol ratios trended slightly in the other direction, with the C22 and C24 to C26 1-alcohol ratio starting lower and trending higher over time, that is, showing a decrease in the C26 homologue relative to the C22 and C24 homologues (Figs. 3 and 4). Even though none of the cultivars displayed a phase shift in their development during our analysis (since all samples were collected from plants in the vegetative phase before flowering), the advancing of their age from early to late vegetative phase, and increasing overall plant size, was associated (except for Margarita) with a shift in the chain-length distribution of their 1-alcohol constituents from shorter to longer chain lengths. The other 1-alcohols that we identified on lettuce, the C20, C25, and C28 1-alcohols remained quite low in relative abundance in all lettuce samples analyzed, and their proportions changed little over time (Figs. 3 and 4). The presence of very high proportions of C22 and C24 1-alcohols in lettuce is a relatively unique chain length profile and raises questions about the biological and ecological significance of high levels of C22 and C24 1-alcohols. Previous studies have shown that docosanol inhibits replication of certain viruses suggesting a potential role as a defense mechanism [54,55]. 1-tetracosanol has been reported to be related to both antimicrobial, antioxidant, and antibacterial activities of soil fungus *Periconia* sp [56,57]. Whether lettuce uses these unique very-long chain C22 and C24 1-alcohols as biologically active compounds to defend its leaves against pathogens or insects, or whether they have other ecological functions such as in heat or drought tolerance, are interesting questions for future studies.

The HTP system was also used to determine the change in Fv/Fm, the ratio of variable fluorescence to maximum fluorescence after dark adaptation, as an indicator of plant photosynthetic performance. Among the measured cultivars, there was a consistent pattern of rapid and near linear increase in Fv/Fm values until day 86 (Fig. 2C). After that point, differences between cultivars became more evident. The values for Fv/Fm peak at an average of 0.81 on day 93 before decreasing until the last measurement date on day 102. The one exception to this trend was Margarita which reached a peak value of 0.83 on day 100, a full week after the other 11 cultivars. Whether this is associated with Margarita's unique change in its 1-alcohol constituent ratios, that is, with its unique decrease in total C26 1-alcohols relative to the C22 and C24 1-alcohols that was not observed in other cultivars, is unknown. Previous reports show that 1-alcohols can have dramatic growth regulator effects on plants, including photosynthesis [58], and future studies would be needed to verify such an effect on our result. It is also noted that previous reports show that cuticular waxes can have a significant impact on light reflectance by the leaf surface and impact the amount of light reaching the chloroplast, and thereby total photosynthesis and fluorescence can be impacted by waxes [59–61]. Comparing wax compositional profiles of the two cultivars within each of the six market classes examined in this study did not reveal any differences directly associated with market class itself. Further, differences in amounts of the total wax classes or specific wax constituents could not be directly associated with market class (Supplemental Fig. 4). Most likely, the differences observed in waxes on cultivars were more closely associated with other ontogenetic variation expressed during vegetative growth than with any specific genetic, phenotypic or morphological determinants of market class.

3. Conclusions

Cuticular waxes serve as the outermost protective coating on plants and are an important adaptation for tolerating many types of environmental stressors such as drought, supra-optimal heat and solar radiation, pathogens, and phytophagous insects [18]. In crops like lettuce and other leafy vegetables, these waxes can also help shed dangerous human pathogens like *E. coli* and *Salmonella* spp [34, 37]. Variation in the cuticle wax phenotype has been an important target for breeding efforts in crops [31] like *Capsicum annuum* L. [62,63], *Camelina sativa* L. Crantz [64], *Leymus angustus* (Trin.) Pilg. [65], *Pisum sativum* L. [66], *Triticum aestivum* L. [67–69], *Sorghum bicolor* L. Moench [70,71] and *Oryza sativa* L. [72], among others. However, efforts to modify the cuticular wax properties on lettuce have not yet been pursued in formal breeding programs. This study represents a first important step toward quantifying and defining the phenotypic diversity of the cuticular wax chemical phenotypes in a diverse collection of 12 lettuce cultivars from different market classes, and under field crop production conditions. We report here that a significantly high degree of variation exists in total wax amounts among lettuce cultivars examined, indicating that selective breeding to modify wax load on lettuce leaves may be possible. Furthermore, we report that the waxes of lettuce possess uniquely abundant 22 and 24 carbon-length 1-alcohols raising new questions about the ecological significance of these wax compounds. Whether these large deposits of shorter than typical 1-alcohol waxes on lettuce leaves provide some unique protection to environmental stress, such as defense to pathogens or phytophagous insects, or protection to supraoptimal temperatures or drought, is a subject for future studies. Studies are also currently underway to advance the use of the HTP platform for phenotyping diverse lettuce populations using hyperspectral imaging, a technique capable of quantifying

the relationship between spectral reflectance and surface wax composition. The application of hyperspectral imaging to quantify wax compounds, if successful, and further studies on the functionality of wax compounds will form the basis of lettuce wax as a target for crop improvement.

4. Experimental

4.1. Plant material and experimental design

For this study, 12 lettuce cultivars were selected as follows: "Iceberg" and "La Brillante", Batavia class; "Margarita" and "Ninja", Butterhead class; "Emperor" and "Salinas", Crisphead class; "Grand Rapids" and "Salad Bowl", Green leaf class; "Lolla Rosa" and "Merlot", Red leaf class; and "Green Towers" and "Valmaine", Romaine class. Each cultivar was grown as part of a randomized incomplete block design with three replicate plots per cultivar at the University of Arizona's Maricopa Agricultural Center (MAC) in Maricopa, Arizona (33°04′24.8″ N 111°58′25.7″ W) in the winter of 2019/20. Raised vegetable beds on 1.02 m row spacing were shaped to have a surface width of 0.56 m with two seed lines per bed spaced at 0.31 m. Experimental plots, measuring 4 m in length, consisted of one individual seed lines per raised bed so that there were two experimental plots per raised bed. Plants were thinned to a density of 10 plants per plot. The soil type is a Casa Grande sandy loam (fine-loamy, mixed, superactive, hyperthermic Typic Natrargids). Standard cultivation practices and agronomic management for lettuce production in the Southwest were followed.

The crop was established using sprinkler irrigation for the first 35 days before switching to subsurface drip irrigation. Pressure compensated drip tape (Model 06D63613.16-12, Netafim, Tel Aviv, Israel), capable of supplying a constant 0.60 L of water per hour, was buried underneath each bed at a depth of 0.20 m. Soil moisture conditions were recorded using a neutron probe (Model 503, Campbell Pacific Nuclear, CPN, Martinez, CA, USA) with readings taken at depths of 10, 30, 50, 70, and 90 cm on a weekly basis. Neutron probe access tubes were distributed throughout the field to capture the volumetric soil water content (VSWC) across the growing period.

4.2. Leaf sampling and harvest

Healthy and disease-free leaves were selected from three individual lettuce plants per plot on 86, 93, 100, 107, and 114 days after planting. All lettuce leaf cuticular wax samples examined were collected from recently developed, fully expanded leaves at approximately 75% of the overall plant height during the vegetative developmental phase. Once the leaf was selected, scissors and tweezers were used, with gloved hands, to extract and place an approximately 2 cm^2 piece of leaf tissue in a 20 mL glass vial that was capped and immediately placed on ice. Once all the samples were collected, they were immediately moved indoors to a laboratory for cuticular wax analysis.

4.3. Cuticular wax analysis

Using the same method as previously described by Tomasi et al. (2021) [64], leaf disks were excised from the plant, placed in scintillation vials, and waxes extracted by 30 s rinses in chloroform. 10 µl internal standard (2 µg hexadecane, Sigma-Aldrich, USA) was added to the wax extracts. The waxes were derivatized using N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, 22 Sigma-Aldrich, USA) as described in Tomasi et al. (2021) [64], and then analyzed using an Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometer for chemical identifications and quantifications. An HP-Ultra 1 capillary column (12 m length, 200 µm inner diameter, 0.33 µm film thickness, Agilent, USA) was used, with helium as the carrier gas at 1.4 mL per min and temperature settings of inlet 350 °C, detector 300 °C, initial oven temperature 50 °C, then increased 20 °C per minute to 260 °C, where it was held for 8 min, then 25 °C per min to 325 °C and held again for 28.9 min for a total run time of 50 min. Molecular identities of compounds were determined by characteristic quadrupole electron impact mass spectra and comparison to the National Institute of Standards and Technology (NIST) library. Compounds missing from the library were compared with previously published spectra or elucidated from their ion chromatograms. A mixture of five external standards at 4 µg of nonadecanoic acid, 10 µg of tetracosane and 20 µg of pentacosanol (LGC Standards, Manchester, NH); 5 µg cholesterol and 4 µg arachidyl stearate (Sigma-Aldrich, USA) were used to determine correction factors. Uncorrected wax quantifications were based on target ions by wax class, Automated Mass Spectral Deconvolution and Identification System (AMDIS) deconvoluted semi-quant target ions were utilized, each relative to the correspondingly added standard class. Leaf sample areas were determined using a flatbed scanner and ImageJ software, and amount of each wax constituent calculated based on internal standard along with external standard correction factors and expressed as a function of leaf surface area.

4.4. Gantry phenotyping

The Field Scanalyzer (FS) is an outdoor HTP platform at the University of Arizona's Maricopa Agricultural Center that collects phenotypic trait data over a total scanning area of 1.11 ha. The scanning area is split into two fields, with a north field at 0.37 ha and a south field at 0.46 ha. The FS is equipped with a ventilated sensor box that holds multiple imagers and cameras but for this study we used the Allied Vision Prosilica GT3300C stereo RGB cameras (RGB, Stadtroda, Germany), LemnaTec photosystem II chlorophyll fluorescence prototype imager (PSII, Aachen, Germany), and a pair of Fraunhofer structured-light laser scanners (3D, Fürth, Germany) The sensor box can be positioned from 0.43 to 6.26 m above ground level to accommodate varying scanning distance requirements for each sensor and to maintain a consistent distance from instrument to plant canopy throughout the growing season.

Point clouds collected over the full south field were collected using the pair of Fraunhofer structured-light laser scanners mounted on the FS. Each data collection was accompanied by metadata files in JavaScript Object Notation (JSON) format containing FS variable position, sensor fixed position (location of sensors within sensor box), preset scanning area, and timestamps. Positioning information is collected by a series of barcodes along the rails (X and Y axes) and a string encoder (Z axis) using a right-handed coordinate system (+X South-to-North, +Y East-to-West, and +Z 0.76 cm above soil upwards). A total of 320 passes were collected for each data collection, collecting data per row at a field of view of 0.85 m and a working distance of 3.5 m above the plant canopy.

Point clouds were generated from depth and reflectance imagery collected by each laser scanner using manufacturer-provided software. In brief, pre-processing of image data to point clouds was performed by PlyWorker before the data was transmitted off-site. The pair of scanners captured the 3D structure of plants from East and West directions, thereby minimizing occlusions. Each capture was accompanied by a metadata JSON file containing FS variable position, sensor fixed position, preset scanning area, and timestamps. The point cloud output of PlyWorker software and metadata JSON files were input into and processed using PhytoOracle [73]. For each data collection, the orientation and scale of 320 pairs of PLY files (covering the full south field) were corrected using the RANSAC algorithm. Co-registration of RGB imagery and 3D point clouds was accomplished by matching landmarks in each data type. Individual plant detections collected from RGB imagery were used to extract individual plants. Soil and plant points were segmented using a Dynamic Graph Convolutional Neural Network (DGCNN), resulting in an individual plant point cloud without soil points. Morphometric values including convex hull volume, axis-aligned and oriented bounding box volumes, height, width, and length were extracted from each individual plant point cloud; in the present study, only convex hull volumes of individually segmented plant point clouds were used for analysis.

4.5. Statistical analysis

A linear mixed model was fitted to each of the total compound amounts as well as the individual compounds, using the MIXED procedure in SAS OnDemand for Academics (SAS Institute, Cary, NC). The objective was to assess the strength of the relationship between the compounds and the explanatory variables of genotype and sampling time. Prior to fitting the mixed linear model, outliers were identified and screened using interquartile range thresholds in R v4.2.0 [74]. Data values that were more extreme than three times the interquartile range (below the first quartile and above the third quartile range) were removed from the data; upon inspection they were often instrument anomalies or mis-entered values. This step resulted in 25 data points being removed from the total number of 5400 (99.54% of data retained). Once outliers were removed, the linear mixed model was fitted to the data, incorporating five individual time points that corresponded to 86, 93, 100, 107, and 114 days after planting. The cultivar and time point were considered as fixed effects, while the GC/MS column liner was considered as a random effect. Time point was a categorical variable in the model. The three-factor mixed model was expressed as:

$$Y_{ijk} = u + C_i + T_j + L_k + \varepsilon_{ijk} \tag{1}$$

in which $i = 1, ..., 12, j = 1, .., 5,$ and $k = 1, ..., 10;$ u is the overall mean; C_i is the fixed effect for the cultivar; T_j is the fixed effect for the time point; $L_k \sim N(0, \sigma_L^2)$ is the random effect for the liner; and $\varepsilon_{ijk} \sim N(0, \sigma_e^2)$ is the random error. The response variable Y_{ijk} represents the wax compound (i.e., 1-alcohols, fatty acids, unknown compounds, and total wax compound) collected for cultivar i at time point j and quantified in liner k. Tests of fixed effects are conducted using Type III tests (F test). To better observe the wax compound changes over time, we also performed a similar procedure at each individual time point, respectively. Best linear unbiased estimators (BLUEs) considering each fixed effect with respect to individual sampling time points for each liner was computed by the LSMEANS procedure in SAS [75]. In addition, BLUEs for each fixed effect for each liner across all time points were also calculated. Variance component estimation was conducted using residual (restricted) maximum likelihood (REML) in the PROC MIXED Procedure in SAS. The Kenward and Rogers approximation was applied in calculating the degree of freedom for the tests of the fixed effects [76, 77]. Model performance was assessed using standard diagnostic procedures including inspection of quantile-quantile (QQ) plots, histogram of the conditional studentized residuals, and scatterplot of conditional studentized residuals versus predicted means. Additionally, all convergence criteria were examined throughout the analysis to ensure reliability and stability of variance component estimation.

Ethics declarations

Informed consent was not required for this study because human subjects were not used.

Funding

Data availability

Data has not been deposited in a repository but will be made available upon request.

CRediT authorship contribution statement

Wenting Luo: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Emmanuel Gonzalez:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Ariyan Zarei:** Writing – original draft, Formal analysis, Data curation. **Sebastian Calleja:** Writing – original draft, Investigation, Formal analysis, Data curation. **Bruno Rozzi:** Writing – original draft, Investigation, Formal analysis, Data curation. **Jeffrey Demieville:** Writing – original draft, Formal analysis, Data curation. **Haiquan Li:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation. **Maria-Jose Truco:** Writing – original draft, Resources, Methodology, Investigation, Data curation. **Dean Lavelle:** Writing – original draft, Resources, Methodology, Investigation, Data curation. **Richard Michelmore:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **John M. Dyer:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Matthew A. Jenks:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Duke Pauli:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27226.

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