University of Nevada, Reno

Camelina sativa: a promising oilseed for producing biofuels on marginal lands: Field production and characterization of a low-pectin seed mutant

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Abstract

Camelina sativa L. Crantz (large-seeded false flax) (Camelina) is a promising oilseed crop for production of edible oil, seed meal for animal feed rations, and/or biodiesel feedstock. Because Camelina does not require prime agricultural land, it does not compete with food crops, and requires limited irrigation and nitrogen inputs.

In 2015 a five-year field trial of eight named varieties at UNR's Valley Road facility was completed, with harvested plants evaluated for total dry biomass, seed production, and oil content of cleaned seeds. Columbia, Cheyenne, Calena, and Blaine Creek were ranked as the top four varieties based on performance stability in high seed yield and calculated oil yield. The yields of this study fall within the ranges reported in both irrigated and rainfed locations of the western United States.

Improving Camelina for cultivation in Nevada includes developing and identifying mutants with desirable phenotypes (e.g., higher oil content per seed, reduced glucosinolates, reduced seed coat mucilage, triacylglycerol desaturation, and shattering of seed pods). More than 4,700 chemically mutagenized (e.g., EMS, ethyl methane sulfonate) M2 *C. sativa* lines were generated and have been screened for phenotypes of interest, *i.e.*, oil content as a % of dry weight (DW), reduced glucosinolates, and seed coat mucilage defects.

Mucilage is a polysaccharide gum composed of rhamnogalacturonan I (RGI), which can interfere with oil extraction. EMS lines with absent or reduced seed coat mucilage were assayed via a high-throughput colorimetric screen using Ruthenium Red staining. To date 250 M3 mutant lines were screened and four promising lines with reduced mucilage were verified for stability and penetrance in the M4 and M5

generations. The overall rate of mucilage defects in this population is approximately 0.05%. Agronomic data was collected comparing the most promising line, Cs98, with Wild-type cv. 'Celine' and their F1 cross, Cross 17.1. Cs98 stocks demonstrated their viability through a successful 2016 field trial. A quantitative spectrophotometric mucilage assay validated the seed coat mucilage content compared to wild-type "Celine."

Mucilage-defect mutant line Cs98 had smaller seeds and less SCM than WT. Oil derived from Cs98 showed significantly higher macromineral levels (K, Ca, Mg, and P) than WT oil. Transesterification of oil into FAMEs reduced macro mineral content by one-to-two orders of magnitude for both WT and Cs98 FAMEs. Cs98 oil showed significantly lower viscosity at 40 °C than WT oil, perhaps due to lower pectin content. A colorimetric assay of the water washes of the two oils showed that Cs98 had only 57.1% of the mucilage and pectic substances compared to WT.

Ongoing research includes characterization of the RGI structure and composition in *C. sativa* Cs98 mucilage defect lines. Characterization includes backcrossing promising lines to clarify genetic background, and mapping the location of the genetic lesions causing the mucilage defects.

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Chapter 1: *Camelina sativa*: a promising oilseed for producing biofuels on marginal lands: Field production and characterization of a low-pectin seed mutant

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Abstract

Camelina (*Camelina sativa* (L.) Crantz)**,** an oilseed also known as false flax, is a member of the Brassicaceae (mustard) family. For the last 25 years, Camelina has been studied as a potential biodiesel feedstock, the main reason it is cultivated in North America. Camelina rapidly matures in 85-120 d. It can be grown in marginal, low-fertility, and saline soils and in semi-arid areas in the western and southwestern United States. Camelina does not require fungicide or pesticide applications and provides an inexpensive cropping system with minimal irrigation and fertilizer inputs.

With petroleum demand steadily increasing, and discovery of new petroleum reserves decreasing, it would be prudent to develop alternatives to petroleum fuels, as reserves may be depleted by 2100. Camelina's seed oil content is approximately 30-45% oil per dry weight (DW). The oil consists of 90% polyunsaturated oil (PUFA), of which the predominant fraction (36-40%) is alpha-linolenic acid 18:3n-3 (ALA). In the United States, soy and canola are the two predominant vegetable oil feedstocks for biodiesel. Although both soy and canola products serve as human foods and animal feed rations, Camelina is not widely consumed in North America. Hence, Camelina avoids the food *vs.* fuel issue in agriculture.

Camelina seed and its protein-rich seed meal (CSM) remaining after oil pressing can supplement animal feed rations. However, due to anti-nutritive glucosinolates and erucic acid in Camelina seed and seed meal, the United States Food and Drug Administration (USFDA) has limited the inclusion of CSM to 10% of the feed ration for both beef cattle and broiler chickens. Besides using CSM to supplement animal feed

rations, ongoing research is exploring other value-added uses of CSM, including resins, thermoplastic films, tackifiers, and adhesives.

Due to its high PUFA content, Camelina biodiesel cannot be used directly as a B100 (100% biodiesel), as it does not meet the American Society for Testing and Materials International (ASTM) D6751 standards. Genetic engineering has been recommended and studied as a strategy to reduce the PUFA content.

The long-term feasibility of planting Camelina is dependent upon developing end uses and infrastructure for processing Camelina seed, meal, and other products. In the United States, growing canola is currently more lucrative than growing Camelina. Areas with developed markets and infrastructure, such as Canada, typically offer crop insurance and value Camelina at \$262-308 $tonne^{-1}$ (\$0.262 to \$0.308 kg^{-1}) compared to \$304-358 per $tonne^{-1}$ of canola (\$0.304 to \$0.358 kg^{-1}).

Long-term breeding goals for Camelina include developing early-maturing accessions with superior seed yield, high seed oil and meal protein contents, increased seed size, resistance to disease and insect pests, and broadleaf herbicide tolerance. These goals may be more quickly realized by the recently developed gene editing technologies than traditional plant breeding methods.

This dissertation contains the results of a five-year field trial evaluating agronomic parameters of eight named cultivars in northern Nevada, in Chapters 2 and 3. In addition, Chapter 4 contains the characterization of the seed, oil, and biodiesel produced by Cs98, a mutant produced by EMS mutagenesis.

Introduction

Camelina (*Camelina sativa* (L.) Crantz)**,** an oilseed also known as false or wild flax, gold of pleasure, and German sesame, is a member of the Brassicaceae (mustard) family. Camelina originated in Northern Europe, the Mediterranean, and Central Asia. Archeological evidence suggests it has been cultivated since Neolithic times as a source of oil for food, lighting, medicine, and as animal feed (Pilgeram, 2007; Putnam et al., 1993; Zubr, 1997b). Camelina arrived in North America and other continents as a "stowaway" seed present in shipments of flaxseed *(Linum usitatissimum)*. As Camelina is a common weed within flaxseed fields, it was called "false flax." For the last 25 years, Camelina has been studied as a potential biodiesel feedstock, which is the main reason it is cultivated in North America (Zubr, 1997b). Camelina continues to be consumed as a salad oil in Europe and North American consumers may purchase edible oil online and in food stores. However, due to anti-nutritives present in the Brassicaceae (*e.g.*, erucic acid, 22:1ω9, and glucosinolates, which affect cardiac and liver functions, respectively) in humans and mammals, consumption must be limited.

Camelina is a short-season species, rapidly maturing in 85-120 d. It is best adapted to cooler climates and is typically planted as an annual or a winter annual crop in traditional flax-growing regions of the upper Midwest (Minnesota, North Dakota, South Dakota, and Montana) and Canada, among many other areas throughout the world (Robinson, 1987). The rapid-cycling oilseed can be used in dual cropping and intercrop rotations, often with soybean (*Glycine max*) or winter wheat (*Triticum aestivam*). Camelina can be grown in marginal, low-fertility, and saline soils and in semi-arid areas (Budin et al., 1995) in the western and southwestern United States (Hunsaker et al.,

2011). Both spring and winter annual accessions of Camelina are cold tolerant, capable of surviving freezes (Gesch and Cermak, 2011; Robinson, 1987), making it a potential oilseed crop for northern Nevada. Additional agronomic traits include a high resistance to insect pests and high nutrient-use efficiency. Camelina does not require fungicide or pesticide applications and provides an inexpensive cropping system with minimal irrigation and fertilizer inputs.

The need for sustainable alternatives to fossil fuels

Decreases in Arctic and Antarctic ice masses, rising ocean levels, and increased coastal flooding are associated with the steady increase in global temperatures. Estimated anthropogenic global warming is currently increasing at 0.2 ℃ per decade due to past and ongoing emissions of greenhouse gases (CO_2, methane, N_2O, fluorinated gases) (IPCC, 2018). In the United States, primary energy consumption by fuel source was estimated at 101.268 quadrillion BTU, with 80.14% coming from fossil fuels (coal, natural gas, and petroleum) (EIA, 2019a). Petroleum fuels provide 92% of energy used for transportation (EIA, 2019b). With petroleum demand steadily increasing, and discovery of new petroleum reserves decreasing, it would be prudent to develop alternatives to petroleum fuels, as reserves may be depleted by 2100 (Minniear, 2009).

Camelina's fatty acid profile

Camelina's seed oil content is approximately 30-45% oil per dry weight (DW) (Radocaj and Dimic, 2013b). The oil consists of 90% polyunsaturated oil (PUFA), of which the predominant fraction (36-40%) is alpha-linolenic acid 18:3n-3 (ALA) and the next largest

fraction (12-20%) is linoleic acid 18:2n-6 (LA) (Nain et al., 2015; Radocaj and Dimic, 2013; Zubr, 1997; Zubr, 2009a). Other PUFA species include (~16%) gondoic acid (20:1n-9) and (~3%) the anti-nutritive erucic acid (22:1n-9) (Zubr, 2009b). As erucic acid has been implicated in myocardial lipidosis in animal studies, limits of 7 mg kg^{-1} body weight were established by the European Union for human consumption (Knutsen et al., 2016). Food oilseeds such as *B. napus* (canola) have undergone extensive breeding to achieve < 2% erucic acid, with many at 0%, whereas Camelina has 2-4% erucic acid (Hrastar et al., 2012). In the United States, low erucic acid canola oil is required to contain a maximum of erucic acid $\leq$ 2% (FDA, 2018). Tocopherol content (90mg/100g unrefined oil) (Zubr, 2009b) is 90% gamma tocopherol (Matthaus, 2004), which preserves unrefined oil stability.

Biosynthesis of seed oil

Plant triacylglycerols (TAGs) in seed oil are synthesized, modified and assembled in three locations, according to the Kennedy pathway (Bates et al., 2013). First, *de novo* synthesis of fatty acids (FAs) and esterification to an acyl carrier protein (ACP) occur in plastids. Second, the cytosol is the repository for the acyl CoA and acyl-lipid pools. Third, the endoplasmic reticulum (ER) modifies lipids and assembles TAGS upon a glycerol backbone using acyl-CoA-dependent diacylglycerol acyltransferase (DGAT) (Dyer et al., 2008). WRINKLED1 (WRI1), one of the APETALA2-ethylene-responsive element binding protein family of transcription factors, activates FA biosynthesis in seeds for TAG production (To et al., 2012). Seed oil content in plants is a quantitative trait

based on several factors including embryo and maternal genetic effects, cytoplasmic effects, and genotype-environment interactions (Weselake et al., 2009).

Besides the Kennedy pathway of assembling *de novo* TAG from diacylglyceride (DAG) via DGAT, two other pathways are known to contribute to TAG accumulation (Bates and Browse, 2012). First, FA's exiting the cytosolic acyl-CoA pool may enter the ER acyl editing cycle, wherein 18:1-CoA is channeled into phosphatidylcholine (PC). In the ER, fatty acid desaturases FAD2 and FAD3 desaturate the 18:1-PC to 18:2 linolenic and 18:3 α-linolenic acid, respectively (Bates and Browse, 2012). The modified FA's reenter the acyl-CoA pool and can then be incorporated into diacylglyceride (DAG) prior to assembly into TAG (Bates and Browse, 2012). DGAT in the ER is the rate-limiting step for assembly of TAG in seed oil.

Desaturation of oleic acid (18:1) to linoleic acid (18:2) and thereafter to α-linolenic acid (18:3) occurs within the ER, with the final rate-limiting step of triacylglycerol ("TAG") synthesis catalyzed by acyl-CoA-dependent diacylglycerol acyltransferase (DGAT) in the Kennedy pathway (Lu et al., 2011). DGAT has been extensively studied, with both DGAT1 and DGAT2 isoforms acting upon diacylglycerol ("DAG") substrate to form TAG (Snyder et al., 2009).

Of the four DGAT isoforms in plants, DGAT1 is a major player in controlling seed accumulation of TAG, as well as determining FA composition (Lu et al., 2011), and is a key target for modifying oil yield through mutagenic and transgenic strategies. Upregulating DGAT1 led to increased seed oil content in four species, including in *Brassica napus* (canola), and *Arabidopsis* (Snyder et al., 2009). Downregulating DGAT1 in tobacco led to 9-49% decrease in seed oil (Taylor et al., 2009). Transgenic *B. napus*

with upregulated DGAT1 showed increases of seed oil up to 14%, as well as partially restoring oil loss due to drought and heat (Weselake et al., 2008).

A second ER DAG synthesis pathway uses phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al., 2009), an enzyme that transfers the PC head group from PC to DAG to modify *de novo* DAG (DAG1) into PC (Bates and Browse, 2012). PDCT also uses PC-modified fatty acid (PC-mFA) substrate to produce the DAG substrate for TAG synthesis (DAG2) (Bates and Browse, 2012). PDCT significantly impacts PUFA content in oil. Analyzing the *pdct* mutant (*rod1)* in *Arabidopsis* showed that the PUFA content of *pdct* seed was reduced to approximately 40% (Lu et al., 2009).

Temperature also affects the fatty acid profile of oil in maturing seeds. In *Borago officinalis* (borage), ALA synthesis declines at temperatures > 25 °C, resulting in an increase in oleic and linoleic acid fractions, due to decreased activity of the Δ15 desaturase (FAD3) converting linoleic to ALA (Gilbertson et al., 2014). One Camelina study indicated a decline of ALA in rainfed *vs.* irrigated plantings, perhaps due to higher temperatures (Pavlista et al., 2016).

In transgenic plants, overexpressing or repressing specific genes encoding enzymes or other proteins involved in TAG biosynthesis and assembly has led to significant increases in seed oil content (Weselake et al., 2009). Metabolic flux studies of the TAG biosynthetic pathways have identified rate-limiting enzymes which, when disabled or overexpressed, significantly affect flux and consequently the percentage of TAG/DW of seed, as well as the fatty acid profile (saturated *vs.* polyunsaturated) of the fatty acid methyl esters (FAME) of the biodiesel produced from the TAG produced

(Weselake et al., 2008). Although genetic modification of a particular pathway enzyme can produce statistically significant results (of greater oil yield or more desirable % PUFA), the complexities of flux in biosynthetic pathways require a more comprehensive approach to pathway manipulation (Taylor et al., 2009).

Camelina compared with soy and canola

In the United States, soy (*Glycine max*) and canola (*Brassica napus*) are the two predominant vegetable oil feedstocks for biodiesel, with 3.07 billion and 7.15 million L reported in 2017, respectively (Hanson and Agarwal, 2018). In Europe, the predominant feedstocks are canola, used cooking oil, palm, and soy, with 7.88, 3.78, 3.49, and 0.91 billion L reported for 2018, respectively (FAS, 2018). Crops produced for human and animal consumption are designated as first generation feedstocks (Atabani et al., 2012), which impact the food *vs.* fuel debate. Briefly, hectares used to grow biodiesel feedstocks may decrease hectares available to grow food crops, thus increasing food prices (Tenenbaum, 2008). Because Camelina is currently not widely consumed in North America, it is regarded as a second generation (inedible) oil feedstock (Atabani et al., 2012). In addition, Camelina does not require the prime farmland and significant irrigation and fertilizer inputs needed by soy and canola. As a biodiesel feedstock, Camelina avoids the food *vs.* fuel debate.

Soybeans and canola seeds have crude protein contents of 42% and 22-23% DW, respectively (Dornbos and Mullen, 1992; Racz and Christensen, 2004). Soy and canola lipid contents are 23% and 44-46% DW, respectively (Dornbos and Mullen, 1992; Racz and Christensen, 2004). After oil pressing, the seed meals used to supplement animal

feed rations have protein contents of 44 and 38% DW for soy and canola, respectively (Nelson and Landblom, 1990). Oil content of soy and canola meal are 0.8 and 3.8%, respectively (Nelson and Landblom, 1990).

Camelina seed and the Camelina seed meal (CSM) remaining after oil pressing can also serve as a nutritious food ration. After oil pressing, CSM contains 4-14% oil and 35-48% protein content per dry weight (Almeida et al., 2013; Colombini et al., 2014a). The mean crude protein (CP) concentration (g kg^{-1} DW) of CSM (457 g kg^{-1} DW) was higher than canola (326 g kg^{-1} DW) and slightly lower than values reported for soybean meal (499 g kg^{-1} DW) (Colombini et al., 2014b). In a case study using a different methodology, CSM also demonstrated a higher CP concentration (385 g kg^{-1} DW) than canola (278 g kg^{-1} DW), respectively (Llewellyn et al., 2015). Using Camelina seed meal as a portion of animal feed ration would provide additional cost justification for growers to produce Camelina (Keske et al., 2013). However, due to anti-nutritive glucosinolates in Camelina seed and seed meal, which affect animal metabolism, the European Union has limited the inclusion of up to 1.5 mmol kg^{-1} glucosinolates in feed for monogastric animals (chicken and pork) (Colombini et al., 2014a). In the United States, Camelina seed meal may consist up to 10% of the feed ration for both beef cattle and broiler chickens (Schill, 2009).

As a legume, soybean generally obtains its N requirement by biological nitrogen fixation (BNF) with additional N absorbed from soil (Mourtzinis et al., 2018). Environmental conditions such as low soil moisture, temperature and soil pH extremes, and soil compaction can limit BNF, with additional N needed in adverse growing conditions or high-yield production (Mourtzinis et al., 2018). A 2018 analysis found that

soy yields responded favorably to fertilization, with a suggested yield apex at 275 kg N ha^{-1} (Mourtzinis et al., 2018). University of Nevada Cooperative Extension (UNCE) recommends an initial application of 112 kg ha^{-1} of 11-52-0 monoammonium phosphate (MAP), followed by a second fertilization of 84 kg N, 17 kg P, and 17 kg K ha^{-1} for soybean in northern Nevada (Davison, 2002).

Canola also requires significant N inputs. Winter canola often starts with 70 kg N ha^{-1}, with more N inputs of 40-80 kg N ha^{-1} prior to flowering (Rathke et al., 2006). For northern Nevada, UNCE recommends a minimum of 84 kg N ha^{-1} for canola (Davison, 2015). Unlike soy and canola, Camelina requires only moderate fertilizer inputs, is drought tolerant, and has very few pests requiring chemical applications (Moser, 2010; Zubr, 1997). Nitrogen is the key input, with optimal fertilizer recommendations of 60 kg ha^{-1} N (Mohammed et al., 2017). Application of P and S increased Camelina seed yield compared with control, and there was no response to K fertilization (Mohammed et al., 2017). Recent studies in northern Nevada show a range of fertilizer inputs for Camelina. Nitrogen inputs via urea to provide inputs of 80 to 120 kg N ha^{-1} were recommended, along with 40 kg ha^{-1} of P as triple super phosphate (Neupane et al., 2018). Another study by Neupane used urea to input 80 kg N ha^{-1}, and 40 kg ha^{-1} of P as triple superphosphate (Neupane et al., 2019). The study described in Chapter 2 of this dissertation used urea at a rate to yield 58.8 kg N ha^{-1}, and achieved seed mass yields comparable to published studies.

Camelina evaluated as a biodiesel feedstock

Due to Camelina's 30-45% oil% DW, its short 12-14 week life cycle, drought and cold tolerance, and adaptability in a wide range of environments, Camelina is viewed favorably as a biofuel feedstock alternative to soy and canola (Moser, 2010). However, due to its 90% PUFA content, Camelina biodiesel cannot be used directly as a B100 (100% biodiesel) fuel or blended with petroleum diesel, as it does not meet the American Society for Testing and Materials International (ASTM) D6751 standards for cetane number, distillation temperature, and oxidation stability (Ciubota-Rosie et al., 2013). Although the cetane number and oxidative stability can be corrected using additives, it would be very difficult to modify the distillation temperature without reducing the high degree of unsaturation and the molecular weight of the oil (Ciubota-Rosie et al., 2013).

Camelina can also be used as a feedstock for hydroprocessed renewable jet (HRJ) fuel. More than 80% of the fatty acyl groups in Camelina TAGs are unsaturated and long-chain (C18-C22), which is undesirable for jet fuel, which is a mixture of medium chain (C10-C14) and short chain (C6-C9) hydrocarbons (Hu et al., 2017). Camelina TAGs require conversion through initial hydrodeoxygenation or hydrotreatment, followed by selective catalytic cracking or hydrocracking and isomeration, and ending with product separation and formulation (Berti et al., 2016). Camelina HRJ fuel has been evaluated favorably as compared to JP-8 (typical jet fuel) (Corporan et al., 2011), and is considered a drop-in replacement jet fuel, which has been tested in blends with JP-8 by the US Air Force and two commercial airlines (Berti et al., 2016).

Other uses of Camelina seed meal and its components

Besides its use as in animal feed rations, ongoing studies are exploring other value-added uses of CSM. Resin derived from CSM seed meal can be combined with recycled newspaper to produce sustainable and biodegradable composite sheets and fibers (Kim and Netravali, 2012). Thermoplastic films developed from grafting CSM with vinyl monomers displayed excellent wet tensile properties (Reddy et al., 2012). CSM can be used as a less expensive tackifier than guar gum in hydromulch for erosion control (Vaughn et al., 2013). Montana State University is investigating the use of the mucilaginous CSM as an herbicidal soil amendment to suppress weeds (McVay and Lamb, 2008). Protein extracted from CSM has been evaluated as an effective base for adhesives (Qi et al., 2016). Ultra-sound treated protein extracted from CSM has been studied as a component for both adhesives and coatings (Zhu et al., 2017).

Camelina as a food supplement for food animals and humans

Ongoing research continues to investigate the effects of CSM as a portion of the feed ration for varied food animals. Glucosinolate content of Camelina seed meal was higher (23.1 mmol kg^{-1}) than canola seed meal (7.2 mmol kg^{-1}) (Colombini et al., 2014). The United States Food and Drug Administration (FDA) has limited CSM to 10% of feed rations for beef cattle and broiler chickens (Schill, 2009).

Besides the crude protein content of 35-48% (Almeida et al., 2013; Colombini et al., 2014) the 36-40% alpha-linolenic fatty acid has been shown to reduced saturated fat and increase PUFA levels in flesh and milk of food animals. CSM has been investigated for growing dairy heifers (Lawrence et al., 2016), beef cattle (Cappellozza et al., 2012),

pigs (Almeida et al., 2013), dairy ewes (Dankow et al., 2015), goats (Pikul et al., 2014), rabbits (Peiretti et al., 2007), broiler chickens (Nain et al., 2015), laying hens for 18:3 enrichment of eggs (Cherian and Quezada, 2016), Japanese quail (Bulbul et al., 2015), farmed salmon (Brown et al., 2016), farmed cod (Hixson et al., 2016), rainbow trout (Collins et al., 2018), and other animals.

The high ALA content of Camelina oil enables it to serve as a dietary supplement for humans. A 2011 study found that rats fed with high fat diets (20% fat, 1% cholesterol) lowered their blood cholesterol when given cold-pressed Camelina oil (Deng et al., 2011). A recent study of human volunteers consuming 10 g Camelina oil day^{-1} showed significant increases in the proportion of ALA content of erythrocyte membranes, plasma phospholipids, cholesterol esters and triglycerides (Manninen et al., 2019). United States consumers can purchase Camelina oil online and in stores, as it is classified as a dietary supplement and not regulated by the FDA. In March 2016, CamStar, LLC (Bigfork, MT) requested that the FDA grant generally recognized as safe (GRAS) status to edible Camelina oil. The FDA issued a response letter indicating the agency had no questions, but had not conducted its own investigation of Camelina oil (FDA, 2016).

Markets for Camelina in North America

Relatively new oilseeds such as Camelina, which are not well established crops, present challenges to farmers in today's economy. The National Agricultural Service (NASS) of the USDA tracked ongoing Camelina production in the states of Arizona, Idaho, Minnesota, Montana, Oregon, Washington, and Wyoming, beginning in 2012, with only Montana continuing to report operations in 2017 (NASS, 2017). Although Montana

reported having 20,000 acres planted in 2007 (McVay and Lamb, 2008), production has declined from 8,256 acres harvested in 2007, as compared with 728 acres harvested in 2012 (Obour et al., 2015).

The long-term feasibility of planting Camelina is dependent upon developing end uses and infrastructure for processing Camelina seed, meal, and other products. Under the United States Renewable Fuel Standard, expanded in 2007 under The Energy Independence and Security Act of 2007 (EISA), biodiesel (such as transesterified Camelina oil) is classified as an advanced biofuel, with an increasing mandate for production through 2022 (Obour et al., 2015).

Economics of growing Camelina *vs.* canola in North America

Farmers and agribusinesses are currently able to obtain crop insurance to protect crop yields in parts of North Dakota and Montana (Diersen and Saleh, 2015). In other states, growers may choose to self-insure, obtain single-peril coverage (e.g., hail), or seek Noninsured Crop Disaster Assistance Program (NCDAP) coverage (Diersen and Saleh, 2015).

In the United States, growing canola is currently more lucrative than growing Camelina. As end uses and supportive infrastructure are established for Camelina products, Camelina's value will increase. Although the National Agricultural Service (NAS) of the USDA does not track the price of Camelina, Oregon prices paid for Camelina in 2014 were estimated at $0.04/lb (Obour et al., 2015), as contrasted to $1.70 to $1.80/lb paid for canola in 2018 (NASS, 2018).

Canada has increased grower incentives to plant Camelina. Although 1,094 ha were planted with Camelina in 2016 (with 80% of the area located in Saskatchewan) (Shumsky, 2018), Saskatchewan acreage doubled to 2,023 ha in 2017 (Arnason, 2017). In Canada, support for growing Camelina has increased grower incentives to plant Camelina. In 2010 Health Canada approved the use of cold-pressed Camelina oil as a food ingredient, noting that Camelina's erucic acid content was less than the maximum limit of 5% (Canada, 2010). Saskatchewan offers crop insurance for Camelina, and values Camelina at \$262-308 $tonne^{-1}$ (\$0.262 to \$0.308 kg^{-1}) compared to \$304-358 per $tonne^{-1}$ of canola (\$0.304 to \$0.358 kg^{-1}) with Camelina being priced at 86.1% of the price of canola (SCIC, 2018). Hail insurance is available for Camelina planted in the provinces of Alberta, Manitoba, and Saskatchewan (CHA, 2019). Canadian production costs for Camelina oil range from \$0.39 to \$1.88 L^{-1} when Camelina meal is valued at \$0.30 per kg^{-1} (Mupondwa et al., 2016).

Due to lack of established markets, United States production costs are more difficult to estimate than Canadian costs. An early model calculated a breakeven price of \$0.83 L^{-1} diesel, with Camelina grown in rotation with wheat, and assuming that both Camelina oil and CSM could be sold readily (Keske et al., 2013). Many studies use the price of canola for economic feasibility (Berti et al., 2016), which probably overvalues Camelina, which is priced at 86.1% of canola in Canada (SCIC, 2018). One estimate determined a breakeven price for winter camelina, used in double or relay cropping with soy in Minnesota, was \$0.65 to \$1.14 kg^{-1} (Gesch et al., 2014).

The conundrum of how to develop infrastructure and markets for Camelina and CSM as commodities in the United States without providing incentives for growers to

raise Camelina may continue until it is identified as a valuable crop for on-farm feed and fuel. The best scenario may be for growers with land resources and livestock to consider growing Camelina both for on-farm feed and fuel use, as the economic benefit of avoiding the purchase and transport of livestock feed is significant (Foulke et al., 2012).

Camelina molecular genetics

Camelina sativa chromosome counts have most commonly been reported as $2n = 40$, suggesting polyploidy (Berti et al., 2016). Due to its large chromosome number, a genome three times larger than that of other Camelina species, and the presence of three functional copies of FAD2 and FAE1 genes resulted in Camelina being described as having an allohexaploid genome (Hutcheon et al., 2010). The reference genome released in 2014 estimated the genome size of Camelina to be ~782 Mb and confirmed hexaploid status, conserved over three sub-genomes (Kagale et al., 2014). Both the reference genome and the *Arabidopsis thaliana* (Arabidopsis) genome are members of Brassicaceae lineage I Camelineae tribe, with a high degree of syntelogs reported between the two genomes (Kagale et al., 2014).

Camelina's leaf transcriptome (from greenhouse grown ecotype MT-5) noted a high degree of sequence identity between Camelina annotated unigenes compared with coding sequences in *A. thaliana* (Arabidopsis), and some unigenes more similar to the diploid *Brassica rapa* (field mustard) (Liang et al., 2013). Nguyen et. al (2013) sequenced the seed transcriptome of 'Suneson' 10 to 20 days after pollination to identify targets to improve CSM and oil and provided a user-friendly database of surveyed sequence alignment of genes relevant to seed lipid metabolism (Nguyen et al., 2013).

Improving Camelina's genome

To improve Camelina it is desirable to alter the genotype to obtain the desired phenotypes through current plant breeding methods that include mutagenic and transgenic strategies. Traditional plant breeding methods focused on detecting desirable phenotypes due to spontaneous mutation and require a long time frame. Mutation rates in *A. thaliana* have been estimated to range from 7×10^{-9} base substitutions per site per generation, evaluated over 30 generations (Ossowski et al., 2010), to 6.95×10^{-9} per site per generation, evaluated over 25 generations (Weng et al., 2019).

Camelina propagates through autogamy (Zubr, 1997) and has very low levels of intraspecific outcrossing (Seguin-Swartz et al., 2013; Walsh et al., 2015; Walsh et al., 2012b). Camelina breeding uses pure line selection; after artificial hybridization, segregating generations use either the pedigree or bulk breeding method (Vollmann and Eynck, 2015). The single-seed descent method to reach homozygosity has been used in Camelina breeding (Seehuber et al., 1987) and to develop recombinant inbred line mapping populations (Gehringer et al., 2006). Mutagenesis has been used to create genetic variation in Camelina, both through ethyl methanesulfonate (EMS), a chemical mutagen, to create herbicide resistance (Walsh et al., 2012a), and through gamma irradiation to modify the fatty acid profile of the oil (Vollmann et al., 1997).

Long-term breeding goals for Camelina include developing early-maturing accessions with superior seed yield high seed oil and meal protein contents, increased seed size, resistance to disease and insect pests, and broadleaf herbicide tolerance (Berti et al., 2016). To compete with other oilseeds (e.g., canola), Camelina's yields need to

increase by a minimum of 20%, to provide equivalent farm revenues, based on lower valuation per tonne, as mentioned above. Other desirable traits include shatterproof pods to reduce yield loss during harvest (Ogutcen et al., 2018) and reducing plant height to increase productivity and reduce lodging (Hirano et al., 2017).

Modern plant breeding includes induced mutagenesis techniques, such as EMS treatment of seeds to create a varied gene pool with random mutations that knockout or knockdown genes resulting in diverse phenotypes. Back crossing is used to incorporate the desired phenotypes into elite cultivars to enhance an already viable cultivar with additional desired traits (Murphy, 2006).

Three projects, including the Cs98 project in Chapter 3 of this dissertation, resulted from EMS treatment of Camelina 'Celine' seeds. M3 seeds were planted at the Nevada Agriculture Experiment Station greenhouse (Building 175) and assayed for phenotypes of interest. The Cs98 project resulted from screening M3 mutants for reduction or absence of pectinaceous seed coat mucilage, a typical Wild-type (WT) phenotype. The second project involved screening M3 seeds for reduced levels of anti-nutritive glucosinolates. The third project involved screening M3 seeds for mean oil% dry weight (DW) $\geq$ 40%, replanting and crossing the high oil lines into WT, and evaluating the oil% DW of the cross progeny for trait stability.

Transgenic techniques can effectively introduce genes from another species, such as *A. thaliana*, into Camelina to produce desirable phenotypes. An *Agrobacterium*-mediated floral dip strategy (Lu and Kang, 2008) is typically used. Transgenic seeds can be identified using seed fluorescence or resistance to antibiotics or herbicides (Berti et al., 2016).

Using a gene from California bay tree (*Umbellularia californica* Nutt.) with a transgenic strategy, a group recently modified Camelina's TAG profile from typically ≥ 80% long-chain (C18-20) fatty acyl moieties to produce up to 43 mol% of three mid-length moieties (lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0), which, after processing, are better feedstocks for jet fuel production. The altered TAG compositions did not affect overall amount of seed oil produced or seed germination (Hu et al., 2017).

Another effort transformed Camelina with heterologous genes in two iterations from several species to produce seeds containing a mean of 24% eicosapentaenoic acid (EPA 20:5n-3) in the first iteration, and means of 11% EPA and 8% docosahexaenoic acid (DHA 22:6n-3) in the second iteration. The transgenic EPA and DHA were reported to be equivalent to the heart-healthy omega-3 long chain PUFAs found in fish oil, thus providing a plant-based source for these fatty acids (Ruiz-Lopez et al., 2014). However, transgenic food crops containing recombinant DNA (rDNA) from other species, are considered Genetically Modified Organisms (GMOs) in the United States, and carry an additional regulatory burden (Demeke et al., 2006).

The newer gene editing technologies (zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9), have two major advantages over conventional plant breeding. First, the time frame to demonstrate phenotype stability is considerably shorter. Second, if the genome-edited crop lacks rDNA, inserted antibiotic markers or marker genes, and does not have pesticidal activity or food safety attributes differing from traditionally bred crops, it is not subject to additional regulatory evaluation (Shah et

al., 2018; Wolt and Wolf, 2018) in the United States. Hence, a crop improved by gene editing can be evaluated and commercialized more quickly than conventionally bred crops with no additional regulatory hurdles.

Modification of Camelina's fatty acid profile through genome improvement methods could solve the problems associated with its 90% PUFA content (Ciubota-Rosie et al., 2013). A transgenic approach targeted the microsomal oleate desaturase (FAD2; EC 1.3.135) through an antisense expression construct, resulting in an increase in 18:1 (oleic acid) content from 13-18% in wild-type Camelina, compared to 38-51% in transgenic seeds, and achieving concomitant decreases in 18:2 and 18:3 PUFAs (Kang et al., 2011).

More recently, gene editing technologies have shown success in modifying Camelina. Because Camelina is an allohexaploid with three highly related and undifferentiated subgenomes (Kagale et al., 2014), three homeologs exist for many genes. All three encoding genes must be targeted to suppress or eliminates endogenous enzyme activity. Using CRISPR/Cas9 targeted mutagenesis of the three delta-12-desaturase (FAD2) genes, resulting in heritable mutations in the three genes evaluated over four generations. Lipid profiles of the Camelina lines created ranged from 30 to 74% oleic acid accumulation and reduced levels of PUFA (Jiang et al., 2017). Another group using CRISPR/Cas9 to target FAD2 achieved a 10 to 62% oleic acid levels and reduced PUFA levels in Camelina (Morineau et al., 2017). A third group successfully mutated three CsDGAT1 genes with Cas9 and a single guide RNA (sgRNA) complementary to a specific DNA sequence (Aznar-Moreno and Durrett, 2017). Phenotypes of reduced oil content and wrinkled seeds were consistent with the modification of the targeted genes.

Seed coat mucilage (SCM) undesirable for a biodiesel feedstock

Plant cell walls contain three predominant polysaccharides: cellulose, hemicellulose and pectin, with cellulose-hemicellulose networks providing tensile strength (Arsovski et al., 2010). Pectin supports cell wall integrity by covalently bonding to the cellulose-hemicellulose networks and by providing cell-to-cell adhesion (Voiniciuc et al., 2018a). The principal pectin in *A. thaliana* primary cell walls is homogalacturonan (HG) (Voiniciuc et al., 2018b).

Pectinaceous seed coat mucilage (SCM) has been reported in myxospermous fruits and seeds in at least 230 angiosperms (Yang et al., 2012), including several Brassicaceae, including *A. thaliana*, Camelina, and *Brassica napus* (canola). Upon hydration, the hydrogel formed by SCM exuded by epithelial cells in the seed coat may retard desiccation, regulate germination, and mediate seed dispersal (Western et al., 2001). SCM is predominantly pectins, acidic polysaccharides, which consist of rhamnogalacturonan I (RGI) and polygalacturonic acid (PGA) (Western et al., 2001).

RGI has a backbone of alternating rhamnose and galacturonic subunits, and is the main component of *A. thaliana* SCM deposited in the apoplast outside the cell wall. Both RGI and HG form ionic cross-links with Ca^{2+} salt bridges (Macquet et al., 2007). Pectins chelate divalent cations such as Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} (Celus et al., 2018), and La^{2+} (McKenna et al., 2010); HG has been more extensively studied than RGI (Celus et al., 2018). Pectins also chelate monovalent cations (e.g., K^{+}), although more weakly than divalent cations (Celus et al., 2018). The stiffness of the hydrogel depend upon ionic bonding between pectin molecules and Ca^{2+} (Western et al., 2001). For oilseeds such as

Camelina and canola, SCM is a sticky contaminant, similar to hydratable gums. Gums are typically removed by degumming pretreatments of unrefined oils using water, acids, or alkali (Dijkstra, 2010; Ohlson, 1992; Segers and van de Sande, 1990).

Making biodiesel

Biodiesel is a sustainable fuel alternative to petroleum diesel containing long-chain alkyl esters (methyl or ethyl esters) derived from renewable feedstocks, such as vegetable oils and animal fats (Salvi et al., 2013). Biodiesel is obtained through the transesterification reaction. During transesterification, an alcohol is chemically reacted with vegetable oil in the presence of a catalyst to produce fatty acid esters and glycerol. Methanol predominates in industrial transesterification, due to its low cost, quick reaction, and ease of dissolving the NaOH catalyst (Ma and Hanna, 1999). Because the reaction is reversible, excess methanol is used to shift the equilibrium to the product side. The catalyst used to improve the reaction rate and yield can be acid or alkali, with alkali catalysts requiring shorter reaction times. Sodium hydroxide (NaOH) or potassium hydroxide (KOH), which have already been dissolved into the alcohol, are the alkaline catalysts most often used (Li and Mupondwa, 2014).

Although Camelina had been grown in rainfed areas of the Western United States (French et al., 2009; Grady and Nleya, 2010; McVay and Lamb, 2008; Robinson, 1987), in 2010 field trials had not yet been conducted for varietal Camelina in northern Nevada. To explore the adaptability of Camelina to the area, and to develop a diverse library of mutants displaying phenotypes that could perform well as biodiesel feedstocks in northern Nevada, the Cushman Lab of the University of Nevada, Reno designed a five-

year field trial of eight named varieties, and generated an EMS mutant library from cv. ‘Celine.’ This dissertation contains the results of the five-year field trial, and the characterization of EMS mutant Cs98, which lacks typical seed coat mucilage.

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Chapter 2: Five-Year field trial of *Camelina sativa* varieties in northern Nevada

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Introduction:

Camelina (*Camelina sativa* (L.) Crantz**),** also known as false or wild flax, is an oilseed species within the Brassicaceae (mustard) family. Camelina, which originated in Northern Europe, has been cultivated as a source of vegetable oil for food, lighting, medicine, and as animal feed (Berti et al., 2016; Murphy, 2016; Putnam et al., 1993; Zubr, 1997). Camelina has also received considerable interest as a promising alternative oilseed crop for biofuel production (Ciubota-Rosie et al., 2013; Moser, 2010b; Shonnard et al., 2010), particularly for on-farm biodiesel production to increase farm income, diversify rural economies, and promote renewable biofuel use (Keske et al., 2013). While Camelina has been grown successfully in Nevada, evaluation of well-adapted varieties to dryland conditions is lacking. Therefore, we report on the performance of eight named varieties grown in Reno, Nevada over a five-year field trial.

Camelina is a rapidly maturing (85-120 day) short-season species typically planted as an annual or a winter annual crop best adapted to cooler climates and is currently grown in traditional flax-growing regions of the upper Midwest (Minnesota, North Dakota, South Dakota, and Montana) and Canada among many other areas throughout the world (Robinson, 1987). The rapid-cycling oilseed can be used in crop rotations or in mixed intercropping systems, often with soybean or winter wheat. Camelina is highly adaptable and can be grown in marginal, low-fertility, and saline soils and in semi-arid areas (Budin et al., 1995). Although typically grown in rain-fed areas, it responds well to applied irrigation. Camelina is quite cold tolerant, capable of surviving late-spring freezes (Robinson, 1987) making it suitable as late-winter, early-spring oilseed crop in northern Nevada.

Additional agronomic attributes include a high level of resistance to insect pest and high nutrient-use efficiency resulting in low-input and inexpensive cropping system with minimal fertilizer inputs without the need for fungicide or pesticide applications. Camelina's nitrogen requirements are modest (54 lb/ac, (Mohammed et al., 2017)) compared to other oilseeds, such as safflower (80 – 161 lb/ac (Haby et al., 1982)) or canola (75 lb/ac (Davison, 2015)).

Areas such as Canada that have developed infrastructures for marketing Camelina products provide crop insurance and value Camelina seed at 86% of the price of canola, with Camelina prices at $0.58 to $0.68 per pound (Corporation, 2018). In the United States, crop insurance is available in North Dakota and Montana. The main incentive in the United States is growing Camelina as a feedstock for biodiesel, classified as an advanced biofuel, with a mandate for increasing production through 2022 (Obour et al., 2015).

Using the protein-rich and oil-rich Camelina seed meal (after oil pressing) as a portion of animal feed rations would provide a cost justification for farmers to produce Camelina (Keske et al., 2013). Ranchers with land resources and livestock are incentivized to grow Camelina both for on-farm feed and fuel use (Foulke et al., 2012).

Background:

Camelina seeds contain 30-45% oil, depending upon the cultivar tested (Gugel and Falk, 2006; Putnam et al., 1993). Greater than 50% of the fatty acids in Camelina oil are polyunsaturated, making it similar to soybean oil in its proportion of saturated to

unsaturated fatty acids with up to 45% omega-3 fatty acids, making it a highly suitable dietary oil. Such a high degree of poly-unsaturation would normally make it susceptible to spoilage and shorten its shelf life (Ciubota-Rosie et al., 2013); however, this is offset partially by its exceptionally high tocopherols (vitamin E) content making it extremely resistant to oxidation and rancidity (Abramovic et al., 2007).

Camelina oil consists of 13-17% oleic (C18:1), 16-23% linoleic (C18:2Ω6), and 39% alpha-linolenic (C18:33Ω3) fatty acids. Linoleic ("omega-6") and alpha-linolenic ("omega-3") fatty acids in Camelina oil make Camelina oil or seed meal an advantageous supplement to animal feed rations. However, when converted to methyl and ethyl esters for biodiesel production, these high percentages of polyunsaturated fatty acids provide for less than ideal oxidative stabilities and high iodine values compared with canola, palm, and soybean oils. However, other fuel properties were similar to these other biodiesels and blends with ultra-low-sulfur diesel fuel including low temperature operability, acid value, octane number, kinematic viscosity, lubricity, sulfur and phosphorous content, and surface tension (Moser and Vaughn, 2010a). The unsatisfactory oxidative stability of biodiesel or diesel blends prepared from Camelina oil can be overcome provided that antioxidant additives are employed (Schober and Mittelbach, 2004).

After oil pressing, Camelina seed meal contains 4-14% oil, 35-48% crude protein, and 10-11% fiber making it well suited as a feed supplement for livestock and poultry (Colombini et al., 2014; Gugel and Falk, 2006; Putnam et al., 1993). Camelina meal has received the FDA "no objection" status to supplement feed rations up to 10% for cattle goats, and poultry (Schill, 2009), and is also approved for feeding many other farmed products including swine, salmon and cod.

While Camelina is grown in Nevada (Neupane et al., 2018; Neupane et al., 2019), selecting varieties that perform well under irrigated dryland conditions has not been fully explored. Therefore, researchers evaluated the field performance of eight named Camelina varieties over a five-year period to identify those that perform well under dryland conditions.

Experimental methods:

The variety trial was conducted at the University of Nevada Valley Road Field Laboratory in Reno, NV. The Natural Resources Conservation Service (NRCS) describes the soils on the Valley Road Field Laboratory (NV628) as Orr sandy loam with 0 to 2% slopes for 87.8% of the area of interest (AOI) and Orr gravelly, sandy loam with 0 to 2% slopes for 12.2% AOI. The planting field (0.19 acre) was rated as prime farmland, if irrigated. The available water-holding capacity for this site is low (approximately 1.5 inches). The normal frost-free period ranges from 109-134 days. The soil is predominantly clay with 1.21% organic matter, and rated as Irrigated Capability Class 2 (moderate limitations), subclass c (very dry climate) (Soil Survey Staff, 2018).

The Camelina varietal trials were planted with six replications per variety, in a pseudo-randomized complete block design to ensure that two plots of the same variety were not planted adjacent vertically, horizontally, or diagonally. The individual plot size was 3.3 by 3.3 feet (1 m^{-2}). The field trials were planted in late winter (March 5-7) from 2011 to 2015. The named Camelina varieties evaluated are listed in Table 1 and were supplied by Russ Karow (Oregon State University). Seeds were planted using hand broadcasting, followed by raking in to a depth of 0.25 inch. In 2011, the seeding rate was

6 pounds per acre (800 seeds m^{-2}). Seeding rates were increased by 5% each year to compensate for possible reductions in germination rates. A perimeter of Camelina was planted to minimize border effects. The plots were weed free at the time of planting.

Prior to planting, the site was fertilized with urea at a rate of 60 pounds per acre, which resulted in 52.5 pounds of nitrogen per acre. The site was irrigated immediately following planting using overhead sprinklers on timers. The site was irrigated regularly until seedlings emerged in seven to ten days. After emergence, the site was inspected weekly for soil dryness and irrigated two to three times per week, to ensure that water was not a limiting factor. Reno's most windy portion of the year (mid-February through the end of June), with average wind speeds of 6.4 mph (Weatherspark, 2019) coincides with the varietal growing season (March through June). Due to wind dispersion and evaporative loss, sprinkler irrigation is not optimal if wind speeds exceed 4.5 mph (Ouazaa et al., 2016). Hence, irrigation was scheduled in the early morning hours when wind speeds are minimal, to reduce loss from evaporation and wind dispersion. Natural precipitation data (Table 6) and average annual precipitation data (Table 7) were obtained from National Weather Service (NOAA National Weather Service, 2018).

Weed control on the Camelina trials consisted of regular cultivation between plots and hand weeding. The primary weed species present were puncture vine (*Tribulus terrestris*), pigweed (*Amaranthus viridis*), and common purslane (*Portulaca oleracea*). The site was enclosed by steel fencing to prevent grazing damage from rabbits. Upon termination of irrigation two weeks prior to harvest, netting was installed to prevent bird sampling in 2013, 2014, and 2015. The field was not treated with any pesticides or

herbicides. No significant insect or microbial disease pests were noted during the course of the study.

The plots were harvested manually on June 29, 2011, June 29 2012, June 21, 2013, June 30, 2014, and July 24, 2015, based on predominance of beige-colored seed pods in the plots. All aerial biomass (including seed pods) was harvested using hedge clippers, stored in paper bags and allowed to dry at 68-81 ° F for four months until air-equilibrated dryness occurred. After drying, the seeds were threshed manually from the aerial biomass and cleaned using an Almaco Air Blast Seed Cleaner (Almaco Seed Co., Model #ABSC, Nevada, IA). The vegetative biomass was stored in labeled bags containing biomass from one plot with the weighed seeds from that plot.

The dry weight mass of seed and aerial biomass was weighed and recorded. Harvest indices were calculated for the seed yield using the formula ((seed weight / (seed weight + biomass weight)). Seed and aerial biomass weights were analyzed using one-way analysis of variance (ANOVA), T-tests, GenStat software (VSNI, 2017), and PROC ANOVA (Table 4) using SAS software 9.4 (SAS Institute Inc., Cary, NC, USA). Mean separation was accomplished using Duncan's HSD at the $p = \leq 0.05$ level. Oil content (%) per dry weight was determined using a Bruker mq20 minispec benchtop Nuclear Magnetic Resonance (NMR) instrument (Bruker Corporation, https://www.bruker.com). The oil yield (Table 5) was calculated as the product of the average seed mass yield for each variety per year, $n = 6$ (Table 1), multiplied by the average oil percentage of dry weight for that variety (Table 4), averaged from 12 replicates per variety per year.

Results:

Seed yield

The average seed yield data for each variety from 2011 to 2015 are shown in Table 1. Although the five-year average seed yields for the eight varieties did not display statistically significant differences, Columbia, Cheyenne, Blaine Creek, and Calena were ranked by GenStat software (VSNI, 2017) as the top four in cultivar superiority due to high seed yield and performance stability. Columbia had the highest average seed yield with 811 lb/acre per year. This variety was followed by Cheyenne at 681 lb/acre per year, Blaine Creek at 663 lb/acre per year, and Calena at 661 lb/acre per year. The highest average yields were in years 2015 (1,412 lb/acre), 2014 (897 lb/acre), and 2011 (670 lb/acre).

Biomass yield

The average biomasses for each of the Camelina varieties from 2011 to 2015 are shown in Table 2. Although the five-year average biomass yields did not display statistically significant differences, Blaine Creek, Columbia, and Calena were ranked by GenStat as the top three in cultivar superiority due to high biomass yield and performance stability. Blaine Creek had the highest average biomass with 4,232 lb/acre per year. The second highest variety was Calena at 4,156 lb/acre per year. Columbia was the third highest at 4,106 lb/acre per year. The highest average yields were in years 2015 (6,181 lb/acre),

2013 (4,030 lb/acre), and 2011 (3,510 lb/acre). Average biomass yields comparing the eight varieties did not show statistical significance in any of the five years.

Harvest index

The harvest index for each of the eight Camelina varieties averaged over five years is shown in Table 3. A harvest index value represents the reproductive efficiency of a crop based on its grain weight to total biomass weight (grain plus aerial biomass) ratio. Cheyenne, Calena, and Suneson were ranked by GenStat as the top three in cultivar superiority due to high harvest indices and performance stability. Columbia had the highest harvest index at 0.1472. This variety was followed by Cheyenne at 0.1312 and Suneson at 0.1305. The five-year averages and individual year averages did not display statistical significance.

Oil content

The oil percent of dry weight summary of the Camelina varieties is shown in Table 4. Although the five-year average oil percentages of dry weight for the eight varieties did not show statistically significant differences, Columbia, Celine, and Ligena were ranked by GenStat as the top three in cultivar superiority due to high oil percentage of dry weight and performance stability. Calena displayed the highest average seed oil percentage at 29.26%. This variety was followed by Columbia at 29.16%, Blaine Creek at 29.08%, and

Suneson at 28.97%. Oil percentages of dry weight comparing the eight varietals displayed statistically significant differences in each of the five years ($p \leq 0.01$).

Oil yield

The oil yields shown in Table 5 were calculated as the product of the average seed mass yield for each variety per year, $n = 6$ (Table 1), multiplied by the average oil percentage of dry weight for that variety (Table 4), averaged from 12 replicates per variety per year. All eight varieties were ranked in descending order, with Columbia having the highest oil yield averaged over five years (32.1 gal/ac), followed by Cheyenne (27.3 gal/ac), Calena (26.4 gal/ac), and Blaine Creek (25.8 gal/ac), with the average being 25.8 gal/ac.

Water inputs

The impact of both natural precipitation and applied irrigation upon seed yield, biomass yield, and oil percentage of dry weight is presented in Table 6. Years with the highest natural precipitation were 2015 (4.60 in) and 2011 (4.59 in). These two years and 2014 also displayed the highest average seed yields and combined harvest indices. With added sprinkler irrigation, the total water received by the varietals for each consecutive trial year was 35.26, 46.05, 52.62, 68.47, and 65.56 inches.

Table 7 displays the average seed yield of the Reno, NV study compared to three other irrigated Camelina varietal field trials in the semi-arid to arid western United States.

The present study had the largest number of trials (40), with the lowest average growing season precipitation and irrigation (13.3 inches) relative to the other studies conducted in NE and AZ. This study also had the widest seed yield range (68-1412 lb/acre) and the lowest average seed yield (658 lb/acre) compared with these other studies.

Discussion:

Availability of soil moisture, judicious application of irrigation, and overall total water applied can dramatically impact yield. Years having higher winter precipitation (2011 and 2015, Table 6) presumably a reserve of soil moisture for plants to draw upon resulting in higher yields. Dry winters (2012, 2013, and 2014, Table 6) require irrigation to obtain significant yields. In 2011 and 2015, natural precipitation of 4.59 and 4.6 inches, respectively, was associated with high average seed yields of 670 and 1,412 lb/acre.

Judicious application of irrigation so that ample water is available to help plants successfully make the transitions from seedlings to well established rosettes to bolting, ensures that adequate foliage is available for flowering and seed development. Irrigation timers, although necessary, cannot substitute for daily inspection of the field to assess soil and foliage conditions. A period of above average temperatures or wind speeds may decrease irrigation efficiency and growers must adapt with increased irrigation.

Irrigation delivery systems and their efficiencies also impact plant growth and yield. The overhead sprinklers used in this study were vulnerable to dispersion due the windy conditions present from mid-February through the end of June. Plots on the

periphery of the field were most affected, with the loss of seed yield in one 3.3 x 3.3 feet square plot in 2011, 2012, 2014, and 2015. Peripheral plots had lower yields than core plots, resulting in data outliers and more variance (data not shown). Increased variance of peripheral plots affects the data for varietals, because one-way ANOVA assumes that a variety's variance is normally distributed around a central value (average), and that variances are equal for all samples. The data were tested for each assumption, and if either assumption failed, ANOVA could not be run and the data column was assigned "n.s." for not statistically significant.

The overhead sprinklers in this study trial were positioned and relocated manually, were not as effective as the irrigation systems used by the other irrigated field trials, and resulted in lower average seed yields (Table 7). Although Reno has an average annual precipitation of 6.5 in, slightly above Maricopa, AZ with 5.4 in, the total water applied (with irrigation) was 13.3 in compared to Maricopa's 13.5 in (Hunsaker et al., 2011). Maricopa's average seed yields were 1.5-fold higher than Reno's. The 2011 Maricopa study used surface irrigation, measured by propeller flow meters, and volumetric soil water contents were monitored by neutron moisture gauges (Hunsaker et al., 2011). Both the 2011 and 2016 Pavlista studies used overhead linear-move sprinkler systems (Pavlista et al., 2016; Pavlista et al., 2011). Water was applied at 17 in per year, per trial, which was considerably higher than the 13.3 and 13.5 in per year for the Reno and Maricopa studies, respectively, and higher yields were obtained.

Insufficient or delayed irrigation during critical stages in plant development can result in fewer pods and lower yields. The average seed yields of two dry years, 2012

and 2013 (68 and 240 lb/ac, respectively) show that irrigation was inadequate or not applied in a timely manner to result in adequate yields. The dry winter of 2012, followed by insufficient irrigation of 43.43 in, produced 68 lb/ac, resulted in the lowest average yield of the trials. After the dry winter of 2013, irrigation was increased to 51.15 in, resulting in a 3.5-fold increase in yield. In 2014, after another dry winter, 66.47 in of irrigation were applied liberally and judiciously, resulting in a 3.7-fold increase over the prior year. In 2015, after a wet winter, 60.96 in of irrigation were applied liberally and judiciously, resulting in a 1.6-fold higher yield than the previous year.

Adequate irrigation levels also impact oil percentage of dry weight, as well irrigated plants produce more oil in their seeds. The years with high natural precipitation (2011 and 2015, Table 6) had average oil percent of dry weights of 29.07 and 30.81, respectively (Table 4). The dry years that had inadequate irrigation (2012 and 2013, Table 6) showed average oil percent of dry weights of 26.65 and 28.05, respectively (Table 4). The dry year with judicious and liberal irrigation (2014, Table 6) showed average oil percent of dry weight of 30.06 (Table 4).

Dry years with poor yields (2012 and 2013, Table 1) and wet years with improved yields (2015) create variance in the data. A low yield year (2012 with 68 lb/ac) or a high yield year (2015 with 1,412 lb/ac) distorts the five-year average, lowering it or raising it, respectively. As seed mass yields (Table 1) are a major factor in calculating harvest indices (Table 3) and calculated oil yields (Table 5), increased variance in seed mass creates a “ripple effect” which affects variance in the other parameters.

Adequate foliar biomass is required for Camelina to flower and produce seed. Biomass is a major factor in calculating the harvest index ratio (seed mass divided by the sum of seed mass and biomass). Varieties such as Columbia, with high seed mass yields and modest biomass yields, have higher harvest indices (Table 3). Aerial biomass can be ground up and be used as a soil amendment to increases organic matter in the soil to increase water absorption and retention capacity, which is highly advantageous in semi-arid areas.

Camelina requires less irrigation than crops such as alfalfa, soy, and canola, and can be grown as a rain-fed crop in semi-arid areas. However, substantially higher yields will result with applied irrigation. The most widely-produced crop in Nevada, alfalfa, requires 1.8 to 3 acre-feet of water per season (Davison et al., 2016). In contrast, this study's Camelina required only 0.074 acre feet, on average, which is approximately 4.1% of alfalfa's seasonal demand.

Summary and Conclusions:

Camelina has several favorable traits that make it a promising alternative oilseed crop for Nevada. Camelina is more cold and drought tolerant than canola and requires lower inputs of irrigation, fertilizer, and pesticides than other conventional crops. Furthermore, Camelina does not require prime agricultural acreage but can be grown on marginal lands. Camelina's seed meal compares favorably with canola and can provide a value-added product as an animal feed ration.

This five-year field trial showed that Camelina can be grown successfully in semi-arid regions with applied irrigation. Overall, Columbia is highly recommended, because it displayed the highest average seed yield of 811 lb/ac (Table 1) and the highest harvest index, 0.1472, (Table 3) over the five-year trial. Columbia also had the second highest seed oil percentage of dry weight at 29.16% (Table 4). However, both Cheyenne and Calena would also be suitable for northern Nevada growing conditions. Cheyenne had the second highest seed yield of 681 lb/ac (Table 1), and the third highest harvest index of 0.1287 (Table 3). Calena's five-year average seed yield of 661 lb/ac (Table 1) exceeded the average of all varieties. Calena also had the fourth highest five-year average harvest index of 0.1266, close to Cheyenne's harvest index of 0.1287 (Table 3).

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Figure 1: A square meter plot of *Camelina sativa*.

Table 1: Seed mass yields (pounds/acre) of eight Camelina varieties grown in Reno, Nevada, 2011-2015.

Variety[1]	2011	2012	2013	2014	2015	Five-year average[2]	Cultivar superiority[3]	Five-year Ranking
Columbia	1,074	78	297	895	1,713	**811**	7,051	**1**
Cheyenne	683	59	118	955	1,589	**681**	29,957	**2**
Calena	717	79	206	742	1,564	**661**	34,611	**3**
Blaine Creek	582	46	215	1,082	1,388	**663**	40,305	**4**
Yellowstone	509	75	244	1,118	1,302	**650**	52,713	**5**
Celine	748	112	225	642	1,198	**585**	64,321	**6**
Suneson	676	61	437	851	1,063	**618**	65,573	**7**
Ligena	368	30	175	888	1,478	**588**	68,245	**8**
Average	**670**	**68**	**240**	**897**	**1,412**	**657**		
Standard error	43.9	7.1	40.7	97.2	115	44.4		
p value[4]	n.s.	0.075	n.s.	n.s.	n.s.	n.s.		
Alpha	0.05	0.05	0.05	0.05	0.05	0.05		

[1]Number of samples: Blaine Creek (*n* = 30), Calena (*n* = 30), Celine (*n* = 30), Cheyenne (*n* = 29), Columbia (*n* = 30), Ligena (*n* = 30), Suneson (*n* = 30), Yellowstone (*n* = 29).

[2]Five-year averages are not significantly different at alpha = 0.05.

[3]Lower score of stability coefficient units indicates higher performance stability over five years.

[4]The *p* value resulting from one-way ANOVA with alpha = 0.05; n.s. (not significantly different) indicates ANOVA not performed as ANOVA assumption tests failed.

Table 2: Biomass yields (pounds/acre) of eight Camelina varieties grown in Reno, Nevada, 2011-2015.

Variety[1]	2011	2012	2013	2014	2015	Five-year average[2]	Cultivar superiority[3]	Five-year ranking
Blaine Creek	3,520	2,784	3,750	3,664	7,442	4,232	145,832	1
Columbia	3,251	2,176	4,553	3,811	6,740	4,106	190,846	2
Calena	3,867	3,091	3,881	2,601	7,332	4,156	236,850	3
Ligena	3,162	2,063	4,058	2,636	6,957	3,774	389,727	4
Yellowstone	3,963	2,289	4,110	3,402	5,401	3,833	547,952	5
Celine	3,653	2,704	3,973	3,280	5,321	3,783	575,396	6
Cheyenne	3,571	2,252	3,091	3,646	5,834	3,679	649,278	7
Suneson	3,092	2,353	4,821	2,947	4,419	3,526	1,119,114	8
Average	**3,510**	**2,464**	**4,030**	**3,248**	**6,181**	**3,886**		
Standard Error	111	183	302	311	422	147.8		
p value [4]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Alpha	0.05	0.05	0.05	0.05	0.05	0.05		

[1]Number of samples: Blaine Creek (*n* = 30), Calena (*n* = 30), Celine (*n* = 30), Cheyenne (*n* = 29), Columbia (*n* = 30), Ligena (*n* = 30), Suneson (*n* = 30), Yellowstone (*n* = 29).

[2]Five-year averages are not significantly different at alpha = 0.05.

[3]Lower score of stability coefficient units indicates higher performance stability over five years.

[4]The p value resulting from one-way ANOVA with alpha = 0.05; n.s. (not significantly different) indicates ANOVA not performed as ANOVA assumption tests failed.

Table 3: Harvest Index of eight Camelina varieties grown in Reno, Nevada, 2011-2015.

Variety[1]	2011	2012	2013	2014	2015	Five-year average[2]	Cultivar superiority[3]	Five-year Ranking
Cheyenne	0.1621	0.0316	0.0344	0.2236	0.2045	0.1312	0.001215	**1**
Calena	0.1542	0.0255	0.0376	0.2367	0.1788	0.1266	0.001256	**2**
Suneson	0.1776	0.0266	0.0540	0.2041	0.1903	0.1305	0.001281	**3**
Columbia	0.2452	0.0313	0.0534	0.1686	0.2375	0.1472	0.001551	**4**
Ligena	0.1034	0.0163	0.0389	0.2929	0.1784	0.1260	0.002156	**5**
Blaine Creek	0.1415	0.0167	0.0381	0.1989	0.1531	0.1097	0.002301	**6**
Yellowstone	0.1078	0.0293	0.0495	0.2159	0.1812	0.1168	0.002545	**7**
Celine	0.1677	0.0397	0.0447	0.1537	0.1594	0.1130	0.002747	**8**
Average	**0.1574**	**0.0271**	**0.0438**	**0.2118**	**0.1854**	**0.1251**		
Standard Error	0.0081	0.0021	0.0040	0.0133	0.0064	0.0059		
p value [4]	n.s.	n.s.	n.s.	0.1745	n.s.	0.773		
Alpha	0.05	0.05	0.05	0.05	0.05	0.05		

[1]Number of samples: Blaine Creek (n=30), Calena (n=30), Celine (n=30), Cheyenne (n=29), Columbia (n=30), Ligena (n=30), Suneson (n=30), Yellowstone (n=29).

[2]Five-year averages are not significantly different at alpha = 0.05.

[3]Lower score of stability coefficient units indicates higher performance stability over five years.

[4]The p value resulting from one-way ANOVA with alpha = 0.05; n.s. (not significantly different) indicates ANOVA not performed as ANOVA assumption tests failed.

Table 4: Oil Percent of Dry Weight yields of eight Camelina varieties grown in Reno, Nevada, 2011-2015.

Variety[1]	2011	Production group[2]	2012	Production group[2]	2013	Production group[2]	2014	Production group[2]	2015	Production group[2]	Five-year average [3]	Cultivar superiority[4]	Five-year ranking
Columbia	29.91	A	28.40	A	27.42	ABC	29.36	AB	30.73	ABC	29.16	1.152	1
Celine	29.25	AB	26.73	AB	27.06	BC	29.92	AB	29.80	C	28.55	1.473	2
Ligena	27.43	C	26.80	AB	29.60	A	28.27	B	31.85	A	28.79	2.093	3
Blaine Creek	28.84	B	25.90	B	28.89	AB	30.50	AB	31.27	AB	29.08	2.281	4
Calena	29.38	AB	26.54	AB	27.84	ABC	31.50	A	31.06	ABC	29.26	2.331	5
Yellowstone	28.71	B	26.50	AB	27.52	ABC	30.66	AB	30.22	BC	28.72	2.849	6
Cheyenne	29.17	AB	26.65	AB	26.29	C	30.87	AB	31.35	AB	28.86	2.899	7
Suneson	29.88	A	25.65	B	29.74	A	29.36	AB	30.23	BC	28.97	3.832	8
Average	**29.07**		**26.65**		**28.05**		**30.06**		**30.81**		**28.93**		
Standard error		0.0990		0.2058		0.2203		0.2155		0.1248	0.2100		
p value [5]		<0.0001		0.0064		<0.0001		0.0065		<0.0001	0.3088		
Alpha		0.05		0.05		0.05		0.05		0.05	0.05		

[1]Number of samples: 12 for all varieties.

[2]Varieties grown within a single year with the same capital letter (A) are not found to differ significantly at alpha = 0.05; varieties followed by a different capital letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of capital letters (e.g., AB and A, or AB and BC) are not found to differ significantly.

[3]Five-year averages are not significantly different at alpha = 0.05.

[4]Lower score of stability coefficient units indicates higher performance stability over five years.

[5]The p value resulting from one-way ANOVA with alpha = 0.05.

Table 5: Calculated Oil Yield (gallons/acre) of Reno, Nevada Camelina field trial, 2011-2015[1]

Variety	2011	2012	2013	2014	2015	Five-year average	Cultivar superiority[2]	Five-year ranking
Columbia	41.9	2.9	10.3	35.0	70.3	**32.1**	16.39	**1**
Cheyenne	25.6	2.0	3.8	39.2	65.8	**27.3**	47.46	**2**
Calena	27.5	2.5	7.3	31.4	63.4	**26.4**	55.36	**3**
Blaine Creek	22.0	1.6	7.5	42.6	55.0	**25.8**	71.00	**4**
Yellowstone	19.3	2.7	7.7	46.7	50.7	**25.4**	94.89	**5**
Ligena	13.4	1.0	6.6	35.5	60.5	**23.4**	111.53	**6**
Suneson	26.0	1.9	15.2	33.4	42.5	**23.8**	120.30	**7**
Celine	28.7	4.0	8.5	24.7	46.9	**22.6**	124.75	**8**
Average	25.6	2.3	8.4	36.1	56.9	**25.8**		

[1]Oil yields calculated by multiplying average seed mass values (n = 6) (Table 1) times average oil percentages of dry weight (n = 12) (Table 4), and converted to gallons/acre.
[2]Lower score of stability coefficient units indicates higher performance stability over five years.

Table 6: Precipitation, irrigation, and average seed and biomass yields of Camelina varietals over five years.

Year (Jan-Jun)	Natural precipitation (in)	Applied irrigation (in)	Total water applied (in)	Average seed yield (lb/ac)	Average biomass yield (lb/ac)	Average seed and biomass yield (lb/ac)	Combined Harvest Indices for all varietals
2011	4.59	30.67	35.26	670	3,510	4,180	0.1603
2012	2.62	43.43	46.05	68	2,464	2,532	0.0267
2013	1.47	51.15	52.62	240	4,029	4,268	0.0561
2014	2.00	66.47	68.47	897	3,247	4,144	0.2164
2015	4.60	60.96	65.56	1,412	6,181	7,593	0.1859

Table 7: Average seed yield of eight Camelina varieties grown in Reno, Nevada, 2011-2015 (this study) compared to other irrigated field trials.

Location	Number of Years (Trials)	Year range	Average annual precipitation (in)	Average growing season precipitation and irrigation (in)	Nitrogen applied (lb/acre)	Seed yield range (lb/acre)	Average seed yield (lb/acre)	Citation
Reno, NV	**5 (40)**	**2011-2015**	**6.5**	**13.3**	**53.4**	**68-1,412**	**658**	**This study**
Scottsbluff, NE	2 (4)	2005-2006	15.8	17	40	495-1,297	947	Pavlista et al., 2011
Maricopa, AZ	2 (4)	2006-2007	5.4	13.5	44.5	914-1,087	1,013	Hunsaker et al., 2011
Scottsbluff/Sidney, NE	2 (4)	2007-2008	14.2	17	37.4	2,265	2,265	Pavlista et al., 2016

Chapter 3: Five-year field trial of eight *Camelina sativa* varieties for biofuel production in Nevada

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Five-year Field Trial of Eight *Camelina sativa* Varieties for Biofuel Production in Nevada

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Abstract

Camelina is a promising oilseed crop used for dietary oil and as a biofuel feedstock. Camelina is a highly adaptable, cool season crop that can be grown on marginal lands with minimal inputs, making it potentially suitable for growth in northern Nevada and other semi-arid areas of North America. A five-year (2011 to 2015) field trial of eight cultivars was conducted to select the best-performing varieties under irrigated dryland conditions. Columbia, Cheyenne, Calena, and Blaine Creek were ranked as the top four varieties due to performance stability in seed yield and calculated oil yield. Overall, Columbia displayed the highest five-year mean seed yield of 909.5 kg ha^{-1} and the highest harvest index of 0.1472. Columbia also had the second highest seed oil percent of dry weight at 29.16%, but the highest calculated oil yield at 300 L ha^{-1}. The highest mean yields of all eight varieties were in years 2015 (1,582 kg ha^{-1}), 2014 (1,005 kg ha^{-1}), and 2011 (751 kg ha^{-1}). The yields of this study fall within the ranges of yields reported in both irrigated and rainfed locations of the western United States. This five-year field trial showed that Camelina can be grown successfully in semi-arid regions with irrigation and ranked eight cultivars by performance stability and oil yield.

Keywords: *Camelina sativa, dryland agriculture, oilseed crops, biodiesel feedstock*

1. Introduction

Camelina (*Camelina sativa* (L.) Crantz)**,** also known as false or wild flax, German sesame, gold of pleasure, and linseed dodder, is an oilseed species within the Brassicaceae (mustard) family. Camelina originated in Northern Europe, the Mediterranean, and Central Asia, where it has been cultivated since Neolithic times as a

source of vegetable oil for food, lighting, medicine, and as an animal feed (Putnam et al., 1993; Zubr, 1997). Camelina has also received considerable interest as an alternative oilseed crop for biofuel production (Moser, 2010a; Shonnard et al., 2010), particularly for on-farm biodiesel production to increase farm income, diversify rural economies, and promote renewable biofuel use (Keske et al., 2013).

Camelina is a rapidly maturing (85-120 d) short-season species typically planted as an annual or a winter annual crop best adapted to cooler climates and is currently grown in traditional flax-growing regions of the upper Midwest (i.e., Minnesota, North Dakota, South Dakota, and Montana) and Canada among many other areas throughout the world including Europe, Asia, and Australia (Robinson, 1987). The rapid-cycling oilseed can be used in crop rotations or in mixed intercropping systems.

Ideal candidate crops for dryland areas demonstrate the ability to adapt to abiotic stress, including drought tolerance, and limited water and fertilizer inputs. Nevada's leading cash crop is *Medicago sativa* (alfalfa), which is valued as $200 million annually from tonnage produced (NASS, 2016) and requires large water inputs (Lindenmayer et al., 2011). Although a legume, alfalfa removes large amounts of minerals from the soil, and may require significant fertilizer inputs for optimum yield (Koenig et al., 1999).

Camelina is highly adaptable and can be grown in in arid and semi-arid areas in the western and southwestern United States (Hunsaker et al., 2013), and on marginal, low-fertility, and saline soils (Budin et al., 1995). Camelina is quite cold tolerant, capable of surviving late-spring freezes (Robinson, 1987) making it potentially suitable as oilseed crop in northern Nevada. Additional agronomic attributes include a high level of resistance to insect pests (Soroka et al., 2015) and high nutrient-use efficiency

resulting in low-input and inexpensive cropping system with minimal fertilizer inputs (Mohammed et al., 2017a) without the need for fungicide (Seguin-Swartz et al., 2009) or pesticide applications.

Camelina seeds contain 30-45% oil, depending on the cultivar tested (Gugel and Falk, 2006; Putnam et al., 1993). About 54% of the fatty acids in Camelina oil are polyunsaturated, which is a highly desirable characteristic for use as a dietary oil. Such a high degree of polyunsaturation would normally make it susceptible to autoxidation and limit its ability to be stored for long periods of time. However, this oxidation potential is offset, in part, by its exceptionally high tocopherols (vitamin E) content making it extremely resistant to oxidation and rancidity (Abramovic et al., 2007).

Camelina oil consists of 13-17% oleic (C18:1), 16-23% linoleic (C18:2Ω6), and 31-39% alpha-linolenic (C18:33Ω3) fatty acids (Radocaj and Dimic, 2013; Zubr, 1997). However, following its conversion to methyl and ethyl esters for biodiesel production, the high proportion of polyunsaturated fatty acids can lead to high degrees of oxidative instability and increase iodine values relative to other biodiesel derived from canola, palm, and soybean oils. However, other fuel properties were similar to these biodiesels and blends with ultra-low-sulfur diesel fuel including low temperature operability, acid value, octane number, kinematic viscosity, lubricity, sulfur and phosphorus content, and surface tension (Moser and Vaughn, 2010b). The unsatisfactory oxidative stability of Camelina biodiesel or methyl ester blends can be overcome easily by the addition of inexpensive and readily available antioxidant additives such as Baynox (Schober and Mittelbach, 2004).

After oil pressing, Camelina seed meal contains 4-14% oil, 35-48% crude protein, and 10-11% fiber making it well suited as a feed supplement for livestock and poultry (Colombini et al., 2014; Gugel and Falk, 2006; Putnam et al., 1993). Camelina meal has received FDA "no objection" approval to supplement feed rations up to 10% for cattle goats, and poultry (Schill, 2009), and is also approved for feeding many other farmed products including swine, salmon and cod.

Several irrigation studies have been completed in the Midwest (Pavlista et al., 2016; Pavlista et al., 2011) and Southwest (Hunsaker et al., 2011) and rainfed studies were completed in the Intermountain west (McVay and Khan, 2011; Mohammed et al., 2017; Obour et al., 2018; Sintim et al., 2015, 2016; Wysocki et al., 2013), the Midwest (Obour et al., 2018; Obour et al., 2017), and the Pacific Northwest (Guy et al., 2014; Schillinger et al., 2012; Wysocki et al., 2013). Two recent Nevada studies reported that Camelina benefits from early season sowing using a seed drill and adequate fertilization rates (Neupane et al., 2018; Neupane et al., 2019). However, comparing varieties that perform well under irrigated rainfed conditions has not been fully explored.

The purpose of the five-year field trial was to determine which Camelina varieties show high yield and performance stability in seed mass, biomass, and calculated oil yields, and high harvest indices when grown in northern Nevada. The null hypothesis was that mean performance would not differ significantly among varieties for the measured parameters. Therefore, the performance of eight named varieties of Camelina were evaluated over a five-year period. This study revealed that Columbia displayed the highest five-year mean seed yield, harvest index, and calculated oil yield among the eight

varieties tested. The yields of all varieties fell within the range of yields reported in both irrigated (Hunsaker et al., 2011; Pavlista et al., 2016; Pavlista et al., 2011) and rainfed (McVay and Khan, 2011; Obour et al., 2018; Obour et al., 2017; Wysocki et al., 2013) locations of the western United States.

2. Material and methods

3.1 Material and methods

The variety trial was conducted at the Nevada Agricultural Experiment Station at the Valley Road Field Laboratory in Reno, NV. The Natural Resources Conservation Service (NRCS) described the soils on the Valley Road Field Laboratory (NV628) as Orr sandy loam with 0 to 2% slopes for 87.8% of the area of interest (AOI) and Orr gravelly, sandy loam with 0 to 2% slopes for 12.2% AOI. The planting field (0.077 hectare) was rated as prime farmland, if irrigated. The available water-holding capacity for this site was low (approximately 3.8 cm). The normal frost-free period ranged from 109-134 days. The soil was predominantly clay with 1.21% organic matter and rated as Irrigated Capability Class 2 (moderate limitations), subclass c (very dry climate) (Soil Survey Staff, 2018).

The Camelina varietal trials were planted with six replications per variety, in a pseudo-randomized complete block design (Yobi et al., 2013) to ensure that two plots of the same variety were not planted adjacent to one another vertically, horizontally, or diagonally. The individual plot size was one m^2. The field trials were planted in late winter (March 5-7) from 2011 to 2015. The named Camelina varieties evaluated are

listed in Table 1 and were supplied by Russ Karow (Oregon State University). Seeds were planted using hand broadcasting, followed by raking in to a depth of 6.35 mm. In 2011, the seeding rate was 6.73 kg ha^{-1} (800 seeds m^{-2}). Seeding rates were increased by 5% each year to compensate for possible reductions in germination rates. The plots were weed free at the time of each planting.

Prior to planting, the site was fertilized with urea at a rate of 67.25 kg ha^{-1}, which resulted in 58.8 kg ha^{-1} N. The site was irrigated immediately following planting using overhead sprinklers on timers. The site was irrigated every other day until seedlings emerged in seven to ten days. After emergence, the site was inspected weekly for soil dryness and irrigated two to three times per week.

Reno's most windy portion of the year (mid-February through the end of June), with mean wind speeds of 2.7 m s^{-1} (Weatherspark, 2019) coincides with the varietal growing season (March through June). Due to wind dispersion and evaporative loss, sprinkler irrigation was not optimal if the wind speeds exceeded 2 m s^{-1} (Ouazaa et al., 2016). Hence, irrigation was scheduled in the early morning hours when wind speeds were minimal, to reduce loss from evaporation and wind dispersion.

Applied irrigation amounts for the months of March through June and the total water applied (the sum of applied irrigation and natural precipitation for the months of January through June) are shown in Supporting Information Table S1. Natural precipitation data (Table S1) and mean annual precipitation data (Table 6) were obtained from the National Weather Service of the National Ocean and Atmospheric Administration (NOAA) (NOAA National Weather Service, 2018).

Weed control on the Camelina trials consisted of regular cultivation between plots and hand weeding. The primary weed species present were puncture vine (*Tribulus terrestris*), pigweed (*Amaranthus viridis*), and common purslane (*Portulaca oleracea*). The site was enclosed by steel fencing to prevent herbivory damage from rabbits. Upon termination of irrigation two weeks prior to harvest, netting was installed to prevent bird sampling in 2013, 2014, and 2015. The field was not treated with any pesticides or herbicides. No significant insect or microbial disease pests were noted during the course of the study.

The plots were harvested manually on June 29, 2011, June 29, 2012, June 21, 2013, June 30, 2014, and July 24, 2015, based on the predominance of beige-colored seed pods in the plots. All aerial biomass (including seed pods) was harvested using hedge clippers, stored in paper bags, and allowed to dry at 23.9 – 27.5°C for four months until air-equilibrated dryness occurred. After drying, the seeds were threshed manually from the aerial biomass and cleaned using an Almaco Air Blast Seed Cleaner (Almaco Seed Co., Model #ABSC, Nevada, IA). The vegetative biomass was stored in labeled bags containing biomass from one plot with the weighed seeds from that plot.

The dry weight mass of seed and aerial biomass was determined using an OHAUS SL Adventurer Laboratory Balance (OHAUS Corp., Parsippany, NJ, USA) and recorded. Oil content (%) per dry weight was determined using a Bruker mq20 minispec benchtop Nuclear Magnetic Resonance (NMR) instrument (Bruker BioSpin Corporation, San Jose, CA, https://www.bruker.com) using a calibration curve derived from purified Camelina seed oil using the manufacturer's instructions.

3.2. Statistical Analysis and calculations

Statistical analysis of seed mass (Table 1), aerial biomass (Table 2), harvest index (Table 3), and oil percentage of dry weight (DW) (Table 4) was performed using analysis of variance (ANOVA). Data from Tables 1 through 4 were tested for ANOVA assumptions for homogeneity of variance (Bartlett's Test) and normality (Shapiro-Wilk Test) at the $p \leq 0.05$ level using GenStat software (VSNI, 2017). If the data failed either test, lack of statistical significance (n.s.) was noted on the table for that particular column or row of means. If the data passed both ANOVA assumption tests, mean separation was performed for Tables 1, 2, and 3 using one-way ANOVA within GenStat software (VSNI, 2017) at alpha = 0.05. The one-way ANOVA *p*-value was noted in the table. Mean separation for Table 4 used PROC ANOVA with SAS software 9.4 (SAS Institute Inc., Cary, NC, USA) followed by Bonferroni's post hoc test at the $p \leq 0.05$ level. As described above, the one-way ANOVA *p*-values were noted in the table.

Harvest indices (Table 3) were calculated for the seed yield using the formula ((seed weight/(seed weight + biomass weight)). The oil yield (Table 5) was calculated as the product of the mean seed mass yield for each variety per year, $n = 6$ (Table 1), multiplied by the mean oil percentage of dry weight for that variety per year, $n = 12$ (Table 4).

To graphically present the performance of each genotype (variety) for each parameter (seed mass, biomass, harvest index, oil percent of dry weight (DW), and calculated oil yield) for each year, mean data of each genotype's performance from Tables 1 through 5 were entered into GenStat software (VSNI, 2017). Genotype and

Genotype-by-Environment (GGE) biplots, a useful visualization technique (Yang et al., 2009) were generated by GenStat software (VSNI, 2017) as shown as Figures 1, 2, 3, 4, 5, and Supporting Information Figures S1, S2, S3, S4, and S5.

As the phenotypic trait of each genotype was determined by both the genotype main effects (G) and the environment, and if these two effects were not additive, genotype-by-environment interactions (GE) were present (Mulualem and Bekeko, 2017). GE analysis to identify the best genotypes was significant when performance ranking of genotypes varied in different environments (Mulualem and Bekeko, 2017). GE analysis programs included additive main effects and multiplicative interactions (AMMI) developed in 1984 (Frutos et al., 2014), and GGE GenStat software (VSNI, 2017) developed as described (Frutos et al., 2014; Yan et al., 2000).

Biplot representation of principal component analysis (PCA) was developed by Gabriel (Gabriel, 1971). Briefly, a set of multi-environment trials (MET) having *g* genotypes tested in each of *e* environments, and each with *r* replications, can be summarized by averaging the phenotypic values of each genotype across *r* replications within each environment, resulting in the *g* X *e* cell means in a two-way table (Yang et al., 2009). The table can be analyzed through ANOVA combined with PCA (Yang et al., 2009). ANOVA or simple linear regression models quantify the complexity of the genotype-by-environment interactions in one dimension (Elias et al., 2016). However, AMMI and GGE GenStat software (VSNI, 2017), are more recent models which decompose genotype-by-environment effects to explain the interaction in more than one dimension (Elias et al., 2016).

PCA, or singular value decomposition (SVD), reduces the dimensionality of the two-way (genotype-by-environment) data matrix (Yan et al., 2000) for the given parameter into two linear constructs called principal components (PCs) (Yeater et al., 2015). The first PC (PC1) represents a vector of best fit for the means data for the given parameter displaying the maximum variability (Yeater et al., 2015) and approximates the genotypic main effects (G) mean performance (Bhartiya et al., 2017; Yan and Tinker, 2006; Yan, 2001). The second PC (PC2) represents a vector displaying the least variability, which approximates the genotype-by-environment (GE) effect (Bhartiya et al., 2017; Yan, 2001), and is orthogonal to PC1 (Starmer, 2018). Ideally, highly stable genotypes score high on PC1 (*e.g.*, high yield) with low scores on PC2 (*e.g.*, high stability) (Yan and Kang, 2002; Yan et al., 2001). Biplots are considered useful if the first two PCs account for > 60% of the sum of (G + GE) variability (Yang et al., 2009). Both genotypic main effects (G) and genotype-by-environment (GE) effects as depicted in biplots are mathematically defined by the SVD of the matrix data they represent and do not have a simple correspondence to the expression of distinct gene clusters (Yan et al., 2007).

Based on differences in the linear models of the older AMMI method and the more recent GGE GenStat software method (VSNI, 2017), GGE GenStat software (VSNI, 2017) provides "which won where" graphical displays of MET, which can help identify genotypes that consistently perform well across multiple environments (Yan et al., 2001; Yan et al., 2000; Yang et al., 2009). AMMI biplots show only GE, whereas biplots generated using GGE GenStat software (VSNI, 2017) represent both the

genotypic main effect (G) plus GE (Yan et al., 2000). GGE GenStat software (VSNI, 2017) "which won where" biplots can provide quantitative comparisons if three caveats are met: (a) adequate sampling of the MET (Yan et al., 2001), (b) genotype PC1 scores having near-perfect correlation ($r > 0.95$) with the genotypic main effects, with very low PC2 scores (Yan et al., 2001), and (c) repeatable "which won where" patterns over multiple years (Yang et al., 2009).

Because the 2011 to 2015 Camelina trials were conducted in the same location, the GenStat software (VSNI, 2017) biplots were modified to represent each trial year as a different "environment," displaying the biplots as "Which Won When" rather than "which won where." As the Camelina trials data did not meet the second caveat, the "Which Won When" biplots for each parameter (Figures S1, S2, S3, S4, and FS5) are included in Supporting Information as qualitative data summaries.

GGE GenStat software (VSNI, 2017) Ranking Biplots (Figures 1, 2, 3, 4, 5) generated genotype scores ("X"), which show performance of varietals in proximity to environments ("+") (years). The blue arrow on AEC (average environment coordinate) abscissa line points in the direction of highest parameter value and ranks the genotypes with respect to the parameter shown (Yan et al., 2007). The circle adjacent to the point of the blue arrow (Yan and Tinker, 2006) represents the mean of all environmental values for the given parameter (*i.e.*, the five-year mean of values for all varietals). The AEC ordinate axis, orthogonal to the AEC abscissa line, represents the contributions of the genotypes to the genotype-by-environment (GE) effect and displays performance stability (Yan et al., 2007). Genotype scores displaying a smaller distance from the blue abscissa

axis indicated higher stability or consistency of performance for all years, with a longer distance indicating lower stability and less consistent performance (Yan et al., 2007). Varietals performed best in the blue years ("environments") closest to them. The sum of the variance accounted for by PC1 + PC2 (genotypic and "genotype-by-environment" (Year) effects was summarized in each ranking plot.

GGE GenStat software (VSNI, 2017) "Which Won When" Biplots (Supporting Information Figures S1, S2, S3, S4, S5) generated genotype (green "X") markers furthest from the plot origin (located at the intersection of the two axes) indicated the best performers for the parameter displayed (Yan et al., 2001). Connecting the markers for best performance formed a convex hull (polygon), which enclosed genotypes that did not perform as well (Yan et al., 2001). Genotypes performed best in the environments (circled years, blue "+") that encompass or are adjacent to genotypes. Genotypes and environments closest to the plot origin indicated low performance of the parameter displayed. Mega-environments are years surrounded by ellipses, which indicated similar performance based on mean yield and stability (Mulualem and Bekeko, 2017); those genotypes within or adjacent to the ellipses displayed the best performance during those years. The sum of the variance accounted for by PC1 + PC2 (genotypic and "genotype-by-environment" (year) effects was summarized in each "Which Won When" plot.

In production agriculture, genotypes are evaluated in terms of production stability (Yan and Kang, 2002). Genotypes that show little variance among varied environments and inputs demonstrate static stability, which is undesirable. Genotypes which demonstrate consistent performance, but show measurable response to varied inputs and

environments, illustrate dynamic stability or the agronomic concept (Becker, 1981; Yan and Kang, 2002). Genotype performance ideally corresponds to an estimated level in specific environments (Becker and Leon, 1988; Yan and Kang, 2002). Over twenty different univariate and multivariate methods have been developed to analyze genotype-by-environment (GE) interactions (Flores et al., 1998), beginning with linear regression analysis (Mooers, 1921) through GGE GenStat software's stability coefficient analysis (Yan and Kang, 2002).

Cultivar superiority units, which provided the five-year rankings of the eight genotypes by high parameter success and performance stability in Tables 1 through 5, were determined by GenStat software (VSNI, 2017). The GGE GenStat software (VSNI, 2017) used a multiplicative model that combined univariate (ANOVA) and multivariate (decomposed by SVD) techniques (Elias et al., 2016).

3. Results

3.1. Seed yield

Although there were no statistically significant differences among the five-year mean seed yields for the eight varieties (Table 1), the GenStat software (VSNI, 2017) ranking biplot ranked Columbia, Cheyenne, and Calena as the top three in cultivar superiority due to seed yield higher than the mean and performance stability (Figure 1). Columbia was the best performer in 2011 and 2015, and Cheyenne performed well in 2012 and 2014, as shown in the GenStat software (VSNI, 2017) "Which Won When" biplot (Figure S1).

The mean seed yield data for each variety from 2011 to 2015 are shown in Table 1. Columbia had the highest mean seed yield with 909.5 kg ha^{-1}. This variety was followed by Cheyenne at 728 kg ha^{-1}, Blaine Creek at 742.7 kg ha^{-1}, and Calena at 741.4 kg ha^{-1}. The highest mean seed yields occurred in years 2015 (1,582 kg ha^{-1}), 2014 (1,005 kg ha^{-1}), and 2011 (751 kg ha^{-1}). Seed yields for the eight varieties did not show statistically significant differences for 2011 through 2015 at alpha = 0.05. However, each individual cultivar demonstrated statistically significant differences when evaluated over the five years ($p < 0.002$).

3.2. Biomass yield

Although the five year mean biomass yields did not display statistically significant differences (Table 2), the GenStat software (VSNI, 2017) ranking biplot ranked Blaine Creek, Calena, and Ligena as the top three in cultivar superiority due to high biomass yield and performance stability (Figure 2). Blaine Creek displayed high biomass production and performance stability in all years except 2013, as shown in the GenStat software (VSNI, 2017) "Which Won When" biplot (Figure S2). Figure S2 also shows that Calena was a highly productive in 2011, 2012, and 2015, and Ligena was highly productive in years 2012, 2013, and 2015. Although Camelina is typically not used for biomass production, biomass data are reported here to evaluate harvest index (see below), which is a performance metric of wide interest.

The mean biomasses for each of the Camelina varieties from 2011 to 2015 are shown in Table 2. Blaine Creek had the highest mean biomass with 4,743.6 kg ha^{-1}. The second highest variety was Calena at 4,656.7 kg ha^{-1}, and Yellowstone was the third highest at 4,296.4 kg ha^{-1}. The highest mean yields were in years 2015 (6,710 kg ha^{-1}), 2013 (4,515.4 kg ha^{-1}), and 2011 (3,934.1 kg ha^{-1}). Mean biomass yields comparing the eight varieties within each year did not display statistical significance. However, Blaine Creek and Ligena demonstrated statistically significant differences ($p < 0.002$) when evaluated over the five years.

3.3. Harvest Index

The harvest index value represents the reproductive efficiency of a crop based on its grain weight to total biomass weight (grain plus aerial biomass) ratio. The GenStat software (VSNI, 2017) ranking biplot ranked Columbia, Cheyenne, and Suneson as the top three in cultivar superiority due to high harvest indices and performance stability (Figure 3). These three varieties exhibited high harvest indices in years 2011, 2012, 2013 and 2015, as shown in the GenStat software (VSNI, 2017) "Which Won When" biplot (Figure S3). The mean harvest index for each of the eight Camelina varieties over five years is shown in Table 3. Columbia had the highest harvest index at 0.1472. This variety was followed by Suneson at 0.1305 and Cheyenne at 0.1287. Mean harvest indices comparing the eight varieties within each year did not display statistical significance. However, all individual cultivars except Cheyenne and Celine demonstrated statistically significant differences ($p \leq 0.001$) when evaluated over the five years.

3.4. Oil content

Although the five-year mean oil percentages of dry weight for the eight varieties did not show statistically significant differences (Table 4), the GenStat software (VSNI, 2017) ranking biplot ranked Columbia, Celine, and Ligena as the top three in cultivar superiority due to high oil percent of dry weight and performance stability (Figure 4). Columbia displayed the highest oil percent of dry weight in years 2011 and 2012, as shown in the GenStat software (VSNI, 2017) "Which Won When" biplot (Figure S4). Figure S4 also shows that both Celine and Ligena displayed high oil percent of dry weight in years 2011, 2013 and 2015. The oil percent of dry weight summary of the Camelina varieties is shown in Table 4. Calena displayed the highest mean seed oil percentage at 29.26%. This variety was followed closely by Columbia at 29.16% and Blaine Creek at 29.08%. Oil percentages of dry weight comparing the eight varietals displayed statistically significant differences in each of the five years ($p \leq 0.0007$). The five-year mean oil percentages of dry weight did not show statistically significant differences. However, all individual cultivars except Celine demonstrated statistically significant differences ($p \leq 0.0001$) when evaluated over the five years.

3.5. Oil yield

The oil yields shown in Table 5 were calculated as the product of the mean seed mass yield for each variety per year, $n = 6$ (Table 1), multiplied by the mean oil percentage of dry weight for that variety, $n = 12$ (Table 4). The GenStat software (VSNI, 2017)

ranking biplot ranked Columbia, Cheyenne, and Calena as the top three in cultivar superiority due to high oil yield and performance stability (Figure 5). The five-year mean oil yields for these three varieties were 299.9, 255.3, and 247.2 liters ha^{-1}, respectively (Table 5). Columbia displayed the highest oil yields in 2011 and 2015, with Ligena showing highest oil yields in 2014, as shown in the GenStat software (VSNI, 2017) "Which Won When" biplot (Figure S5).

3.6. Other field trials

Table 6 displays the mean seed yield of the Reno, NV study compared to three other irrigated Camelina varietal field trials and twelve other rainfed Camelina varietal field trials in the arid western United States. The present study had the largest number of trials (40), with the second lowest mean annual precipitation (165.1 mm), relative to the other fifteen studies. This study also had the widest seed yield range (76 - 1,583 kg ha^{-1}) and the third lowest mean seed yield (736.4 kg ha^{-1}) compared with these other studies.

4. Discussion

Due to the low rates of natural precipitation in semi-arid northern Nevada, supplementary irrigation is required to maximize oilseed crop yields. The Reno field trial site in northern Nevada, received 165.1 mm mean annual precipitation (NOAA, 2018) for the 2011-2015 field trial years (Table 6). Supplementary irrigation (Table S1) was applied each year. Total water applied in 2014 (1,739.14 mm) was slightly more than in 2015 (1,665.22 mm). However, in 2015 the mean seed yield of 1,582.4 kg ha^{-1} was 1.6-

fold higher than the 2014 mean seed yield, likely due to the greater amount of natural precipitation in 2015 (Table S1). The two years with the highest natural precipitation (2011 and 2015) resulted in high mean seed yields and harvest indices (all eight varieties combined). Natural precipitation provided the soil base with ample moisture prior to planting, which was reinforced by ensuing irrigation. In 2011 and 2015, natural precipitation totals of 116.59 and 116.84 mm, respectively, were associated with mean seed yields of 751 and 1,582.4 kg ha^{-1}. The driest years were 2012 - 2014. In 2012 and 2013, the lowest mean seed yields of 75.7 and 268.5 kg ha^{-1}, respectively, were obtained. However, in 2014 seed yields improved as more irrigation was applied, which resulted in a mean seed yield of 1005.2 kg ha^{-1}, which exceeded the yield in 2011, a wet year.

Seed mass yields were higher in years with high natural precipitation (2011 and 2015) and the highest irrigated year (2014) (Table S1), with mean seed masses of 751, 1,582 and 1,005 kg ha^{-1} respectively (Table 1). The years with low natural precipitation and with lower irrigation levels (2012 and 2013) (Table S1) showed mean seed masses of 76 and 269 kg ha^{-1} respectively (Table 1).

While all eight cultivars produced substantial seed mass yields in 2011, 2014, and 2015, a few varieties exceeded mean yields in the two drier years (2012 and 2013). Cultivars that demonstrated above average seed mass yield in 2012 were Columbia, Calena, Yellowstone, and Celine, with yields of 87, 89, 84, and 126 kg ha^{-1} respectively (Table 1). Cultivars that exceeded mean seed mass yield in 2013 were Columbia, Yellowstone, and Suneson, with yields of 333, 274, and 490 kg ha^{-1} respectively (Table 1).

In evaluating genotypes for dynamic production stability, Columbia was the leader, as it exceeded mean seed yields in each of the five trial years (Table 1). Based on seed mass yield and stability over the five years, both Cheyenne and Calena were ranked second and third by GenStat software (VSNI, 2017) (Figures 1 and S1). However, Yellowstone's performance in both 2012 and 2013, illustrates the resilience of that cultivar in exceeding mean seed yields for both of the drier years (Table 1).

Biomass production results showed that Blaine Creek produced the highest mean aerial biomass of 4,743.6 kg ha^{-1} over five years (Table 2). The next highest biomass producers were Calena and Columbia, with aerial biomass of 4,656.7 and 4,248.6 kg ha^{-1}, respectively. These three varieties were ranked highest in terms of biomass yield and performance stability. Aerial biomass can provide benefits as a soil amendment that increases organic matter in the soil, which will increase water absorption capacity, advantageous in arid areas.

The harvest index provides a useful measure to compare yields based on reproductive efficiency, as it gives the ratio of seed yield to combined seed and biomass yields. The high seed yields in 2011 and 2015 resulted in combined harvest indices of 0.1574 and 0.1850, respectively (Table 3). In 2014, the highest level of applied irrigation (1,739.14 mm) (Table S1) resulted in a harvest index of 0.2117, which exceeded the 2011 and 2015 harvest indices. The improved 2014 harvest index as a result of applied irrigation shows the positive impact of irrigation upon improving reproductive efficiency.

Columbia, Cheyenne, and Suneson exceeded mean harvest indices in four, four, and three of the five trial years (Table 3) and were ranked as the top three cultivars by

GenStat software (VSNI, 2017) (Figures 3 and S3). However, the harvest index values of Celine and Yellowstone in both 2012 and 2013 illustrate the resilience of those cultivars in exceeding mean harvest index for both of the drier years (Table 3).

Mean oil percentage of dry weight values were highest in 2011, 2014, and 2015, with means of 29.07, 30.06 and 30.81 respectively (Table 4). Table S1 shows that 2011 and 2015 had the greatest natural precipitation and 2014 had the greatest total water applied. Columbia, Celine, and Ligena exceeded the mean 2012 oil percentage, and Ligena, Blaine Creek, and Suneson exceeded the mean 2013 oil percentage. Ligena's performance in 2012, 2013 and 2015 (the two drier years and one wet year) showed the strength of that cultivar in both dry and wet years.

Columbia, Cheyenne, and Calena are the top three varietals in Calculated Oil Yield (Figures 5 and S5) and follow the rankings in seed mass (Figures 1 and S1), due to the calculation of oil yield. Varietals that exceeded the 2012 and 2013 means were Columbia, Calena, Yellowstone, and Celine; and Columbia, Suneson, and Celine, respectively. Both Columbia's and Celine's performance in exceeding the means of each of the two drier years showed the strengths of those cultivars.

The year effect, as shown in the statistical significance of each individual variety evaluated over the five years of the study (Tables 1 through 4) illustrates the sensitivity of each parameter to the varying levels of natural precipitation and total water applied (Table S1). Biomass displayed the most variance and showed the least statistical significance, perhaps due to the dynamic response of vegetative growth to water availability and genotypic differences in water uptake and use efficiency.

The yields of this study fall within the ranges of yields reported in both irrigated and rainfed locations of the western United States (Table 6). When compared with three other irrigated field trials, the results of this study showed mean seed yields of 736.4 kg ha^{-1}. Although Reno has an mean annual precipitation of 165.1 mm, slightly above Maricopa, AZ with 137.2 mm, the total water applied (with irrigation) was 337.8 mm compared to Maricopa's 342.9 mm (Hunsaker et al., 2011). Maricopa's mean seed yields were 1.5-fold higher than Reno's, perhaps due to the irrigation methods used. The 2011 Maricopa study used surface irrigation, measured by propeller flow meters and volumetric soil water contents monitored by neutron moisture gauges (Hunsaker et al., 2011). Both the 2011 and 2016 irrigated Nebraska studies used overhead linear-move sprinkler systems (Pavlista et al., 2016; Pavlista et al., 2011). Water was applied at 431.8 mm per year, per trial, which was 1.3-fold higher than the 337.8 and 342.9 mm per year for the Reno and Maricopa studies, respectively, and higher yields were obtained.

In contrast to Camelina, Nevada's leading cash crop, *Medicago sativa* (alfalfa), requires large water inputs. Alfalfa's water demand, using flood irrigation in Reno, has been estimated as a mean of 7,790 mm per season (Davison et al., 2016). In contrast, this study's Camelina required a mean of 338 mm or approximately 4.3% of alfalfa's seasonal water demand. These differences show the value of developing alternative crops (*e.g.*, Camelina) to modify habitual practices of growing high input crops that require high levels of scarce water resources. However, a minimum level of irrigation above 1,000 mm is suggested to realize respectable seed yields and economic return on investment.

5. Conclusion

This five-year field trial showed that Camelina can be grown successfully in semi-arid regions with applied irrigation. Overall, Columbia is highly recommended for use as an oilseed crop in arid lands, because it displayed the highest mean seed yield of 909.5 kg ha^{-1} (Table 1) and the highest harvest index, 0.1472, (Table 3) over the five-year trial. Columbia also had the second highest seed oil percentage of dry weight at 29.16% (Table 4). However, both Cheyenne and Calena would also be suitable for northern Nevada growing conditions. Cheyenne had the second highest seed yield of 728.0 kg ha^{-1} (Table 1), the second lowest biomass weight of 4,038 kg ha^{-1} (Table 2) and the third highest harvest index of 0.1287 (Table 3). Calena's five-year mean seed yield of 741.4 kg ha^{-1} (Table 1) exceeded the mean. Calena also had the fourth highest five-year mean harvest index of 0.1266, close to Cheyenne's harvest index of 0.1287 (Table 3). Blaine Creek is another productive variety for northern Nevada. Although it had the second highest seed yield (742.7 kg ha^{-1} (Table 1), its harvest index was the lowest at 0.1097 (Table 3), due to its high biomass weight of 4,743.6 kg ha^{-1} (Table 2). Despite these weaknesses, due to high performance stability in seed yield and calculated oil yield, GenStat software (VSNI, 2017) ranked Blaine Creek above the remaining four varietals.

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Table 1: Seed mass yields (kg ha^{-1}) of eight Camelina cultivars grown in Reno, Nevada, 2011-2015.

Cultivar	2011[1]	2012[1]	2013[1]	2014[1]	2015[1]	Five-year mean[2]	SEM[3]	One way ANOVA, Cultivar *p* value[4]	Cultivar superiority[5]	Five-year Ranking
Columbia	1,204$^{(ab)}$	87$^{(b)}$	333$^{(b)}$	1,004$^{(ab)}$	1,921$^{(a)}$	909.5	±165.3	<0.001	8,858	1
Cheyenne	766$^{(bc)}$	66$^{(c)}$	132$^{(bc)}$	1,070$^{(ab)}$	1,781$^{(a)}$	728.0	±145.9	<0.001	37,365	2
Calena	803$^{(b)}$	89$^{(c)}$	231$^{(bc)}$	832$^{(b)}$	1,753$^{(a)}$	741.4	±126.7	<0.001	43,482	3
Blaine Creek	653$^{(abc)}$	52$^{(c)}$	241$^{(bc)}$	1,213$^{(ab)}$	1,555$^{(a)}$	742.7	±143.6	0.0010	50,635	4
Yellowstone	571$^{(abc)}$	84$^{(c)}$	274$^{(bc)}$	1,254$^{(ab)}$	1,459$^{(a)}$	710.1	±145.6	0.0010	66,224	5
Celine	839$^{(ab)}$	126$^{(b)}$	253$^{(ab)}$	720$^{(ab)}$	1,343$^{(a)}$	656.0	±140.1	0.0181	80,807	6
Suneson	758$^{(ab)}$	68$^{(b)}$	490$^{(ab)}$	954$^{(ab)}$	1,191$^{(a)}$	692.2	±118.7	0.0107	82,379	7
Ligena	412$^{(b)}$	34$^{(b)}$	196$^{(b)}$	995$^{(ab)}$	1,657$^{(a)}$	658.8	±147.2	<0.001	85,736	8
Mean	**751**	**76**	**269**	**1,005**	**1,582**	**729.9**				
Standard error[6]	±49.2	±7.9	±45.6	±107.8	±127.2	±49.8				
One way ANOVA										
Year *p* value[4]	n.s.	0.0747	0.2125	n.s.	n.s.	0.9316				
Alpha	0.05	0.05	0.05	0.05	0.05					

[1]Cultivars grown across five years with the same lower case letter (a) are not found to differ significantly at alpha = 0.05; varieties followed by a different lower case letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of lower case letters (e.g., ab and bc, or c and abc) are not found to differ significantly.
[2]Five year means were not found to differ significantly at alpha = 0.05.
[3]SEM calculated for five-year means of individual cultivar yields.
[4]One way ANOVA p value run with alpha = 0.05; n.s. indicates ANOVA not performed as ANOVA assumption tests failed. *P* values have been adjusted for multiple comparisons.
[5]Lower score of stability coefficient units indicates higher performance stability over five years.
[6]Standard errors calculated for mean of yields of eight cultivars grown in individual years.

Table 2: Biomass yields (kg ha^{-1}) of eight Camelina cultivars grown in Reno, Nevada, 2011-2015.

Cultivar	2011[1]	2012[1]	2013[1]	2014[1]	2015	Five-year mean[2]	SEM[3]	One way ANOVA, Cultivar *p* value[4]	Cultivar superiority[5]	Five-year Ranking
Blaine Creek	3,946$^{(b)}$	3,121$^{(b)}$	4,203$^{(b)}$	4,107$^{(b)}$	8,341$^{(a)}$	4,744	±501.0	0.0050	183,209	1
Columbia	3,644	2,439	5,103	4,272	5,785	4,249	±392.5	n.s.	239,761	2
Calena	4,335	3,465	4,350	2,915	8,218	4,657	±517.7	n.s.	297,557	3
Ligena	3,544$^{(ab)}$	2,312$^{(b)}$	4,548$^{(ab)}$	2,955$^{(ab)}$	7,797$^{(a)}$	4,231	±595.7	0.0173	489,617	4
Yellowstone	4,442	2,566	4,607	3,813	6,054	4,296	±402.5	n.s.	688,396	5
Celine	4,095	3,031	4,453	3,666	5,964	4,242	±480.2	0.3518	722,874	6
Cheyenne	4,002	2,524	3,455	4,087	6,539	4,038	±438.8	0.0609	815,693	7
Suneson	3,465	2,638	5,403	3,303	4,953	3,952	±420.0	n.s.	1,405,951	8
Mean	**3,934.1**	**2,762.0**	**4,515.4**	**3,639.7**	**6,710.1**	**4,301**				
Standard error[6]	±124.4	±204.9	±338.4	±348.2	±468.1	±165.7				
One way ANOVA Year *p* value [4]	n.s.	n.s.	n.s.	n.s.	n.s.	0.9244				
Alpha	0.05	0.05	0.05	0.05	0.05					

[1]Cultivars grown across five years with the same lower case letter (a) are not found to differ significantly at alpha = 0.05; varieties followed by a different lower case letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of lower case letters (e.g., ab and bc, or c and abc) are not found to differ significantly.

[2]Five year means were not found to differ significantly at alpha = 0.05.

[3]SEM calculated for five-year means of individual cultivar yields.

[4]One way ANOVA *p* value run with alpha = 0.05; n.s. indicates ANOVA not performed as ANOVA assumption tests failed. *P* values have been adjusted for multiple comparisons.

[5]Lower score of stability coefficient units indicates higher performance stability over five years.

[6]Standard errors calculated for mean of yields of eight cultivars grown in individual years.

Table 3: Harvest Index of eight Camelina cultivars grown in Reno, Nevada, 2011-2015.

Cultivar	2011[1]	2012[1]	2013[1]	2014[1]	2015[1]	Five-year mean[2]	SEM[3]	One way ANOVA, Cultivar *p* value[4]	Cultivar superiority[5]	Five-year Ranking
Cheyenne	0.1621	0.0316	0.0344	0.2236	0.2045	**0.1287**	±0.0166	n.s.	0.001215	1
Calena	0.1542$^{(b)}$	0.0255$^{(c)}$	0.0376$^{(c)}$	0.2367$^{(a)}$	0.1788$^{(ab)}$	**0.1266**	±0.0166	<0.001	0.001256	2
Suneson	0.1777$^{(a)}$	0.0266$^{(b)}$	0.0540$^{(b)}$	0.2041$^{(a)}$	0.1903$^{(a)}$	**0.1305**	±0.0156	<0.001	0.001281	3
Columbia	0.2452$^{(a)}$	0.0313$^{(b)}$	0.0534$^{(b)}$	0.1686$^{(a)}$	0.2375$^{(a)}$	**0.1472**	±0.0186	<0.001	0.001551	4
Ligena	0.1034$^{(bc)}$	0.0163$^{(c)}$	0.0389$^{(bc)}$	0.2929$^{(a)}$	0.1784$^{(ab)}$	**0.1260**	±0.0234	<0.001	0.002156	5
Blaine Creek	0.1415$^{(a)}$	0.0167$^{(b)}$	0.0381$^{(b)}$	0.1989$^{(a)}$	0.1531$^{(a)}$	**0.1097**	±0.0166	<0.001	0.002301	6
Yellowstone	0.1078$^{(b)}$	0.0293$^{(c)}$	0.0495$^{(bc)}$	0.2159$^{(a)}$	0.1812$^{(b)}$	**0.1168**	±0.0149	<0.001	0.002545	7
Celine	0.1677	0.0397	0.0447	0.1537	0.1594	**0.1130**	±0.0136	n.s.	0.002747	8
Mean	**0.1574**	**0.0271**	**0.0438**	**0.2117**	**0.1850**	**0.1295**				
Standard error[6]	±0.0081	±0.0021	±0.0040	±0.0134	±0.0133	±0.8095				
One way ANOVA										
Year *p* value [4]	n.s.	n.s.	n.s.	0.1745	n.s.	0.8797				
Alpha	0.05	0.05	0.05	0.05	0.05					

[1]Cultivars grown across five years with the same lower case letter (a) are not found to differ significantly at alpha = 0.05; varieties followed by a different lower case letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of lower case letters (e.g., ab and bc, or c and abc) are not found to differ significantly.

[2]Five year means were not found to differ significantly at alpha = 0.05.

[3]SEM calculated for five-year means of individual cultivar yields.

[4]One way ANOVA p value run with alpha = 0.05; n.s. indicates ANOVA not performed as ANOVA assumption tests failed. *P* values have been adjusted for multiple comparisons.

[5]Lower score of stability coefficient units indicates higher performance stability over five years.

[6]Standard errors calculated for mean of yields of eight cultivars grown in individual years.

Table 4: Oil percent of dry weight of eight Camelina cultivars grown in Reno, Nevada, 2011-2015.

Variety	2011[1,2]	2012[1,2]	2013[1,2]	2014[1,2]	2015[1,2]	Five-year mean[3]	SEM[4]	One way ANOVA, Cultivar *p* value[5]	Cultivar superiority[6]	Five-year Ranking
Columbia	29.91$^{(A, ab)}$	28.40$^{(A, bc)}$	27.42$^{(ABC, c)}$	29.36$^{(AB, abc)}$	30.73$^{(ABC, a)}$	**29.16**	±0.2984	<0.0001	1.152	**1**
Celine	29.25$^{(AB)}$	26.73$^{(AB)}$	27.06$^{(BC)}$	29.92$^{(AB)}$	29.80$^{(C)}$	**28.55**	±0.2280	n.s.	1.473	**2**
Ligena	27.43$^{(C, bc)}$	26.80$^{(AB, c)}$	29.60$^{(A, ab)}$	28.27$^{(B, bc)}$	31.85$^{(A, a)}$	**28.79**	±0.3250	<0.0001	2.093	**3**
Blaine Creek	28.84$^{(B, b)}$	25.90$^{(B, c)}$	28.89$^{(AB, b)}$	30.50$^{(AB, a)}$	31.27$^{(AB, a)}$	**29.08**	±0.2802	<0.0001	2.281	**4**
Calena	29.38$^{(AB, ab)}$	26.54$^{(AB, c)}$	27.84$^{(ABC, ab)}$	31.50$^{(A, a)}$	31.06$^{(ABC, a)}$	**29.26**	±0.3466	<0.0001	2.331	**5**
Yellowstone	28.71$^{(B, bc)}$	26.50$^{(AB, d)}$	27.52$^{(ABC, cd)}$	30.66$^{(AB, a)}$	30.22$^{(BC, ab)}$	**28.72**	±0.2685	<0.0001	2.849	**6**
Cheyenne	29.17$^{(AB, b)}$	26.65$^{(AB, c)}$	26.29$^{(C, c)}$	30.87$^{(AB, a)}$	31.35$^{(AB, a)}$	**28.86**	±0.3238	<0.0001	2.899	**7**
Suneson	29.88$^{(A, a)}$	25.65$^{(B, b)}$	29.74$^{(A, a)}$	29.36$^{(AB, a)}$	30.23$^{(BC, a)}$	**28.97**	±0.2899	<0.0001	3.832	**8**
Mean	**29.07**	**26.65**	**28.05**	**30.06**	**30.81**	**28.93**				
Standard error[7]	±0.099	±0.206	±0.220	±0.216	±0.125	±0.210				
One way ANOVA Year *p* value[5]	<0.0001	0.0064	<0.0001	0.0065	<0.0001	0.3088				
Alpha	0.05	0.05	0.05	0.05	0.05					

[1]Varieties grown within a single year with the same capital letter (A) are not found to differ significantly at alpha = 0.05; varieties followed by a different capital letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of capital letters (e.g., AB and A, or AB and BC) are not found to differ significantly.

[2]Varieties grown across five years with the same lower case letter (a) are not found to differ significantly at alpha = 0.05; varieties followed by a different lower case letter are not found to differ significantly at alpha = 0.05. Two varieties having an overlap of lower case letters (e.g., ab and bc, or c and abc) are not found to differ significantly.

[3]Five year means were not found to differ significantly at alpha = 0.05.

[4]SEM calculated for five-year means of individual cultivar yields.

[5]One way ANOVA *p* value run with alpha = 0.05; n.s. indicates ANOVA not performed as ANOVA assumption tests failed. *P* values have been adjusted for multiple comparisons.

[6]Lower score of stability coefficient units indicates higher performance stability over five years.

[7]Standard errors calculated for mean of yields of eight cultivars grown in individual years.

Table 5: Oil yield of eight Camelina cultivars grown in Reno, Nevada, 2011-2015[1].

Variety	2011	2012	2013	2014	2015	Five-year mean	Cultivar superiority[2]	Five-year ranking
Columbia	391.7	27.6	96.0	327.1	657.4	299.9	1,434	**1**
Cheyenne	239.7	18.8	35.5	367.1	615.5	255.3	4,153	**2**
Calena	257.2	23.8	68.6	293.6	593.0	247.2	4,844	**3**
Blaine Creek	206.1	14.8	70.6	398.9	514.1	240.9	6,212	**4**
Yellowstone	180.9	25.0	72.2	437.0	474.3	237.9	8,302	**5**
Ligena	125.5	9.4	61.3	331.9	566.1	218.9	9,759	**6**
Suneson	243.4	17.8	142.4	312.8	397.7	222.8	10,526	**7**
Celine	268.9	37.6	79.1	231.3	438.9	211.2	10,915	**8**
Mean	**239.2**	**21.8**	**78.2**	**337.5**	**532.1**	**241.8**		

[1]Oil yields calculated by multiplying mean seed mass (Table 1) values times mean oil percentages of dry weight (Table 4) and converted to liters ha^{-1}.

[2]Lower score of stability coefficient units indicates higher performance stability over five years.

Table 6: Mean seed yield of eight Camelina varieties grown in Reno, Nevada, 2011-2015 (this study) compared to other field trials.

Location	Number of Years (Trials)	Year range	Mean annual precipitation (mm)	Mean growing season precipitation and irrigation (mm)	Nitrogen applied (kg ha^{-1})	Seed yield range (kg ha^{-1})	Mean seed yield (kg ha^{-1})	Citation
Hays, KS	3 (3)	2013-2015	541.0	350.5*	56.0	447.2	447.2	Obour et al., 2017
Hays, KS	2 (2)	2014-2015	431.8	353.1*	44.8	424-908	666.9	Obour et al., 2018
Reno, NV	**5 (40)**	**2011-2015**	**165.1**	**337.8**	**58.8**	**76-1,583**	**736.4**	**This study**
Sheridan, WY	2 (2)	2014-2015	513.1	251.5*	44.8	852-975	913.5	Obour et al., 2018
Scottsbluff, NE	2 (4)	2005-2006	401.3	431.8	44.8	555-1,454	1061.4	Pavlista et al., 2011
Maricopa, AZ	2 (4)	2006-2007	137.2	342.9	49.9	1,026-1,221	1135.4	Hunsaker et al., 2011
Mocccasin/Pendroy, MT	3 (7)	2013-2015	386.1	223.5*	44.8	1,095-1,258	1212.8	Mohammed et al., 2017
Sheridan, WY	2 (2)	2013-2014	411.5	228.6*	44.8	988.6	988.6	Simtim et al., 2016
Sheridan, WY	3 (3)	2013-2015	414.0	248.9*	56.0	789-1,539	1107.4	Simtim et al., 2015
Huntley, MT	2 (2)	2008-2009	393.7	226.1*	34.0	972-1,684	1327.1	McVay and Khan, 2011
Pendleton, OR	3 (6)	2008-2010	421.6	198.1*	44.8	1,296-1,769	1543.4	Schillinger et al., 2012
Corvalis, OR	3 (3)	2008-2010	990.6	320.0*	44.8	1,561-1,593	1577.0	Wysocki, et al., 2013
Pendleton, OR	3 (3)	2008-2010	421.6	188.0*	45.0	1,628-1,707	1658.3	Guy et al., 2014
Moscow, ID/Pullman,WA	3 (3)	2008-2010	736.6	322.6*	44.8	1,658-1,697	1677.9	Wysocki, et al., 2013
Pendleton, OR	3 (3)	2008-2010	421.6	215.9*	50.0	1,760-1,791	1775.5	Wysocki, et al., 2013
Scottsbluff/Sidney, NE	2 (4)	2007-2008	360.7	431.8	41.9	2538.7	2538.7	Pavlista et al., 2016

*Rainfed

Table S1: Precipitation and irrigation for Camelina varietals over five years.

Year (Jan-Jun)	Natural precipitation (mm)	Applied irrigation (mm)	Total water applied (mm)
2011	116.59	779.02	895.60
2012	66.55	1,103.12	1,169.67
2013	37.34	1,299.21	1,336.55
2014	50.80	1,688.34	1,739.14
2015	116.84	1,548.38	1,665.22

Figure 1. Seed mass ranking biplot. Genotype scores ("X") show performance of varietals in proximity to years ("+") (Environment scores). The blue arrow on AEC (average environment coordinate) abscissa line points in direction of highest trait value (greatest seed yield). The circle adjacent to the point of the blue arrow represents the five-year mean of values for all varietals. A smaller distance from blue abscissa axis indicates higher stability or consistency of performance for 2011 – 2015 (*e.g,*. Columbia had greater seed yield than Calena, with Calena more stable than Columbia). Varietals performed best in the blue years closest to them (*i.e.*, Columbia performed best in 2011 and 2015). Genotype and Genotype x Environment (Year) effects accounted for 82.9% of trait performance.

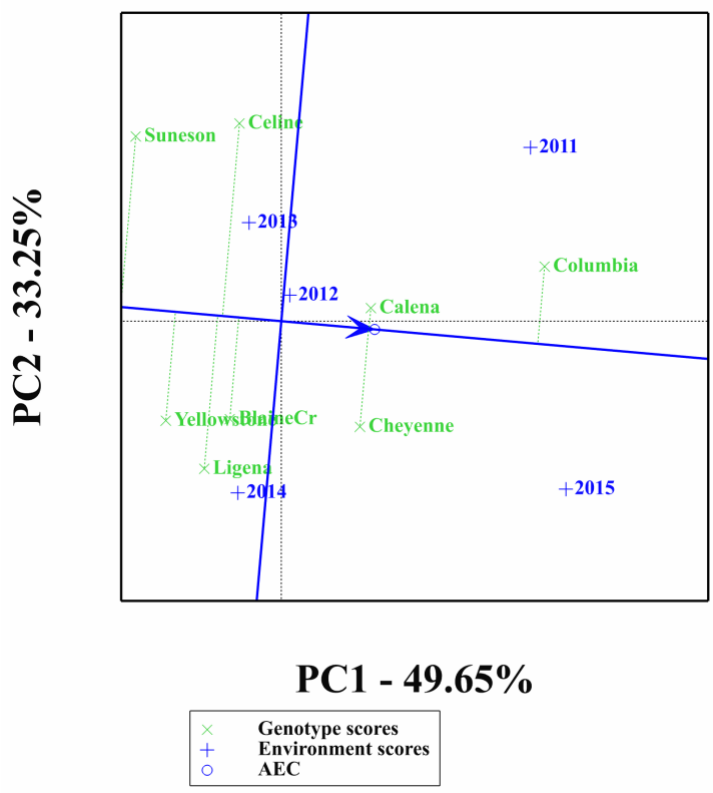


Figure 2. Biomass ranking biplot. Genotype scores ("X") show performance of varietals in proximity to years ("+") (environment scores). Blue arrow on AEC (average environment coordinate) abscissa line points in direction of highest trait value (greatest biomass yield). The circle adjacent to the point of the blue arrow represents the five-year mean of values for all varietals. A smaller distance from the blue abscissa axis indicates higher stability or consistency of performance for 2011 – 2015 (*e.g.*, Blaine Creek had greater biomass yield than Calena and Ligena, with Blaine Creek and Calena more stable than Ligena). Varietals performed best in the blue years closest to them (*i.e.*, Columbia performed best in 2012 and 2013, and better than Suneson, which performed best in 2013). Genotype and Genotype x Environment (Year) effects accounted for 80.80% of trait performance.

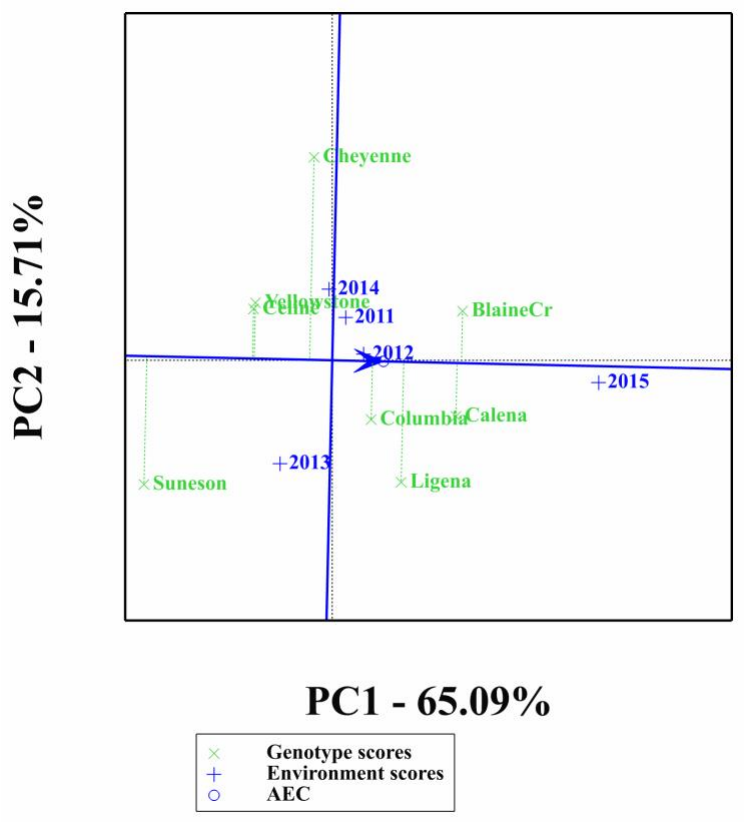


Figure 3. Harvest Index ranking biplot. Genotype scores ("X") show performance of varietals in proximity to years ("+") (environment scores). Blue arrow on AEC (average environment coordinate) abscissa line points in direction of highest trait value (greatest harvest index). The circle adjacent to the point of the blue arrow represents the five-year mean of values for all varietals. A smaller distance from blue abscissa axis indicates higher stability or consistency of performance for 2011 - 2015 (*e.g.*, Columbia had a higher harvest index than Cheyenne, with Cheyenne more stable than Columbia). Varietals performed best in the blue years closest to them (*i.e.*, Columbia performed best in 2011, and better than Cheyenne, which performed best in 2015). Genotype and Genotype x Environment (Year) effects accounted for 95.26% of trait performance.

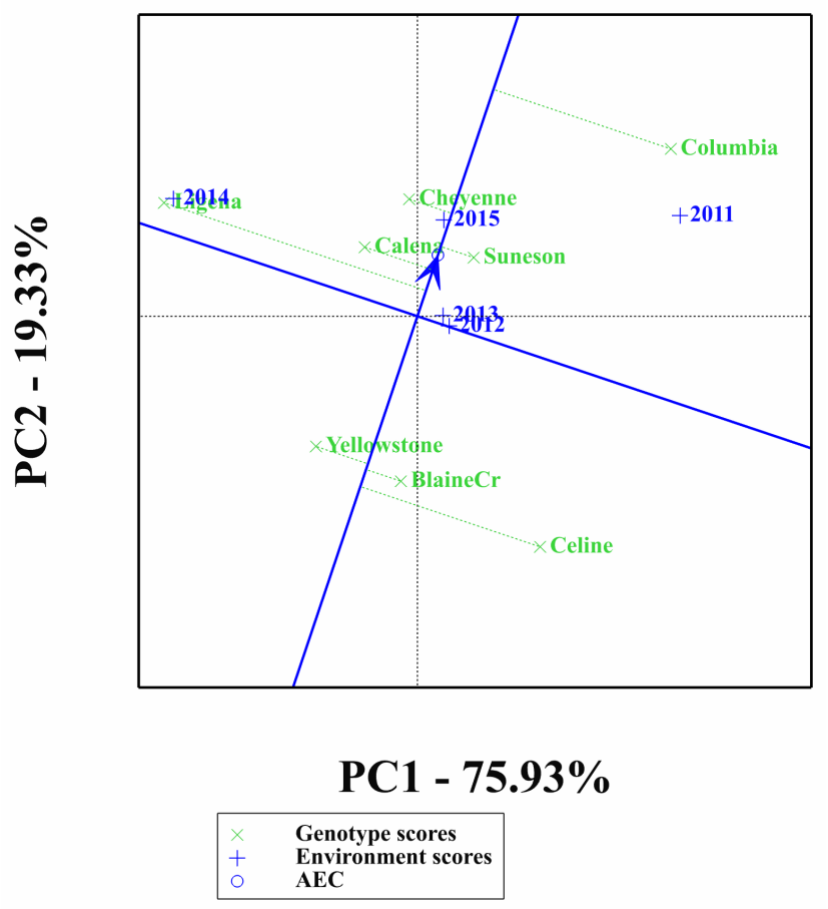


Figure 4. Oil percent of dry weight ranking biplot. Genotype scores ("X") show performance of varietals in proximity to years ("+") (environment scores). Blue arrow on AEC (average environment coordinate) abscissa line points in direction of highest trait value (greatest oil percentage). The circle adjacent to the point of the blue arrow represents the five-year mean of values for all varietals. A smaller distance from blue abscissa axis indicates higher stability or consistency of performance for 2011 - 2015 (*e.g.*, Columbia had higher oil percentage than Ligena, with Columbia more stable than Ligena). Varietals performed best in the blue years closest to them (*i.e.*, Columbia performed best in 2012, and better than Cheyenne, which performed best in 2014 and 2015). Genotype and Genotype x Environment (Year) effects accounted for 82.46% of trait performance.

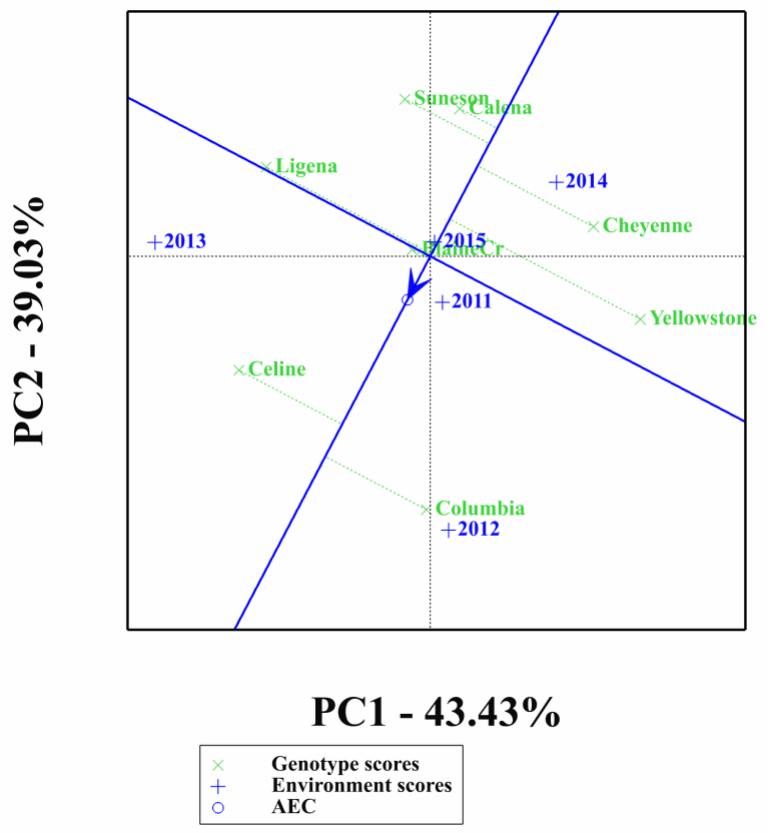
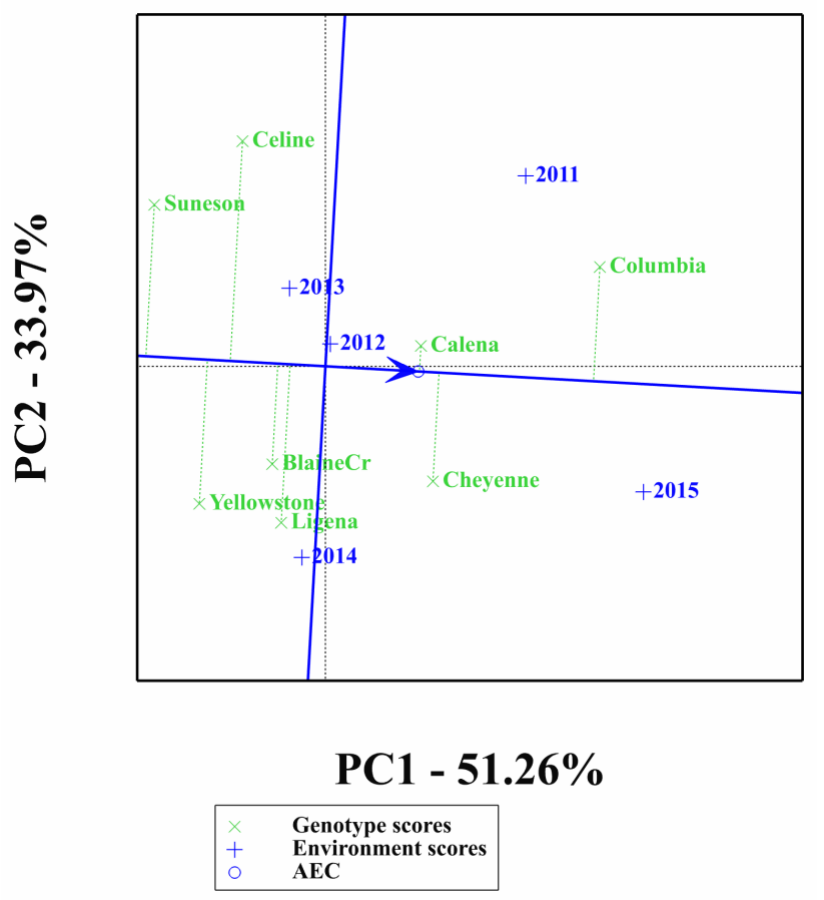
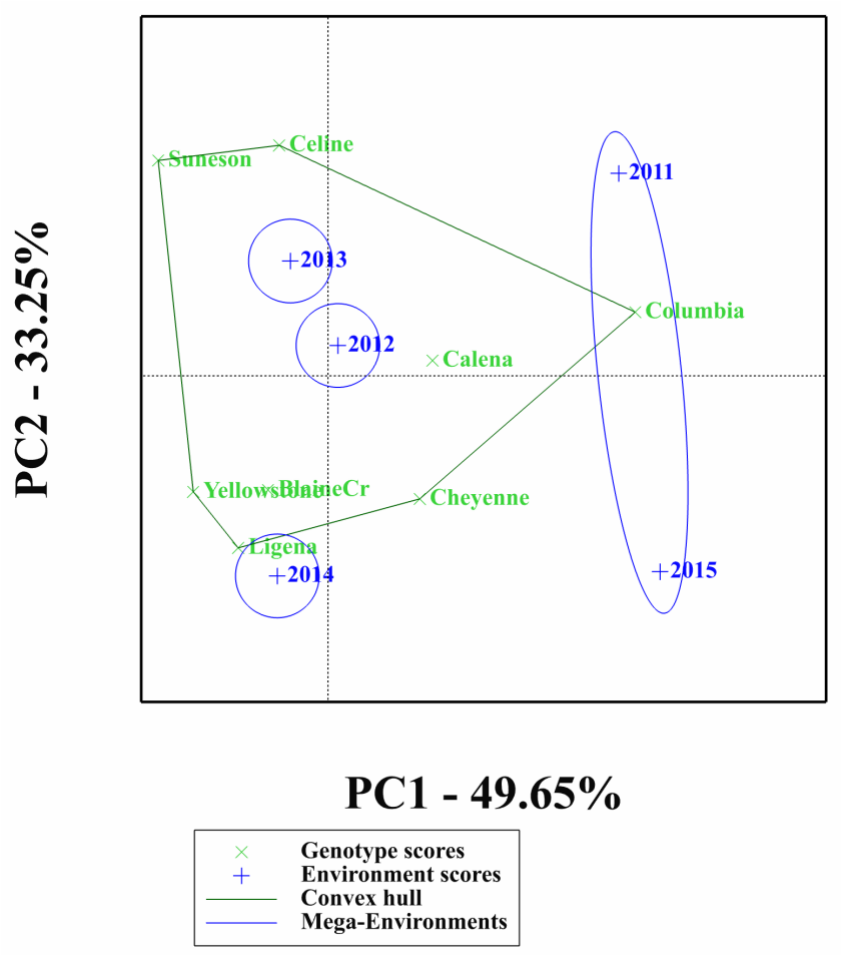


Figure 5. Calculated oil yield ranking biplot. Genotype scores ("X") show performance of varietals in proximity to years ("+") (environment scores). Blue arrow on AEC (average environment coordinate) abscissa line points in direction of highest trait value (greatest seed yield). The circle adjacent to the point of the blue arrow represents the five-year mean of values for all varietals. A smaller distance from blue abscissa axis indicates higher stability or consistency of performance for 2011 - 2015) (*e.g.*, Columbia had greater seed yield than Calena, with Calena more stable than Columbia). Varietals performed best in the blue years closest to them (*i.e.*, Columbia performed best in 2011 and 2015, and better than Celine, which performed best in 2012 and 2013). Genotype and Genotype x Environment (Year) effects accounted for 85.23% of trait performance.

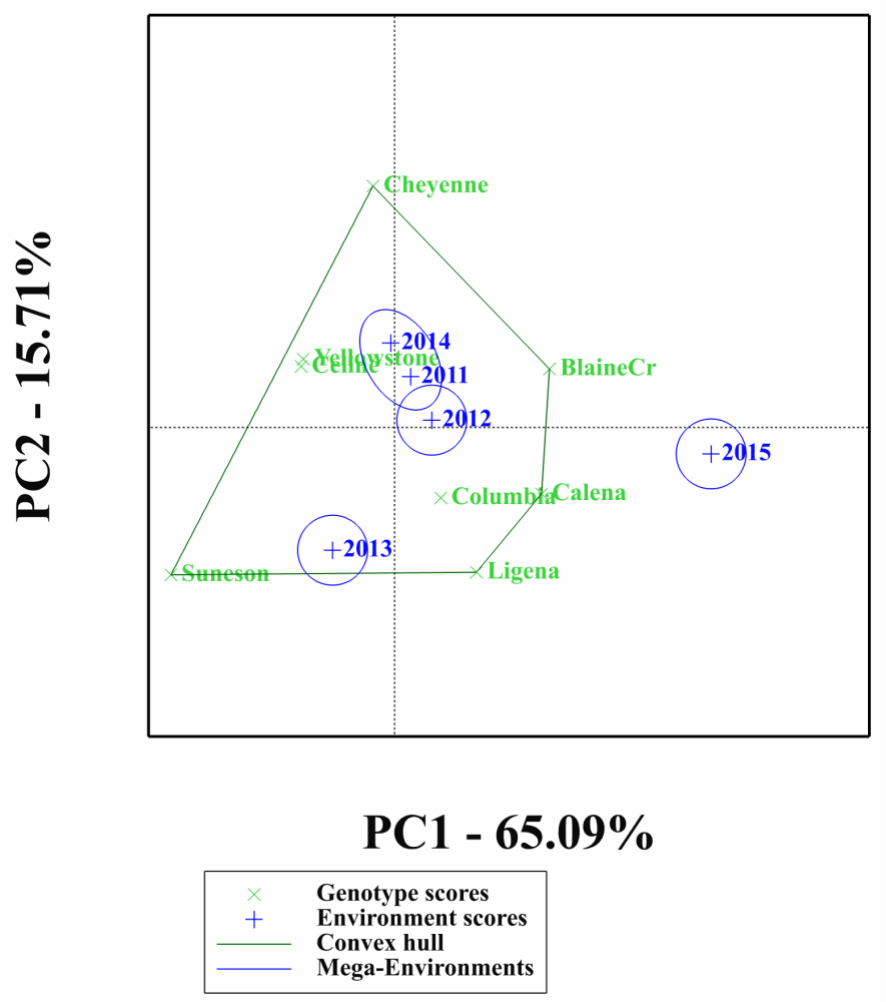


Supporting Information Figure S1. Seed mass "Which Won When" biplot.

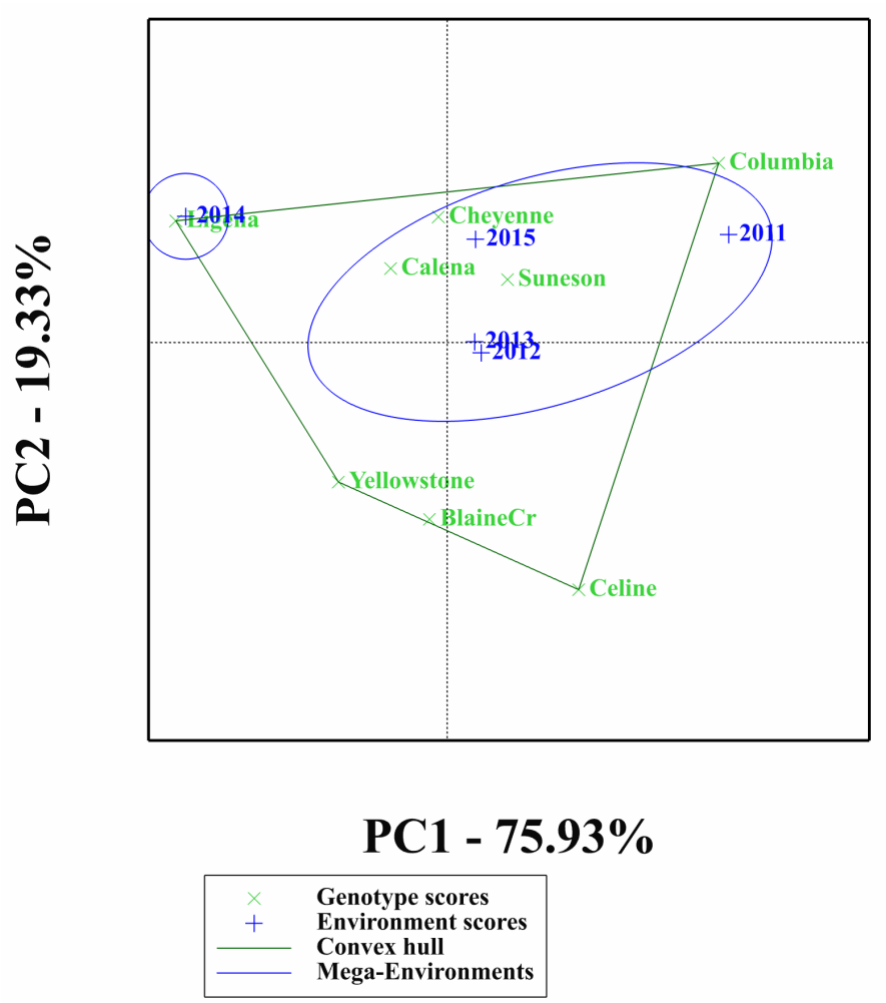
Genotypes ("X") or named varietals (green) at vertices of the polygon are the best performers for the mega-environments (circled years, blue) that encompass names (*e.g.* Columbia performed best in 2011 and 2015). Varietals performed best in those years closest to varietal names (*e.g.*, Celine and Suneson performed best in 2013, with Celine performing better than Suneson, indicated by closer distance to years indicated). Varietals within the polygon (*e.g.*, Blaine Creek) did not perform as well as vertex varietals. Genotype and Genotype x Environment (Year) effects accounted for 82.9% of trait performance.



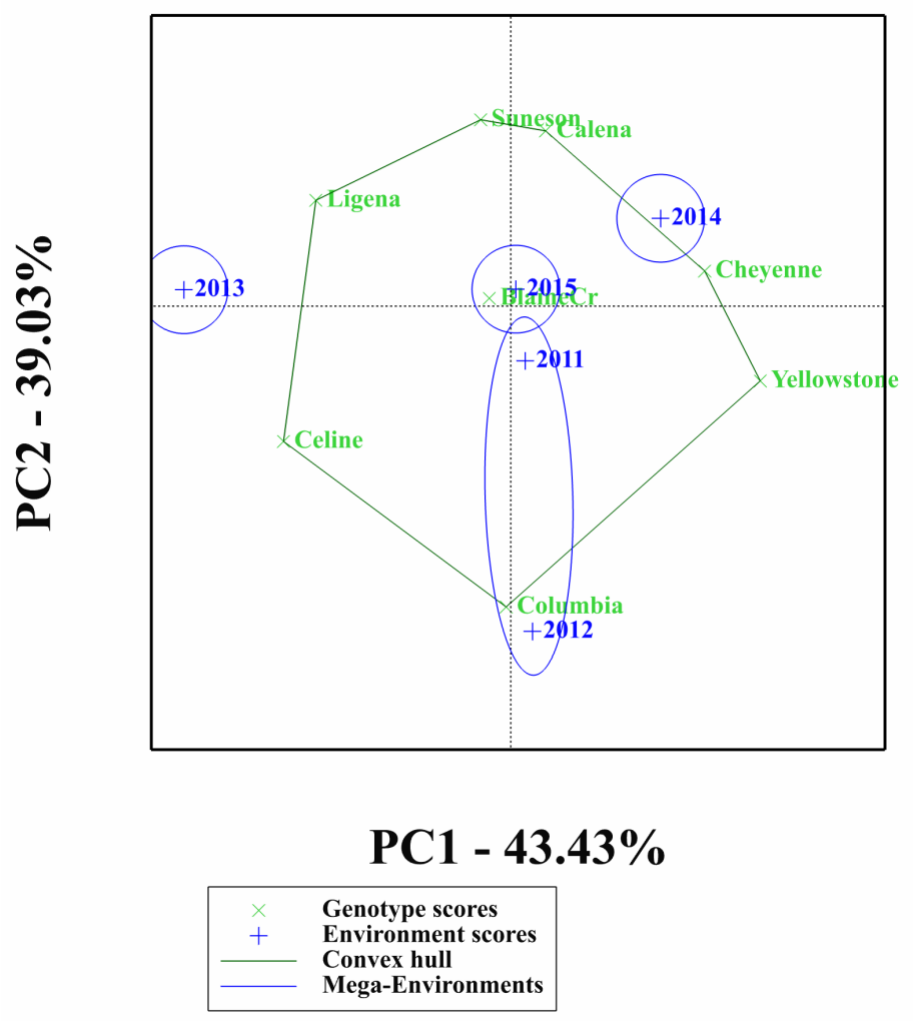
Supporting Information Figure S2. Biomass "Which Won When" biplot. Genotypes ("X") or named varietals (green) at vertices of the polygon are the best performers for the mega-environments (circled years, blue) adjacent to names (*e.g.*, Blaine Creek and Calena performed best in 2012 and 2015). Varietals performed best in those years closest to varietal names (*e.g.*, Suneson and Columbia performed best in 2013). Varietals within the polygon (*e.g.*, Celine, Columbia, Yellowstone) did not perform as well as vertex varietals. Genotype and Genotype x Environment (Year) effects accounted for 80.80% of trait performance.



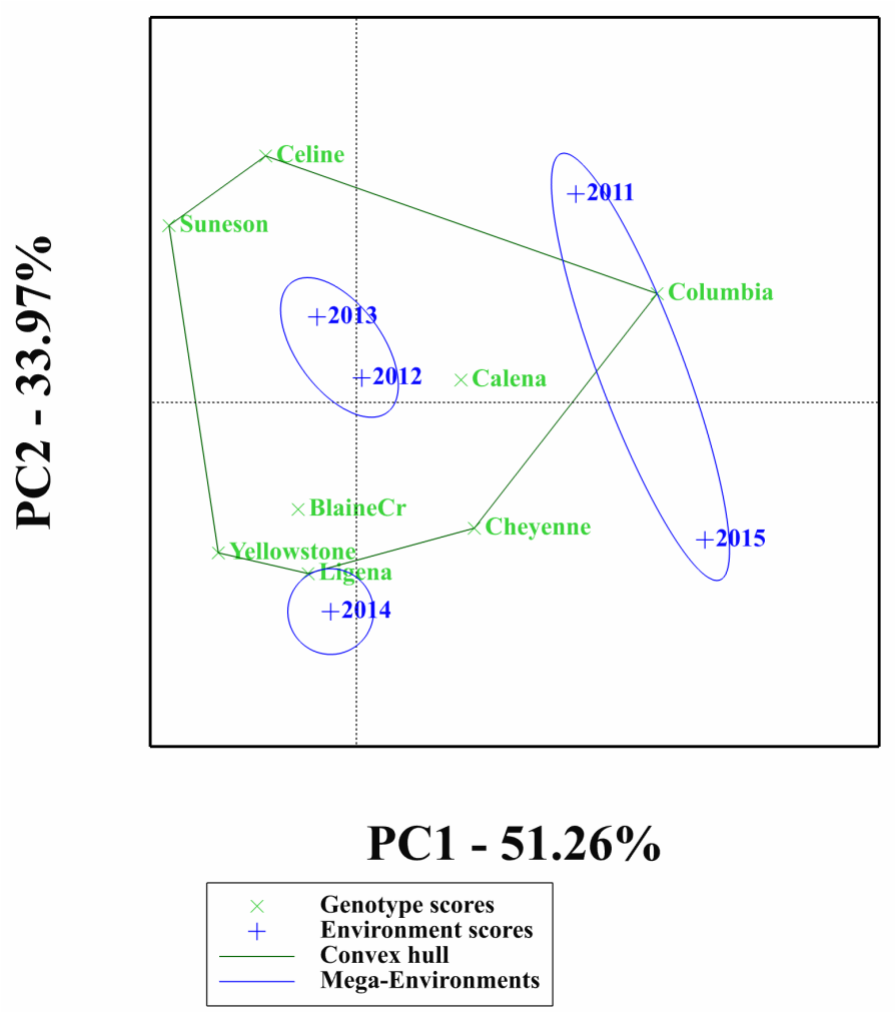
Supporting Information Figure S3. Harvest Index "Which Won When" biplot. Genotypes ("X") or named varietals (green) at vertices of the polygon are the best performers for the mega-environments (circled years, blue) that encompass names (*e.g.* Columbia performed best in the mega-environment of 2011, 2012, 2013, and 2015). Varietals performed best in those years closest to varietal names (*e.g.*, Ligena performed best in 2014). Columbia performed better in 2011 and 2015, then Yellowstone, Blaine Creek, and Celine, indicated by closer distance to years indicated. Varietals within the polygon (*e.g.*, Calena) did not perform as well as vertex varietals. Genotype and Genotype x Environment (Year) effects accounted for 95.26% of trait performance.



Supporting Information Figure S4. Oil percent of dry weight "Which Won When" biplot. Genotypes ("X") or named varietals (green) at vertices of the polygon are the best performers for the mega-environments (circled years, blue) adjacent to names (*e.g.* Columbia performed best in the mega-environment of 2011 and 2012, while Celine and Ligena performed almost the same in 2013). Varietals performed best in those years closest to varietal names (*e.g.*, Cheyenne performed best in 2014). Varietals within the polygon (*e.g.*, Blaine Creek) did not perform as well as vertex varietals. Genotype and Genotype x Environment (Year) effects accounted for 82.46% of trait performance.



Supporting Information Figure S5. Calculated oil yield "Which Won When" biplot. Genotypes ("X") or named varietals (green) at vertices of the polygon are the best performers for the mega-environments (circled years, blue) adjacent to names (*e.g.* Columbia performed best in the mega-environment of 2011 and 2015). Varietals performed best in those years closest to varietal names (*e.g.*, Ligena performed best in 2014). Varietals within the polygon (*e.g.*, Calena) did not perform as well as vertex varietals. Genotype and Genotype x Environment (Year) effects accounted for 85.23% of trait performance.



Chapter 4: Characterization of seed oil and fatty acid methyl esters (FAMEs) from wild-type and an ethyl methanesulfonate (EMS) mutant of *Camelina sativa* with reduced seed-coat mucilage

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Abstract

Camelina sativa L. Crantz (large-seeded false flax) is a promising oilseed crop for the production of edible oil and biodiesel. *C. sativa* can be grown on marginal land, not competing with food crops, and requires minimal inputs, including little or no irrigation and limited nitrogen inputs. More than 4,700 chemically mutagenized using ethyl methanesulfonate (EMS) M_2 *C. sativa* lines were generated and screened for seed coat mucilage (SCM) defects. EMS lines with absent or reduced seed coat mucilage were identified using Ruthenium Red colorimetric staining. Of the 250 M3 mutant lines screened, four lines with reduced mucilage were identified indicating an 0.05% overall rate of mucilage defects in this population. Compared with wild-type (WT) plants, the mucilage-defect mutant line Cs98 had smaller seeds and significantly less SCM. Cs98 greenhouse grown plants were significantly taller than WT. In addition, Cs98 plants had significantly greater aerial biomass and number of seed pods, but these differences were not significant. The seed mass and oil content of the seeds of the Cs98 mutant were significantly lower than WT plants; however, the Cs98 had a significantly higher crude protein and starch contents, but a lower neutral detergent fiber (NDSF) fraction, which contains pectin. Evaluation of the seed, oil, and FAMEs derived from the SCM mutant revealed that the seeds of the mutant showed reduced pectin content as well as reduced oil viscosity. Although Cs98 seed contained significantly higher mineral contents for various minerals, these differences were not observed in Cs98 or WT seed oil or biodiesel, which passed all American Society for Testing and Materials (ASTM) standards for macro- and micro-mineral content and viscosity, pH, and tubidity. Notably,

the Cs98 oil and biodiesel had significantly reduced viscosity than WT, indicating that Cs98 oil and biodiesel had improved flow characteristics likely a result of significantly lower pectin residues. Assaying the water washes of WT and Cs98 oils showed that Cs98 reconstituted residues contained 57.1% of the mucilage content compared to WT residues. Although the ratio differed from the calculated NDSF fraction in the ground seed assays, it confirmed the hypothesis that Cs98 had significantly less mucilage and pectic substances than WT.

1. Introduction

Camelina (*Camelina sativa* (L.) Crantz)**,** also known as false or wild flax, and gold of pleasure, is an oilseed species within the Brassicaceae (mustard) family. Camelina originated in Northern Europe, the Mediterranean, and Central Asia, where it has been cultivated since Neolithic times as a source of vegetable oil for food, lighting, medicine, and as animal feed (Putnam et al., 1993; Zubr, 1997). Camelina has received considerable interest as an alternative oilseed crop for biofuel production (Moser, 2010a; Shonnard et al., 2010), particularly for on-farm biodiesel production to increase farm income, diversify rural economies, and promote renewable biofuel use (Keske et al., 2013).

Camelina oil consists of 13-17% oleic (C18:1), 16-23% linoleic (C18:2w6), and 31-39% alpha-linolenic (C18:33w3) fatty acids (Radocaj and Dimic, 2013; Zubr, 1997). However, following its conversion to fatty acid ethyl esters or methyl esters (FAMEs) for biodiesel production, the high proportion of polyunsaturated fatty acids can lead to increased of oxidative instability and iodine values relative to biodiesel derived from

canola, palm, and soybean oils. However, other fuel properties were similar to these biodiesels and blends with ultra-low-sulfur diesel fuel including low temperature operability, acid value, octane number, kinematic viscosity, lubricity, sulfur and phosphorus content, and surface tension (Moser and Vaughn, 2010b). The unsatisfactory oxidative stability of Camelina biodiesel can be overcome easily by the addition of inexpensive and readily available antioxidant additives such as Baynox (Schober and Mittellbach, 2004). After oil pressing, Camelina seed meal contains 4-14% oil, 35-48% crude protein, and 10-11% fiber making it well suited as a feed supplement for livestock and poultry (Colombini et al., 2014; Gugel and Falk, 2006; Zubr, 1997). Camelina meal has received Food and Drug Administration (FDA) 'no objection' status to supplement feed rations up to 10% for cattle, goats, and poultry (Schill, 2009), and is also used for feeding many other farmed products including swine, salmon and cod.

Plant cell walls consist of three predominant polysaccharide carbohydrates: cellulose, hemicellulose, and pectin, which along with cellulose-hemicellulose networks providing tensile strength (Arsovski et al., 2010). Pectin supports cell wall integrity by covalently linking to the tensile polysaccharides and by providing cell-to-cell adhesion (Voiniciuc et al., 2018a). The principal pectin in *Arabidopsis* primary cell walls is homogalacturonan (HG) (Voiniciuc et al., 2018b). The three major pectic structures are branched rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), and non-branched homogalacturonan (HG). RGI and HG form cross links with ionic Ca^{2+}, and RGII binds covalently to B. Knocking down the expression of pectin biosynthesis genes galacturonosyltransferase 4, (GAUT4) reduced both HG and RGII in cell walls, as well as cell wall Ca^{2+} and B (Biswal et al., 2018). Poplar and switchgrass engineered to

express less GAUT4 improved both the cell wall extractability of sugars, as well as increased growth in greenhouse and field grown plants (Biswal et al., 2018). With less constriction from crosslinked cell wall glycans, transgenic plants showed significantly greater heights, stem diameter, and overall biomass, perhaps due to greater cellular expansion properties (Biswal et al., 2018)

Pectinaceous seed coat mucilage (SCM) has been noted in myxospermous fruits and seeds in at least 230 genera of angiosperms (Yang et al., 2012), including many members of the Brassicaceae, (*e.g., Arabidopsis thalina* (Arabidopsis), *Camelina sativa* (Camelina), and *Brassica napus* (canola)). The hydrogel formed by SCM exuded by epithelial cells in the testa upon hydration may retard desiccation, regulate germination, and mediate seed dispersal (Western et al., 2001). SCM is composed of predominantly pectins, which are acidic polysaccharides consisting mainly of rhamnogalacturonan I (RGI) and polygalacturonicacid (PGA) (Western et al., 2001).

Rhamnogalacturonan I (RGI), which has a backbone of alternating rhamnose and galacturonic subunits, decorated with side chains of other pectic polysaccharides such as HG, is the main component of *Arabidopsis* SCM deposited in the apoplast outside the cell wall. Both RGI and HG form ionic cross-links with Ca^{2+} salt bridges, with rhamnogalacturonan II covalently bonding with B (Macquet et al., 2007a). Pectins chelate divalent cations such as Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+}, Sr^{2+}, As^{2+}, Cd^{2+} (Celus et al., 2018) and La^{2+} (McKenna et al., 2010), with HG more extensively studied than RGI or RGII (Celus et al., 2018). Pectins also chelate monovalent cations (e.g.$^{+}$), although more weakly than divalent cations (Celus et al., 2018). The stiffness and

rheological properties of the hydrogel are dependent upon ionic bonding between pectin molecules and Ca^{2+} (Western et al., 2001). For oilseeds such as Camelina and canola, SCM is a sticky contaminant, similar to hydratable gums, which are usually removed by degumming pretreatments of crude oils using water, acids, or alkali in oil processing (Dijkstra, 2010; Ohlson, 1992; Segers and van de Sande, 1990).

Within the Brassicaceae Camelina is closely related to *Arabidopsis thaliana* (Arabidopsis), and shares a high (81% average) sequence identity with Arabidopsis proteins (Nguyen et al., 2013). In Arabidopsis, approximately 54 genes have been identified, which are necessary for seed coat mucilage synthesis and release (Francoz et al., 2015). In addition, 27 transcription factors have been identified as controlling mucilage production (Golz et al., 2018).

Ethyl methanesulfonate (EMS) is an alkylating agent, preferentially modifying guanine residues, and inducing 2 to 10 mutations Mb^{-1} of diploid DNA (Till et al., 2007). Guanine modification results in knock out and knock down of gene expression. Treating seeds with varying levels of EMS can produce a library of mutants with varying phenotypes. EMS was used to treat *Camelina sativa* 'Celine' to create the mutant library, which included EMS mutant Cs98.

Here we report on the characterization of a mucilage-deficient mutant (Cs98), which had significantly less SCM than wild-type Camelina seeds. The Cs98 mutant plants showed significantly greater plant height and greater aerial biomass and number of seed pods than wild-type plants, whereas the seeds were significantly smaller with a lower oil content. Detailed analysis of the seed revealed that the Cs98 mutant displayed

statistically significant differences in crude protein, lipid content, macro and mineral content, and seed carbohydrate fractions, including pectic substances.

2. Methods

2.1 Creation of EMS mutant library

Ethyl methanesulfonate (EMS) was used as a chemical mutagen. *Camelina sativa* 'Celine' seeds were soaked for 16 h in 0.5%, 1%, and 2% (v/v) EMS solutions. After washing with distilled water, treated seeds (M_0 generation) were planted in flats containing Sunshine MVP/LA4 soil mix (Sun Gro Horticulture, Bellevue, WA, USA) and grown in a greenhouse in the Valley Road Greenhouse Complex of the Nevada Agricultural Experiment Station (Reno, NV). Natural lighting was provided at 250 μmole m^{-2} s^{-1} with mean of 14 h light (22 °C)/10 h dark (18 °C). Seeds were harvested from the M1 plants. Approximately 27,000 M1 seeds from 0.5% and 0.1% EMS treatments were sown in two plots (3 x 10 m) at Valley Road Test Plot Facility on June 5, 2010. Plots were watered by sprinkler. Approximately 4,725 individual lines of M2 plants were harvested in November, 2010 (Acharjee, 2012).

2.2 Growing M3 EMS mutants and Wild-type in greenhouse

M_2 seeds were sown 13 pot^{-1}, in two-gallon pots containing Sunshine MVP/LA4 soil mix (Sun Gro Horticulture, Bellevue, WA, USA) in the Valley Road Greenhouse Complex under standard greenhouse conditions with natural light in the range of 1,100-1,500 μmol

$m^{-2}s^{-1}$ and temperature at 22-24 °C day/16-18 °C night. Pots were watered twice a week and treated after germination with Osmocote Plus® fertilizer (The Scotts Company, Marysville, OH, USA). Plants were treated with insecticide (Acephate 97/UP, United Phosphorus Inc., King of Prussia, PA, USA) according to manufacturer's instructions. M_3 seeds were harvested and cleaned with an Almaco Air Blast Seed Cleaner (Almaco Seed Co., Model #ABSC, Nevada, IA). Phenotypes of interest were recorded during harvesting.

2.3 Screening for mucilage defect seeds

Twelve seeds per line were placed in individual wells of 96 well plates, immersed in 100 µl of 0.02% Ruthenium Red dye (R2751, ammoniated ruthenium oxychloride) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in deionized water produced by Sybron Barnstead Nanopure II water purification system (APS Water Services Corporation, Lake Balboa, CA). Seeds were hydrated overnight (15 h) at 4° C. Phenotypes were scored, recorded, and photographed. Seed lines of interest were bulked under greenhouse conditions as described above.

2.4 Agronomic data collection and calculations for Figure 3

Six seeds pot^{-1} were sown from *Camelina sativa* 'Celine' (WT), EMS mutant Cs98, and Cross 17.1 (F1 cross of male Cs98 into female WT) in two-gallon pots per 2.2 Methods (above), in multiple pots, in May, August, and December 2016. Irrigation was withdrawn

approximately 14 weeks after germination. Plants were air dried for eight weeks and were hand harvested.

Agronomic parameters measured included height (cm), seeds pod^{-1}, 100-seed weights, total seeds plant^{-1} (g), and aerial biomass plant^{-1} (g). An OHAUS AS313 Adventurer SL mass balance (OHAUS Corp., Parsippany, NJ, USA) was used for all weights. Total biomass was calculated as the sum of total seed mass plus total plant aerial biomass. Harvest indices were calculated for the seed yield using the formula ((seed weight/(seed weight + biomass weight)). Seed and aerial biomass weights were analyzed using one-way ANOVA and unpaired T-tests, using online GraphPad software (https://www.graphpad.com/quickcalcs/ttest1/?Format=C)

The Bruker mq20 minispec NMR (Bruker Corporation, https://www.bruker.com) was used to obtain values for oil % of DW. Three glass probes, each containing one cm^3 of seed, were assayed per line for each planting date to obtain means ± SE. Seed mass probe^{-1} was recorded (approximately 0.380 g). A standard curve for *Camelina sativa* 'Celine' seed oil was created for the Bruker mq20 minispec according to manufacturer's instructions for evaluating oil content. Data from the Bruker mq20 was validated in December 2017 by a commercial lab (POS Bio-Sciences, Saskatoon, Saskatchewan, Canada) using a gravimetric wet chemistry petroleum ether extraction method.

2.5 Methods for proximate analysis of seed fractions (Figure 4) and carbohydrate fractions (Figures 5 and 6)

To compare the seed fraction components of field-grown Cs98 compared with field-grown WT (see 2.7 below for field planting), ~150 g of each accession (153.86 g WT and 152.49 g Cs98) were submitted for complete proximate and complete mineral analyses to Northwest Labs, LLC (Jerome, ID). After receiving the two samples, each sample was ground and split into three smaller samples to create three technical replicate samples for each accession.

The analyses for ether extraction (EE), amylase-treated neutral detergent (aNDF), acid detergent fiber (ADF), and neutral detergent insoluble crude protein (NDICP) were performed by Bar Diamond (Parma, ID). The EE samples were double extracted with ether (AOAC method 920.39) and then the "defatted" remainder was used to analyze aNDF (AOAC method 2002.04) and ADF (AOAC method 973.18). NDICP values were obtained from the residue remaining after the crude protein (CP) assay, which was determined by the Kjeldahl wet chemistry method (Kjeldahl, 1883).

Northwest Labs LLC performed the remaining analyses and calculated values. These included crude protein (CP) (AOAC method 2001.11), dry matter (DM) (NFTA 2.1.4 (AOAC method 935.29)), ash (AOAC method 942.05), starch (AOAC method 2014.10), moisture (AOAC method 935.29), water-soluble Carbohydrates (WSC) using the phenol sulfuric acid assay (DuBois et al., 1956), and total digestible nutrients (TDN) for rumen of dairy cattle (calculated) (Weiss et al., 1992).

Several carbohydrate fractions were assayed to enable Northwest Labs to calculate NDSF (Figure 5). First, the neutral detergent insoluble crude protein (NDICP) was assayed to determine the non-fibrous Carbohydrate (NFC) calculation: NFC = 100 –

(CP + (NDF – NDICP) + EE + Ash). Second, the total non-structural carbohydrates (TNC) were calculated as the sum of water-soluble carbohydrates (WSC) and starch. Lastly, with NFC and TNC calculated, the neutral detergent soluble fiber (NDSF) was calculated as the NDSF = NFC – TNC. The NDSF fraction contained the pectic substances.

2.6 Methods to obtain macro and mineral values of ground seed (Tables 1 and 2)

Mineral analyses were performed by SDK Laboratories (Hutchinson, KS). Phosphorus (P) was analyzed by spectrophotometry (AOAC method 965.17). Potassium (K), calcium (Ca), and sodium (Na) were analyzed gravimetrically (AOAC method 956.01). Note: because sodium was not detected, it was omitted from the data tables. Magnesium (Mg) was analyzed by flame atomic absorption spectroscopy (FAAS) (AOAC method 968.08). Aluminum (Al), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), and sulfur (S) were analyzed by ICP-OES (AOAC method 985.01).

2.7 Field planting of Wild-type and Cs98

Wild-type and Cs98 seeds were raised under greenhouse conditions as described above. Seeds were bulked up by planting outdoors in the University of Nevada Valley Road Field Laboratory in Reno, NV. The Natural Resources Conservation Service (NRCS) described the soils on the Valley Road Field Laboratory (NV628) as Orr sandy loam with 0 to 2% slopes for 87.8% of the area of interest (AOI) and Orr gravelly, sandy loam with

0 to 2% slopes for 12.2% AOI. The planting field (0.077 hectare) was rated as prime farmland, if irrigated. The available water-holding capacity for this site was low (approximately 3.8 cm). The normal frost-free period ranged from 109-134 days. The soil was predominantly clay with 1.21% organic matter, and rated as irrigated capability class 2 (moderate limitations), subclass c (very dry climate) (Soil Survey Staff, 2018). Prior to planting, the site was fertilized with urea at a rate of 67.25 kg ha^{-1}, which resulted in 58.8 kg ha^{-1} N.

WT and Cs98 seed were planted outdoors in June 2016. Seeds were planted in 1.5 m x 14.5 m plots using hand broadcasting, followed by raking in to a depth of 6 mm. Due to hot temperatures and low germination rates, WT was replanted in July 2016. Additional Cs98 seed was planted in the greenhouse as described above in July 2016. Plants were harvested in October 2016, dried to air-equilibrium dryness, and cleaned with the Almaco seed cleaner as described above.

2.8 Processing Wild-type and Cs98 seeds into oil, FAMEs, and FAMEs derived from sorbent-treated oil

Seeds for oil pressing were derived from both field-grown and greenhouse WT and Cs98 plants. Oil was collected from seeds using Nutrichef PKOPR15 countertop kitchen seed oil press (Nutrichef Kitchen, Brooklyn, NY, USA) at "raw" (unheated) setting for Black Sesame. Oil was centrifuged for 30 min on Beckman Allegra 6R at 2,800 *x g*, and stored at -20 °C until submission to Cashman Fluid Analysis lab for analyses.

To treat oils prior to transesterification, the method of Wang and Johnson, 2001 was followed, with modifications (Wang and Johnson, 2001). Approximately 40 g of each oil were decanted into 50 ml polypropylene tubes (T5000, Argos Technologies, Cole-Palmer, Inc., Vernon Hills, IL). Three percent (w/w) of deionized water was added to each tube, which was then shaken for 1 h at 200 RPM on a New Brunswick™ Innova® 4000 incubator/shaker (Eppendorf North America, Hauppauge, NY) at 25 °C. Water-treated oil was then centrifuged for 30 m at 2,800 x *g*, frozen to immobilize the aqueous pellet, and water-treated oil was decanted into another 50 ml tube.

While Wang and Johnson used magnesium silicate for adsorbent treatment (Wang and Johnson, 2001), this study used Sorbsil© R92 (sodium silicate, PQ Corp., Joliet, IL, USA), due to superior pellet stability. Sorbsil© R92 was added to each water-treated sample to a final percentage of 1.5% (w/w). The tubes were then shaken for 20 min at 200 RPM on a New Brunswick™ Innova® 4000 incubator/shaker at 25 °C. Pretreated oil was then centrifuged for 30 min at 2,800 x *g* to immobilize the pellet and decanted for storage at -20 °C until transesterified. FAMEs were transesterified from oil pretreated with 3% water and 1.5% adsorbent or from oil with no pretreatments.

Transesterification followed the method of Yang et al. (2016) with modifications. Oils to be transesterified were heated on a Cimarec SP131635 heating stir plate (ThermoFisher Scientific, Inc., Waltham, MA) to 40 °C. Methanol with 1.66% (wt. %) 99.99% sodium hydroxide (Alfa Aesar, Tewksbury, MA, USA) was added at a 6.9:1 methanol:oil ratio. Molar mass of Camelina oil was calculated as 285.5489, based on data characterizing Camelina biodiesel (Ciubota-Rosie et al., 2013). When the

oil/methanol mixture attained 40 °C, the mixture was stirred at 900 RPM, maintaining temperature at 40 °C for 50 min.

After transesterification, the FAMEs/glycerol mixture was decanted into 50 ml tubes and allowed to sit until clear separation of the glycerol fraction. FAMEs were decanted into beakers, and washed with autoclaved deionized water with previously recorded pH for 1 h. After each h, water wash was removed and fresh water added. When water washes were transparent, pH was measured. When pH of output wash was $\leq$ pH of input wash, the FAMEs were decanted into beakers for desiccation with 0.006% (w/w) anhydrous $CaCl_2$ (EMD Millipore Corp., Billerica MA, USA). The FAMEs were stirred at 300 RPM for 15 min, followed by centrifugation for 30 min at 2,800 x *g* to immobilize the pellet, and then decanted into glass jars for storage at 4 °C until delivered to Cashman Fluid Analysis for assays.

2.9 Hydratable carbohydrate analysis from aqueous washes of oils (Figures 7, 8A, 8B)

To remove all hydratable carbohydrates from oils to determine carbohydrate masses and pectin content, a separate experiment used substrates of both WT and Cs98 oils pressed as stated above. The water degumming method of Wang and Johnson, 2001 was followed with further modifications (Wang and Johnson, 2001). Approximately 20.5 g of each oil were decanted into 50 ml polypropylene tubes (T5000, Argos Technologies, Cole-Palmer, Inc., Vernon Hills, IL). One hundred percent (w/w) of deionized water (wash A) was added to each tube, which was then shaken for 1 h at 200 RPM on a New

Brunswick™ Innova® 4000 incubator/shaker (Eppendorf North America, Hauppauge, NY) at 25 °C. Water-treated oil was then centrifuged for 30 m at 2,800 x *g*, frozen to immobilize the aqueous pellet, and water-treated crude was decanted into another 50 ml tube. Two additional washes (washes B and C) of the water-treated crude using deionized water were used, following the above method. Aqueous pellets from wash A were consolidated in 35 ml volumes within a 50 ml polypropylene tube, frozen, lyophilized on the Labconco Freezone 18 (Kansas City, MO), weighed on an aeADAM Mass Balance 100L 2524T, and recorded. Aqueous pellets from washes B and C followed the same procedure. Unpaired t-tests were run to compare consolidated WT A B C aqueous residues with those of Cs98, using online calculator at https://www.graphpad.com/quickcalcs/ttest2/.

Mucilage was extracted from WT Camelina seeds to provide a standard curve to quantitate the pectin in the reconstituted lyophilized residues from aqueous pellets of water washes of WT and Cs98. The method of Macquet et al., 2007 was followed with modifications (Macquet et al., 2007a). Two hundred ml of deionized water was added to approximately 10 g of WT Camelina seeds in an Ehrlenmeyer flask and shaken overnight on a New Brunswick™ Innova® 4000 incubator/shaker at 25 °C. The mixture of seeds and solution was put into Olympus Plastics 50 ml screwtop tubes and centrifuged on a Sorvall RC-5B centrifuge at 8000 x *g* for 5 min. Supernatants were recovered and decanted into Sartorius Vivaspin 15R 10,000 MWCO HY spin tubes and centrifuged at 3,000 x *g* for 8 min. Supernatants were decanted into new Sartorius Vivaspin 15R 10,000 MWCO HY spin tubes and centrifuged at 4,000 x *g* for 10 min. Retentate was washed

with deionized water and centrifuged again at 4,000 x *g* for 10 min. Retentate was decanted into 15 ml high-clarity Falcon polypropylene conical tubes (Corning Science, Mexico) and lyophilized on a Labconco Freezone 18 lyophilizer. Mucilage residue was stored in paraffin-sealed 15 ml polypropylene tubes in air-tight jars with Drierite (W.A. Hammond Drierite Co., Xenia, OH).

Lyophilized mucilage and residues from aqueous pellets were reconstituted by adding deionized water to residues. Mucilage was dissolved overnight with stirring in a beaker on a Corning Stir plate Model PC-410 (Corning International, Corning, NY) at 550 RPM. Aqueous pellet residues were shaken in 50 ml polypropylene tubes for 2 h at 450 RPM on a New Brunswick™ Innova® 4000 incubator/shaker at 37 °C.

Four hundred forty microliters of reconstituted aqueous washes or mucilage extracts were pipetted into 1.7 ml Olympus Plastics clear polypropylene microfuge tubes Cat #22-281 (Genesee Scientific Corp., https://geneseesci.com/). An equal amount of 0.0125% Ruthenium Red dye made up in deionized water was added to each 1.7 ml microfuge tube, which was then vortexed for 10 sec on a Fisher Vortex Genie 2, Cat #12-812 (Fisher Scientific, Lenexa, KS). The wash/dye solution was incubated for 30 min, and then centrifuged at 20,200 x *g* at 25 °C on an Eppendorf 5417R centrifuge (Eppendorf North America, Inc., Westbury, NY) for 20 min. Two hundred microliters of supernatants from the reconstituted aqueous washes and mucilage extracts were pipetted separately onto Greiner Bio-One 96 well microplates Cat #655101 (Greiner Bio-One North America Inc., Monroe NC). The 96 well microplate was assayed on a Perkin Elmer Victor 3V™ 1420 Multilabel Counter (PerkinElmer, Santa Clara, CA) at 530 nm.

2.10 Oil macro and mineral analyses, and viscosity, TAN and pHLip assays

Cashman Fluid Analysis (Sparks, NV) analyzed the six substrates (WT and Cs98 oils, WT and Cs98 FAMEs, and WT and Cs98 FAMEs derived from sorbent-treated oil) by siphoning out three samples from each substrate jar. Jars were stored at 21.9 °C. The following methods were used: Mineral analyses used inductively coupled plasma (IP) atomic absorption spectroscopy, ASTM D5185 ICP (ASTM, 2015). SULU Sulfur was determined by UV Fluorescence, ASTM D5453 (ASTM, 2015). Viscosity at 40 °C used ASTM D445 and Total Acid Number (TAN) used ASTM D664 (ASTM, 2015). The oil and FAMEs were added to the pHLip assay (Cytoculture International Inc, Point Richmond, CA, USA) vials following pHLip instructions (http://www.phliptest.com/instructions.html).

Supplemental Table 1 compares the viscosity, Na + K values, Ca + Mg values, S values, P values, and TAN values of the four FAMEs with the ASTM D6751 specifications given in (Hoekman et al., 2012).

2.11 Primer design to probe mucilage-defect genes in Camelina

To identify candidate genes responsible for the reduced SCM phenotype, flowering tissue was collected from WT, Cross 17.1, and Cs98 lines at 0, 5, 10, 15, 20 and 24 DAF, in liquid N_2. Tissue was ground under liquid N_2 and RNA was extracted from WT, Cross 17.1, and Cs98. RNA was stored at -80 °C.

Primers were designed using CLC Main Workbench 7 software (Qiagen, Redwood City, CA) based on phenotypes of thirteen *Arabidopsis* mucilage defect lines closest to the Cs98 phenotype. The phenotypes of three *Arabidopsis* mucilage defect lines closest to the Cs98 phenotype due to mutated transcription factors are TTG2 (Gonzalez et al., 2016), TT2 MYB5 (Gonzalez et al., 2009), and GL2 (Walker et al., 2011). The phenotypes of ten *Arabidopsis* mucilage defect lines closest to the Cs98 phenotype due to mutated genes are PMEI6 (Saez-Aguayo et al., 2013), SBT1.7 (Rautengarten et al., 2008), BGAL6 (Macquet et al., 2007b), RHM2 (Dean et al., 2007), GAUT11 (Caffall and Mohnen, 2009), EXO70 (Synek et al., 2006), SEC8 (Kulich et al., 2010), RSW3 (Burn et al., 2002), PER36 (Saez-Aguayo et al., 2013), and IRX14 (Voiniciuc et al., 2015; Wu et al., 2010). See Supplemental Table 2 for primer sequences.

3. Results

3.1. Phenotypic and agronomic differences between Wild-type c.v. 'Celine' and Cs98

Wild-type (WT) and EMS mutant Cs98 (Cs98) plants exhibited several characteristic differences when hydrated in 0.02% Ruthenium Red dye (Figure 1). WT seeds exuded copious seed coat mucilage in contrast to the scanty mucilage displayed by Cs98 seeds. WT seeds were approximately 2 mm in length and slightly larger than Cs98 seeds likely due to reduce seed coat mucilage content (Figure 2).

Differences in seven agronomic parameters were observed among greenhouse-grown WT, Cs98, and the selfed F1 cross (17.1 Cross) plants (Figure 3). Mean plant heights for WT, 17.1 Cross, and Cs98 plants were 84.83, 103.06, and 105.21 cm, respectively. The 17.1 Cross and Cs98 plants showed significantly taller plant stature compared with WT controls (Figure 3A). Consistent with greater plant height, the Cross 17.1, and Cs98 plants showed greater aerial biomass (Figure 3B) than WT plants. Mean plant biomass for WT, 17.1 Cross, and Cs98 plants were 2.98, 3.26, and 3.34 g, respectively, although differences were not statistically significant.

The 17.1 Cross and Cs98 plants showed significantly greater number of seeds pod^{-1} than WT plants (Figure 3C). Mean seeds pod^{-1} for WT, 17.1 Cross, and Cs98 plants were 7.33, 9.02 and 8.73, respectively. Consistent with greater numbers of seeds pod^{-1} , the 17.1 Cross, and Cs98 plants showed a significantly lower 100-seed weight pod^{-1} for 17.1 Cross and Cs98 plants compared to WT control (Figure 3D). One-hundred-seed weights for WT, 17.1 Cross, and Cs98 seeds were 0.094, 0.078 and 0.074 g, respectively. However, the mean total seeds $plant^{-1}$ did not differ significantly among the three lines with WT, 17.1 Cross, and Cs98 plants displaying means of 1.46, 1.41, and 1.58 seeds $plant^{-1}$, respectively (Figure 3E). The harvest index $plant^{-1}$ of WT, 17.1 Cross, and Cs98 were 0.3061, 0.2865, and 0.3192, with the 17.1 Cross showing a significantly lower index compared with the WT control and Cs98 (Figure 3F). Lastly, mean oil content expressed as percent of dry weight (DW) of Cs98 was significantly lower than that of either of the WT or the 17.1 Cross with WT, 17.1 Cross, and Cs98 displaying means of 29.47, 29.33, and 27.28%, respectively (Figure 3G).

3.2 Proximate analysis of WT and Cs98 seeds

To compare the major compositional differences between WT and Cs98 seeds, a complete proximate analysis was performed (Figure 4). Mean Cs98 Crude Protein was significantly higher that WT seed value, 37.14% compared with 33.36% DW (Figure 4A). In contrast, Cs98 total lipid content was significantly lower than in WT seed, with Cs98 containing 26.63% DW compared with 30.18% DW (Figure 4B). The 3.8% difference in lipid values was consistent with the lower oil content of Cs98 compared with WT seed (Figure 3G). Ash content was significantly higher in Cs98 seeds than in WT seeds, with values of 8.83% and 6.65% DW, respectively (Figure 4C). In addition, Cs98 seeds displayed significantly higher moisture content (5.75% DW), compared to WT seeds (5.04% DW) (Figure 4D). Lastly, the WT seeds displayed a 9.6% increase in Total Digestible Nutrients (TDN) calculated for dairy cattle (113.51% DW) compared with that of Cs98 seeds (103.83% DW) (Figure 4E).

3.3 Carbohydrate fractions from proximate analysis

To compare the major carbohydrate content differences between WT and Cs98 seeds, a complete proximate analysis was performed (Figure 5 and 6). Overall content and cell wall carbohydrates fractions assayed in order to determine the calculated neutral detergent soluble fiber (NDSF) fraction of interest, which contains pectin, are shown in Figure 5. Significant differences between WT and Cs98 carbohydrate fractions, which resulted in a significant difference in NDSF are shown in Figure 6.

All cell wall components measured showed significant differences between WT and Cs98. Cs98 seed acid detergent fiber (ADF) values for were significantly higher (13.05% DW) than those determined for WT seed (10.46% DW) (Figure 6A). The calculated neutral detergent insoluble crude protein (NDICP) mean values for Cs98 seed were significantly lower (1.98% DW) than the WT seed (2.95% DW) (Figure 6B). However, Cs98 seed had significantly higher amylase-treated neutral detergent (aNDF) fraction content (19.41% DW), which contains all structural cell wall carbohydrates except for pectic substances, than the WT seed (17.00% DW) (Figure 6C).

Next, the cell content carbohydrates were assayed, with Cs98 containing significantly higher water-soluble carbohydrates (WSC) (10.32% DW), than WT (11.10% DW) (Figure 6D). In contrast to WSC, Cs98 seed showed a significantly higher starch content (0.38% DW), than WT seed (0.22% DW) (Figure 6E). Consistent with the lower WSC content, Cs98 seed showed a lower calculated total nonstructural carbohydrate (TNC) (10.70% DW) compared with WT seed (11.32% DW), but this difference was not statistically significant (Figure 6F). Similarly, Cs98 seed showed a significantly lower calculated non-fibrous carbohydrate (NFC) value (12.11% DW) than WT seed (14.63% DW) (Figure 6G). With the cell wall and cell content carbohydrates assayed, the neutral detergent fiber (NDSF) fraction containing pectin was calculated. As expected, the Cs98 contained less than half the NDSF (1.41% DW) than WT seed (3.31% DW) (Figure 6H).

3.4 Mineral nutrition of seeds

In addition to the feed characteristics of the seed meal, the mineral content of seed meal can also be important for overall animal nutrition. Thus, both the major (macro) and minor (micro) mineral contents of both WT and Cs98 ground seed were analyzed (Tables 1 and 2). Cs98 seed had lower potassium content than WT seed, but the difference was not statistically significant differences. However, Cs98 seed did show significantly lower calcium and phosphorus content (Table 1). In contrast, Cs98 seed had higher magnesium and sulfur content (Table 1). Cs98 seed had statistically significant higher aluminum, copper, iron, manganese, and zinc content than WT seed (Table 2). Notably, aluminum and iron content was 2.4-fold and 2.6-fold greater in Cs98 seed than in WT seed.

3.5 Macro and micro mineral content from ICP analysis of crude oils and FAMEs

In order to determine if mineral content differences translated to unacceptable levels in oil or fatty acid methyl esters (FAMEs) derived from WT and Cs98 seed, the mineral contents of these produced were analyzed and compared (Table 3). To ensure that the FAMEs would pass ASTM standards, the pretreated FAMEs were also treated with 3% (wt%) deionized water and a sorbent that was known to reduce mineral contents. Oil derived from Cs98 showed significantly greater potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P) content than WT oil (Table 3). Notably, Cs98 crude oil contained significantly lower sulfur (S) content than WT oil.

Transesterification of oil into FAMEs reduced macro mineral content by one-to-two orders of magnitude for both WT and Cs98 FAMEs. Cs98 FAMEs showed significantly lower calcium and sulfur content than WT FAMEs (Table 3). However, the Cs98 FAMEs and WT FAMEs showed no statistically significant differences in K, Mg, and P content. Pretreatment of WT and Cs98 oils followed by transesterification also reduced macro mineral content in the FAMEs by one-to-two orders of magnitude. Mean values of pretreated WT and pretreated Cs98 FAMEs were similar to or lower than the mineral content of WT and Cs98 FAMEs. Cs98 FAMEs derived from adsorbent-treated oil displayed the lowest calcium values of the four FAMEs, whereas WT FAMEs derived from adsorbent-treated oil showed the lowest S and P values of the four FAMEs. All macro mineral content values met or exceeded the acceptable ASTM testing standards.

Crude oil derived from WT and Cs98 seeds and FAMEs derived from untreated or adsorbent-preteated oils were also analyzed for micro mineral content (Table 4). Notably, Cs98 oil contained significantly higher aluminum (Al) and silicon (Si) content than WT crude oil. However, the Al content was only 0.8-fold greater in the Cs98 oil compared with the WT oil. In contrast, Cs98 oil contained significantly lower iron (Fe) and Manganese (Mn) contents compared with WT oil. No significant differences in zinc (Zn), Molybdenum (Mo), or Antimony (Sb) (Table 4). No copper (Cu) was detected in any of the samples tested.

Transesterification of crudes into WT and Cs98 FAMEs reduced micro mineral content by two-to-three orders of magnitude for Al, Fe, and Mn, and a three-fold reduction in Zn content. WT and Cs98 FAMEs did not show significant differences in

Al, Fe, Zn, Mo, and Sb content (Table 4). The 11.21 mg kg^{-1} mean Si value for WT FAMEs appears to be an artifact. FAMEs derived from sorbent-treated oil also displayed reduced micro mineral content by two-to-three orders of magnitude for Al, Fe, Mn, and Si. Mean values of micro minerals of pretreated WT and pretreated Cs98 FAMEs were similar to or lower than means of WT and Cs98 FAMEs. Cs98 FAMEs derived from sorbent-treated oil displayed the lowest Al, Zn, Si, and Sb contents of the four FAMEs, whereas WT FAMEs derived from sorbent-treated oil showed the lowest Mo content of the four FAMEs (Table 4). All micro mineral content values met or exceeded the acceptable ASTM testing standards.

In order to determine if the oil and FAMEs produced from both WT and Cs98 meet current quality standards for biofuel production, differences in kinematic viscosity, total acid number (TAN), and pHLip assays were assessed (Table 5). Cs98 oil showed significantly lower viscosity at 40 °C than WT oil (Table 5). Kinematic viscosity is a critical parameter associated with the pumpability of biodiesel at operating temperatures. Transesterification of untreated and sorbent treated oil resulted in a reduction of viscosity by one order of magnitude, but no significant differences among the four FAMEs were noted.

Total acid number (TAN) is a measure of the total acidity and is used to assess corrosion potential within a sample. The Cs98 FAMEs and Cs98 FAMEs derived from sorbent-treated oil both showed significantly lower TAN values than WT FAMEs (Table 5). WT FAMEs and WT FAMEs derived from sorbent-treated oil did not show significantly different values.

The pHLip assay was used to detect turbidity, contaminant traces (catalysts, glycerin, soaps, acids, oxidized fuel), and incorrect pH. All four FAMEs passed the three pHLip criteria of brightness, mirror interface, and neutral pH (Table 5). Photos of the pHLip assays are shown in Supplemental Figure 1.

All four WT and Cs98 FAMEs derived from untreated and sorbent-treated oil passed the ASTM D6751 specifications for viscosity, Na + K metals, Ca + Mg metals, total sulfur, phosphorus, and TAN (Supplemental Table 1).

3.6 Carbohydrate content of water washes of WT and Cs98 oils

To determine the amounts of carbohydrate present in the WT and Cs98 oils, each oil was washed three times with deionized water, with aqueous residues from washes A, B, and C lyophilized and weighed. Figure 7 shows that WT consolidated residues contained 19.3% more aqueous resides than Cs98 consolidated residues with means of 0.2608 and 0.2104 mg ml^{-1}, respectively. However, this difference was not statistically significant, with $p = 0.4252$.

3.7 Mucilage content of water washes of WT and Cs98 oils

To determine the amounts of mucilage present in the WT and Cs98 oils, each oil was washed three times with deionized water, with aqueous residues from washes A, B, and C lyophilized and weighed. WT A, B, and C reconstituted washes were assayed

colorimetrically and compared with Cs98 A, B, and C reconstituted washes. Figure 8 shows that Cs98 consolidated washes contained 57.1% mucilage compared to WT, with means of 8.198 E^{-2} mg ml^{-1} and 14.369 E^{-2} mg ml^{-1} mucilage, respectively, with statistical significance at $p < 0.0001$.

4. Discussion

4.1 Phenotypic and agronomic data

Cs98 seed exhibited significantly less seed coat mucilage than WT plants (Figure 1). This deficiency in seed coat mucilage was likely responsible for the smaller seed size of Cs98 compared with WT (Figure 2) and was correlated with a greater number of seeds pod^{-1} and smaller 100-seed weights (Figure 3). The smaller seed weight phenotype was also found in 17.1 Cross. The smaller seeds for Cs98 and 17.1 Cross resulted in significantly higher seeds pod^{-1} compared to the WT control (Figure 3). Even though Cs98 and 17.1 displayed a higher number of seeds pod^{-1} (Figure 3C) and a higher number of seeds per plant in the case of Cs98, this did not compensate for the smaller seed size, and higher overall seed production per plant (Figure 3D). Thus, in terms of overall seed production, the Cs98 mutant was considered less desirable than WT plants.

Harvest index is defined as the weight of the harvested seed as a percentage of the total weight of the harvested crop. Interestingly, Cs98 (and 17.1 Cross) plants displayed greater aerial biomass than WT plants, and Cs98 showed a higher harvest index $plant^{-1}$ than WT plants, but these differences were not statistically significance (Figure 3F). The

harvest index for 17.1 Cross was significantly lower than that of WT plants. The observed increase in biomass might be related to the observed defect in pectin biosynthesis. Recent studies of galacturonlytransferase (GAUT) gene family expressing pectin biosynthetic enzymes indicate that cell wall pectin may be a limiting factor in cell expansion and tissue growth (Biswal et al., 2018). GAUT4 knockdown of switchgrass increased biomass yield six-fold compared with control, with increased growth and taller plants (Biswal et al., 2018). Further studies are needed to determine if a defect in pectin biosynthesis is responsible for the increased biomass productivity observed in the Cs98 mutant of Camelina.

As Camelina is an oilseed crop, seed oil content is a critical parameter when evaluating the value different varieties. However, the Cs98 mutant displayed a significant 2.2% reduction in oil seed content compared to WT plants (Figure 3G). The mean oil percent of DW for 17.1 was lower, but not significantly different from WT. Although Shi *et al.* (2012) found that *Arabidopsis* glabra2 transcription factor mutants deficient in seed coat mucilage produced more oil, this correlation was not shown in Cs98 or 17.1 Cross in Camelina.

In examining the significant differences between the WT, 17.1 Cross, and Cs98 lines for the seven agronomic parameters, the F1 backcross (17.1 Cross) only exhibits intermediate values between WT and Cs98 for 100 seed weight (Figure 3C). The 17.1 Cross means for plant heights (Figure 3A), seeds pod^{-1} (Figure 3B), and aerial biomass (Figure 3E) are close to the Cs98 values with significant differences from WT in plant heights and seeds pod^{-1}. The remaining parameters, total seeds $plant^{-1}$ (Figure 3D),

harvest index plant^{-1} (Figure 3F) and oil percentage of DW (Figure 3G) showed the 17.1 Cross means as not differing significantly from WT in total seeds plant^{-1} and oil percentage of DW. Harvest index plant^{-1} shows 17.1 Cross values as being significantly lower than WT control. From the 17.1 Cross means, it is apparent that the F1 cross phenotypes do not show simple dominant or recessive expression. Unlike the phenotypic differences exhibited in diploid species such as *Arabidopsis, Camelina* appears to be an allohexaploid (Hutcheon et al., 2010; Kagale et al., 2014), with three gene copies compared to the single copy found in diploids.

4.2 Seed fractions from ground seed

Seed coat mucilage is a considerable metabolic investment by the plant and ranges from approximately 2% of *Linum usitatissimum* (flax) seed mass (Naran et al., 2008) to 3% in *Arabidopsis thaliana* (North et al., 2014). Photosynthate not used for mucilage production may be shunted into other storage compounds (*e.g.*, lipid, protein or starch). In *A. thaliana*, seed coat mucilage defects can result in seed oil content increases (Shi *et al.* 2012), but this was apparently not the case in the Cs98 mutant, which showed more than a 2.0% lower seed oil content (Figure 3G) and a 3.5% lower total lipid content (Figure 4B). However, the Cs98 mutant seed did contains significantly more crude protein (4%) than WT plants (Figure 4A). Also, the Cs98 mutant contained contains significantly more starch (0.16 %) than WT plants (Figure 6E). The observed increases in protein and starch content of the Cs98 seeds would be advantageous for use as a seed meal.

Ash content is a measure of the inorganic mineral content remaining after ground seeds are combusted (Van Saun, 2013). Cs98 showed significantly higher mineral content (2.18%) than WT (Figure 4C). The origin of the more than 2% increase in ash content in the Cs98 lines is unknown. However, the higher mineral content of Cs98 was confirmed by the significantly higher macro minerals Mg and S (Table 1) and micro minerals Al, Cu, Fe, Mn, and Zn than WT (Table 2). Notably, the Cs98 mineral content was lower in macro minerals K, Ca, and P than WT (Table 1).

Ground Cs98 seed displayed significantly higher moisture compared to WT (Figure 4D). The origin of the 0.7% increase in moisture content in the Cs98 lines is unknown. Exuded seed coat mucilage in myxospermous species might facilitate germination due to its hydrophilicity (Huang et al., 2015). However, prior to hydration, SCM is a physical barrier that regulates water and oxygen diffusion to the seed, and might inhibit germination (Huang et al., 2015). Because the dry mucilage in Camelina acts as a barrier to external humidity entering the seed, the moisture content of WT should be lower than that of Cs98 with scanty SCM. However, without the humidity barrier, Cs98 seed might be more prone to absorbing humidity from its storage environment. Both WT and Cs98 seeds were harvested and stored in Reno, NV, having a mean humidity of 41.7% (2019). The seed samples were shipped to Jerome, ID where they were ground within 24 h to obtain seed fraction data, including moisture content. Jerome, ID has a mean humidity of 54.75% (2019). Thus, Cs98 might have absorbed more moisture from the humid Jerome environment, which might explain the significant increase in moisture content as an artifact.

The origin of the more than 9.5% increase in TDN in the WT lines is unknown (Figure 4E). TDN is calculated as the sum of the digestible fiber, protein, lipid, and carbohydrate components of a feedstuff or diet and is directly related to digestible energy and is often calculated based on ADF. As lipids, protein, and carbohydrates have 9, 4, and 4 Calories g^{-1}, respectively (Youdim, 2019), feedstocks with lower lipid content will have lower TDN values. Cs98 ground seed has contains significant differences in seed and carbohydrate fractions, which contribute to its lower TDN value. The concentration of lipid and crude protein (CP) contents are positively related to TDN (Weiss, 1998). Thus, lower TDN of Cs98 was likely attributable to the significantly lower lipid content (Figure 4B). ADF values present in the CS98 seed were actually higher than the values obtained for WT seed (Figure 6A). The lower TDN of the Cs98 seed was apparently not offset by its significantly higher CP content (Figure 4A) and the significantly lower neutral detergent insoluble crude protein (NDICP) compared to WT (Figure 6B).

The significantly higher ash content (Figure 4C), ADF content (Figure 6A), and aNDF content (Figure 6C) of the Cs98 seed would also contribute to its lower TDN score compared with WT as ash and some carbohydrates (*e.g.*, lignins) are considered indigestible with 0 DE and detract from the TDN score (Weiss, 1998). However, rumen bacteria and protists help cattle digest cellulose, some hemicellulose, and fructans (Demeyer, 1981). Fiber concentrations (ADF and aNDF) are negatively related to TDN, as fibrous carbohydrates are less digestible than non-fibrous carbohydrates (Weiss, 1998).

4.3 Carbohydrate fractions

The fibrous cell wall carbohydrates (lignins and hemicellulose) are less digestible than fibrous cellulose and the soluble carbohydrates. The least digestible plant components, include cellulose and lignin. ADF values are inversely related to digestibility, so feed with low ADF concentrations are usually higher in energy. Therefore, the lignins and cellulose content found in ADF (Figure 6A) are redundant to those measured by aNDF (Figure 6C). Thus, aNDF is a more accurate measure in estimating the indigestible cell wall carbohydrates as they contribute to the TDN calculated values (Figure 4E).

Cs98's NDICP values (Figure 6B) were significantly lower than WT, which was likely correlated with its higher CP values (Figure 4A). Furthermore, NDICP was assayed in order to remove the insoluble portion of CP before calculating the nonfibrous carbohydrate (NFC) values in Figure 6G. NFC was a calculated value, starting with unity (100%) and subtracting out the other reported seed fractions (CP, EE, Ash, and the difference between NDF and NDICP).

Water soluble carbohydrates (WSC) (Figure 6D) and starch (Figure 6E) were assayed to obtain their sum as the calculated total nonstructural carbohydrate (TNC) fraction (Figure 6F). Subtracting the TNC values from the calculated NFC determined the calculated neutral detergent soluble fiber (NDSF) (Figure 6H), which contains pectic substances. The NDSF values showed a significant difference between WT and Cs98 pectic substances with Cs98 having less than half the WT values. This provided a quantitative confirmation of the visual qualitative and quantitative differences noted between the SCM displayed by WT and Cs98 seeds (Figure 1, Figure 8).

4.4 Mineral values in seed meal

One of the most striking mineral value differences between the Cs98 pectin mutant and WT seed was the observation that Cs98 ground seed had 9.3% less Ca than WT (Table 1). In *Arabidopsis* SCM, which is composed of the predominant pectin (rhamnogalacturonan I (RGI) and homogalacturonan (HG)), form ionic cross-links with Ca^{2+} salt bridges (Macquet et al., 2007a). As Cs98 has significantly less SCM (Figure 1) and pectic substances (Figure 6H), it follows that Cs98 would have significantly lower Ca values.

Camelina seed meal (CSM), the proteinaceous byproduct remaining after oil pressing, has potential use in animal feed ration. Both WT and Cs98 ground seed showed acceptable K, Ca, and Mg values that are lower than the maximum tolerable level (MTL) for beef cattle feed rations (Council, 2005). However, the macro mineral values of both ground WT and Cs98 seed for some mineral macronutrients presented challenges for using CSM. For example, the S and P values for both WT and Cs98 ground seed exceed the 400 mg kg^{-1} and 700 mg kg^{-1} (0.4% and 0.7% DW) MTL for those two minerals (Council, 2005). In 2009, the U.S. FDA raised the limit of CSM as a feedlot beef cattle feed ration to 10% from 2% (Schill, 2009). Feedlot beef cattle consume feed ration supplements between 1.0 to 1.5% of body weight, ranging from 3.4 kg to 9.75 kg as they finish weight gain to attain > 500 kg weight prior to slaughter (Davis, 2002). The S and P values (see Table 1) would require livestock producers to decrease Camelina feed rations to less than 10% to limit overexposure to high S and P.

As with some macro minerals, several micro minerals present in ground WT and Cs98 seed also presented challenges for using CSM. For example, for Cs98, the Al and Fe values were 2.4-fold and 2.7-fold higher, respectively, than WT values (Table 2). Such high Al values would limit the use of Cs98 for CSM in animal feed rations, as the MTL for Al is 1000 mg kg^{-1} (Council, 2005). Although Cs98's high Fe levels exceeded the 500 mg kg^{-1} MTL for Fe (Council, 2005), this might provide an opportunity for Cs98 to serve as a feedstock where iron-enrichment is needed for animal feed rations. In addition, the high Al sequestration ability of Cs98 suggests that it might be used to phytoremediate aluminum-rich soils (Pilon-Smits, 2005); however, this speculation would require additional study.

4.5 Mineral values in oil and FAMEs

The macro mineral values of Cs98 oil showed significantly higher K, Ca, Mg, and P content and significantly lower S content than WT oil (Table 3). These higher mineral levels correlated with the higher ash content present in ground seed (Figure 4C). In contrast to the macro mineral values found in ground seed (Table 1), the macro mineral values in oil were found to be within the MTL limits (Table 3), suggesting that most of the macro minerals were removed from the oil. Pectins such as homogalacturonan (HG) and rhamnogalacturonan (RGI) chelate divalent cations (*e.g.*, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, and Cu^{2+}) (Celus et al., 2018). Pectins also chelate monovalent cations (*e.g.*, K^{+}), although more weakly (Celus et al., 2018). As suggested by the high mineral values (Tables 1 and

2), the mineral content of both crude oils probably originated from the ground seed meal after oil pressing.

The macro mineral values of WT and Cs98 FAMEs and FAMEs derived from sorbent-treated oil showed the impact of transesterification and sorbent treatment followed by transesterification on reducing macro mineral content of crudes. Sorbent treatment followed by transesterification reduced S values significantly more than K, Ca, Mg, and P values. Sodium silicate adsorbent has been used successfully to significantly reduce Ca, Mg, and P in edible oil applications (Nock, 1996). Consistent with this use, FAMEs derived from the sorbent-treated oils showed significantly reduced Ca content, but not reduced Mg and P values compared to control FAMEs (Table 3). Note that the reporting lab routinely rounded ICP mineral values up to 1 mg kg^{-1} if ≥ 0.5, and down to 0 mg kg^{-1} if ≤ 0.5, for K, Mg, and P raw values reported for FAMEs and FAMEs derived from the sorbent treated oils (Table 3).

Micro mineral values of Cs98 crude oil showed significantly higher mineral levels for Al, Si, and Mo, with the remaining micro minerals showing lower values than WT oil (Table 4). In contrast macro minerals, Cs98 did not appear to contain micro minerals than WT oil. As with the macro mineral content, sorbent treatment of the WT and Cs98 oils reduced the micro mineral contents in the WT and Cs98 FAMEs Except for Zn, Mo, and Sb, sorbent treatment of the oil followed by transesterification reduced micro minerals in the FAMEs to almost zero. These results showed that the sorbent treatment of the oils did not reduce the values significantly of most micro minerals compared to non-treated oils. Note that the reporting lab routinely rounded ICP mineral values up to 1

mg kg^{-1} if $\geq$ 0.5, and down to 0 mg kg^{-1} if $\leq$ 0.5, for Mn, Zn, Mo, and Sb raw values reported for FAMEs and FAMEs derived from the sorbent treated oils (Table 4).

The only significant difference between the micro mineral profiles of WT and Cs98 FAMEs was an elevated Si content in the WT FAMEs (Table 4). As the FAMEs were not subjected to sodium silicate pretreatment, this value is likely artifactual, as transesterification generally reduced mineral values (Tables 3 and 4). Also, an elevated Si value did not appear in the FAMEs derived from sorbent-treated oils. The cause of this elevated Si value remains unknown.

4.6 Viscosity, TAN, and pHLip

In order to confirm that both WT and Cs98 oils and FAMEs were suitable for biodiesel use, they were tested for viscosity, TAN, and pHLip. The Cs98 oil had a significantly lower viscosity than WT (Table 5). Because the Cs98 seed displayed less SCM (Figure 1) than WT, and the ground Cs98 seed contained significantly less pectic substances (Figure 6H) than WT, this reduced viscosity might reflect the lower amount of pectic substances present in Cs98 oil.

Alternatively, Cs98 pectin may exhibit different structural properties compared with WT pectin. The principal pectin in *Arabidopsis* primary cell walls is homogalacturonan (HG) (Voiniciuc et al., 2018b). Rhamnogalacturonan I (RGI), which has a backbone of alternating rhamnose (Rha) and galacturonic acid (GalA) subunits, decorated with side chains of other pectic polysaccharides such as HG, is the main

component of *Arabidopsis* SCM deposited in the apoplast outside the cell wall (Haughn and Western, 2012). Due to structural differences, RGI is more flexible than HG, with a lower viscosity than HG (Ralet et al., 2008). While *Arabidopsis* SCM is well-characterized in the literature, and closely reflects the 1:1 ratio of Rha:GalA (Vasilevski et al., 2012), only preliminary data have been published regarding the monosaccharide fractions of Camelina SCM (Pattathil et al., 2010). Those data showed a 2:1 ratio of Rha:GalA in Camelina SCM, which is questionable, as the same study reported a 2:1 ratio of Rha:GalA in Arabidopsis SCM (Pattathil et al., 2010). Further study is needed to determine if the viscosity differences between Cs98 and WT oil are merely due to the relative amounts of pectic substances present in each or whether significant structural differences exist between the pectic fractions of Cs98 and WT. However, the lower total soluble pectin content derived from the washed seeds indicates that the observed differences in viscosity arose from less total pectin content with the seed coat (Figures 7 and 8). The lower viscosity of the Cs98 oil and biodiesel also suggests that biodiesel derived from Cs98 will have superior flow characteristics than biodiesel derived from WT plants, particularly at lower temperatures. However, additional testing will be necessary to confirm this hypothesis.

Total acid number (TAN) of Cs98 FAMEs and FAMEs derived from sorbent-treated oil are significantly lower than WT FAMEs and Cs98 FAMEs, respectively (Table 5). The differences in TAN values might be artifactual. Both the Cs98 and WT FAMEs and FAMEs derived from sorbent-treated oils were subjected to washes of acidic (~pH 6.0) nanopure water to remove soaps. WT FAMEs, Cs98 FAMEs, and WT FAMEs

and Cs98 FAMEs derived from sorbent-treated oils were washed 370, 811, 613, and 491 minutes, respectively. These data indicated a very weak correlation between the number of minutes the FAMEs were washed in acidic nanopure water (for water pretreatment plus time washing the FAMEs) and TAN value ($R^2 = 0.0316$). Washes of acidic nanopure water might strip out mineral cations from the oils and FAMEs, and might have contributed to the low K, Mg, and P values in Table 3, and the low Al, Fe, Mn, Zn, Mo, and Sb values in Table 4. Si would not have been affected, as it usually does not ionize in water (LennTech, 2019) unless the pH is > 13 (Wulfsberg, 1987).

The four FAMEs were also assayed using the pHLip assay (Table 5), a quick visual qualitative assay to evaluate B99/B100 biodiesel (von Wedel, 2015). All four FAMEs passed the criteria to evaluate turbidity from glycerides and glycerin, contaminants (*e.g.*, unreacted mono-, di- and triglycerides, sterol glucosides, alkaline soaps), and neutrality (acidic aged fuel turns the solution orange or yellow) (von Wedel, 2015) (Supplemental Figure 1). The pHLip assays confirmed the efficacy of the lab-scale protocols in producing high quality biodiesel. However, because all four FAMEs passed the criteria, it did not support the speculation that biodiesel derived from mucilage-deficient Cs98 might be superior to that derived from WT for turbidity and neutrality.

The macro and micro mineral values obtained in this study compared favorably with literature values (Table 6). The 2013 Camelina biodiesel characterization by Ciubota-Rosie et al. contains that study's values, along with values from other studies to date (Ciubota-Rosie et al., 2013). Ciubota-Rosie et al's values were V40 viscosity (4.3 $mm^2\ s^{-1}$), Group I metals (Na + K) (0.11 mg kg^{-1}), Group II metals (Ca + Mg) (0.16 mg

kg^{-1}), total sulfur (0.57 mg kg^{-1}), phosphorus (<0.1 mg kg^{-1}), and TAN (0.15 mg KOH g^{-1}) (Ciubota-Rosie et al., 2013). This study had equivalent V40 viscosity values, lower Group I metal values, higher Group II metal values, higher total sulfur, higher phosphorus, and equivalent TAN values.

4.7 Carbohydrate and pectin content of water washes of WT and Cs98 oils

The calculated neutral detergent soluble fiber (NDSF) (Figure 6H) from ground seed, which contains pectic substances, showed that Cs98 had less than half the pectic substances of WT. To verify this, the oils were degummed with three deionized water washes, and the lyophilized aqueous residues of WT and Cs98 were weighed, and masses compared. Although WT residue masses were 19.3% greater than Cs98 residues (Figure 7), the difference was not statistically significant. The purpose of degumming in edible oil and biodiesel feedstock preparation is to remove gums, principally hydratable phosphatides found in cell walls (principally phosphatidyl choline and phosphatidyl inositol) (Dijkstra, 1998) to facilitate oil processing. Hence, the residues obtained from water washing contained a mixture of species, including hydratable phosphatides and pectins.

Reconstituting the aqueous residues of WT and Cs98 and incubating them with Ruthenium Red dye, which binds to rhamnogalacturonan I, the pectic principal component of Arabidopsis mucilage (Macquet et al., 2007a; Western et al., 2000) showed a statistically significant difference between WT and Cs98 (Figure 8A and 8B). Cs98

reconstituted residues contained only 57.1% mucilage compared to WT. Although the ratio differed from the 1:2 ratio of pectin calculated in the NDSF fraction in the ground seed assays (Figure 6H), it confirmed the hypothesis that Cs98 has significantly less mucilage, and hence less pectin than WT.

5. Future directions

The genetic lesions responsible for the Cs98 phenotypes (Figures 1 and 3) require further investigation. However, mutations in regulatory factors or pectin biosynthetic enzymes are likely responsible for the reduced SCM phenotype. A candidate gene approach could be used to identify the genetic lesions. In *Arabidopsis thaliana* approximately 54 genes have been identified that are necessary for SCM synthesis and release (Francoz et al., 2015). In addition, 27 transcription factors have been identified as controlling mucilage production (Golz et al., 2018). Within the Brassicaceae Camelina is closely related to *A. thaliana*, and shares a high (81% average) sequence identity with *A. thaliana* proteins (Nguyen et al., 2013). The high sequence identity between the two species has been used for RNAi engineering of high-oleic lines in Camelina using *A. thaliana FAD2* and *FAE1* sequences (Nguyen et al., 2013). More recently, a seed coat-specific promoter from *A. thaliana* MUCILAGE-MODIFIED4 (*MUM4*) gene was used to effectively direct reporter gene expression in both *A. thaliana* and Camelina (Dean et al., 2017).

In an effort to identify candidate genes responsible for the reduced SCM phenotype, flowering tissue was collected from WT, Cross 17.1, and Cs98 lines. The

next step would be to create cDNA from RNA, amplify it, and probe it with primers based upon *A. thaliana* mucilage-defect genes. An initial set of twelve primers was designed (Supplemental Table 2).

Once the lesion(s) have been characterized, it would be desirable to conduct further backcrossing of 17.1 Cross to determine if WT alleles will extinguish the 17.1 Cross phenotypic expression, in order to elucidate the mechanism of expression of the three gene copies or their co-expression in 17.1 Cross and clarify genetic background. Several backcrosses are desirable, with the number varying by species and the number of loci involved (Hallauer et al., 1988).

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Figure 1: Seed phenotypes of A) Wild-type 'Celine' and B) Cs98 seeds. Seeds were hydrated in 0.02% Ruthenium Red dye, displaying copious and scanty seed coat mucilage, respectively. Scale bar = 2 mm.

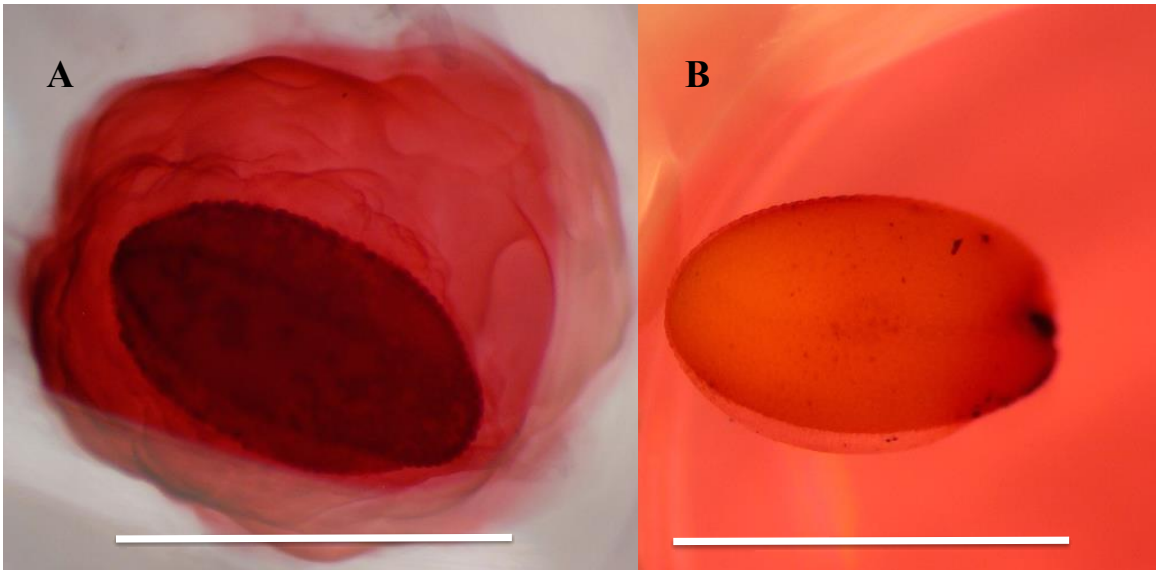


Figure 2: Seed sizes of A) Wild-type ‘Celine’ and B) Cs98 seeds shown with 2 mm scale bar, at 100 X magnification. Note: WT seeds are slightly larger than Cs98 seeds.

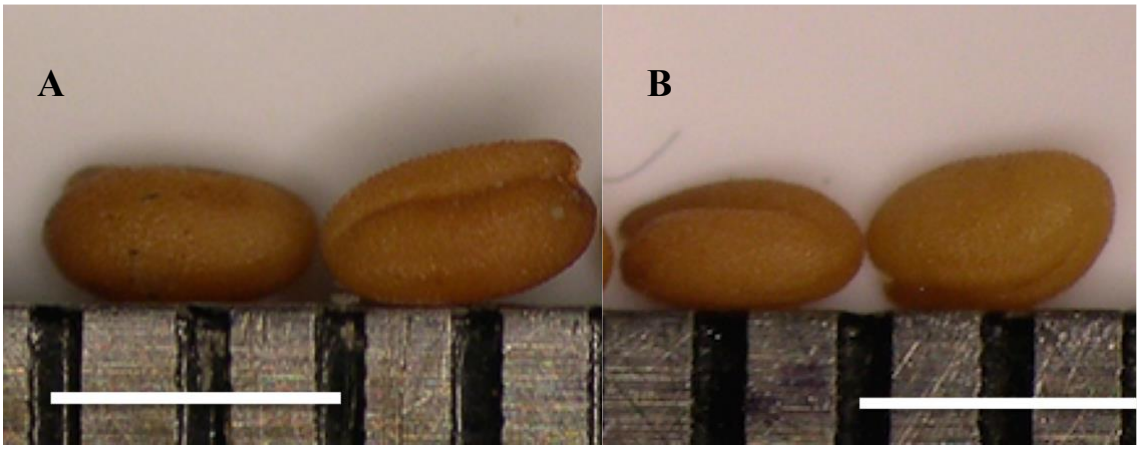


Figure 3: Agronomic traits of Wild-type, 17.1 Cross (F1 hybrid) and Cs98 mutant Camelina plants, significance: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. A) Plant heights, Wild-type n = 108; 17.1 Cross n = 102; Cs98 n = 95; B) Seeds pod^{-1}, Wild-Type n = 90; 17.1 Cross n = 90; Cs98 n = 90; C) 100 seed weights, Wild-Type n = 99; 17.1 Cross n = 100; Cs98 n = 90; D) Total seeds plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 94; E) Aerial biomass plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 90; F) Harvest index plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 90; G) Mean Oil % Dry Weight, Wild-Type n = 6; 17.1 Cross n = 9; Cs98 n = 9.

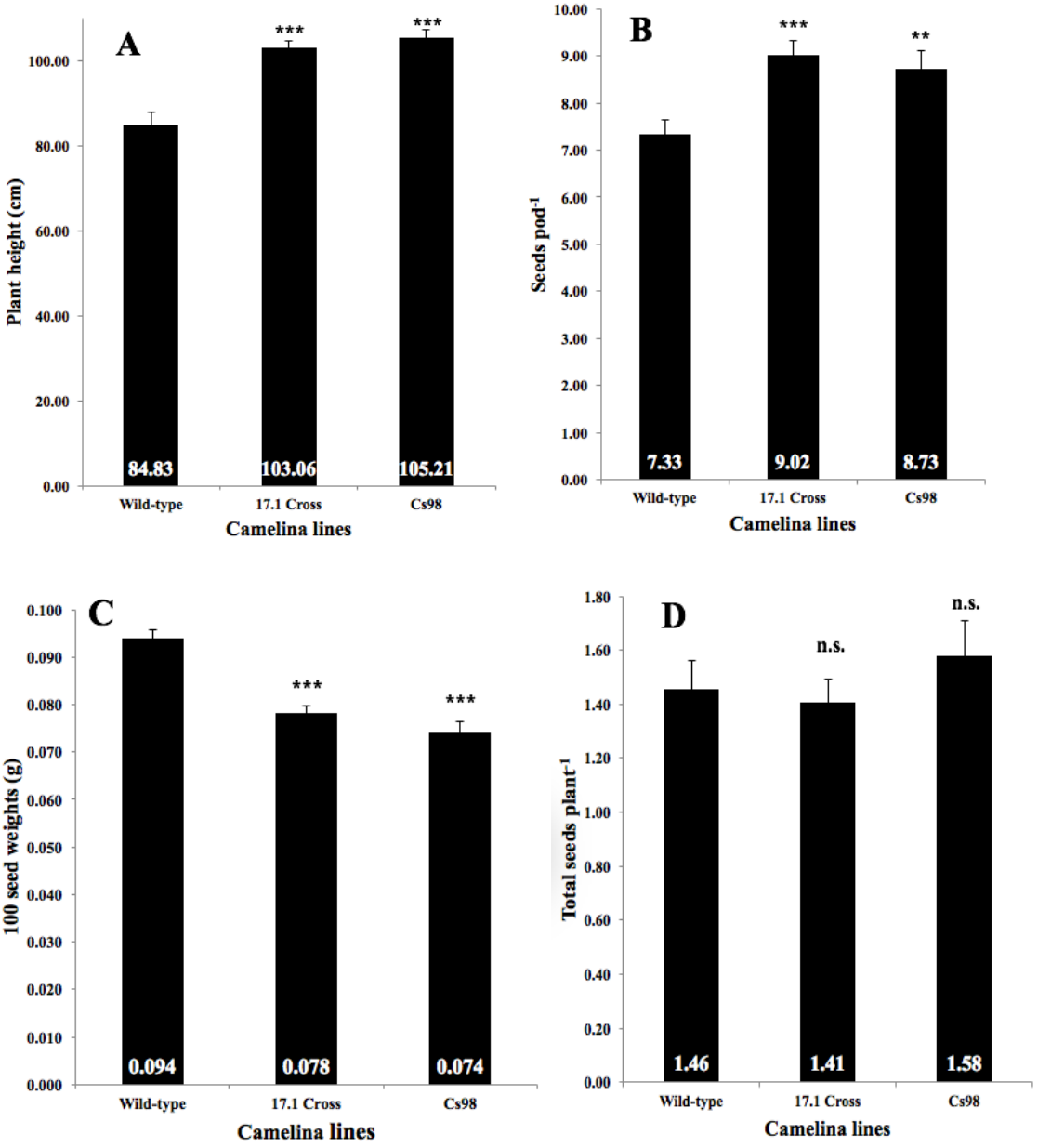


Figure 3: Agronomic traits of Wild-type, 17.1 Cross (F1 hybrid) and Cs98 mutant Camelina plants, significance: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. A) Plant heights, Wild-type n = 108; 17.1 Cross n = 102; Cs98 n = 95; B) Seeds pod^{-1}, Wild-Type n = 90; 17.1 Cross n = 90; Cs98 n = 90; C) 100 seed weights, Wild-Type n = 99; 17.1 Cross n = 100; Cs98 n = 90; D) Total seeds plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 94; E) Aerial biomass plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 90; F) Harvest index plant^{-1}, Wild-Type n = 107; 17.1 Cross n = 100; Cs98 n = 90; G) Mean Oil % Dry Weight, Wild-Type n = 6; 17.1 Cross n = 9; Cs98 n = 9.

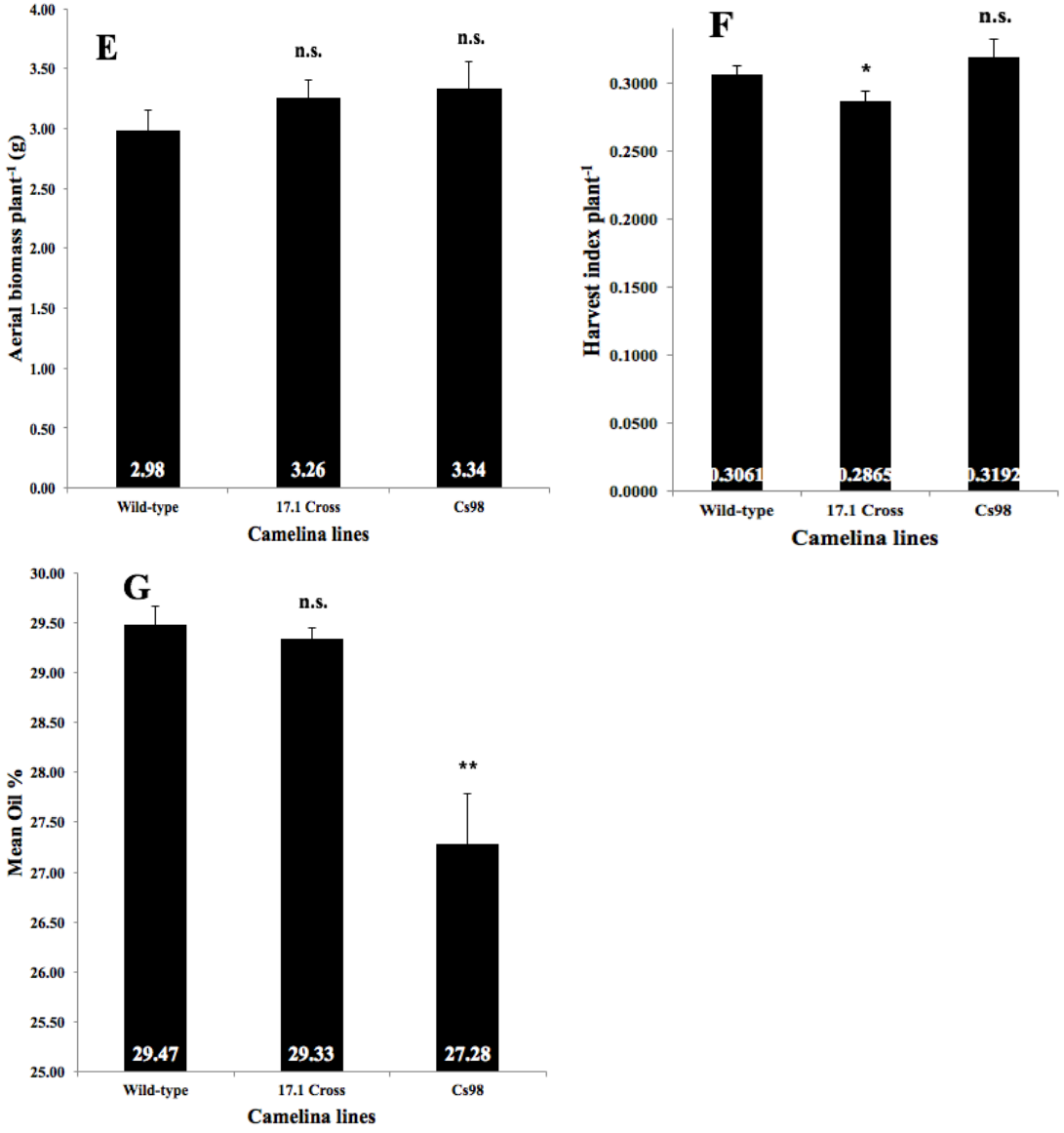


Figure 4: Proximate analysis of seed composition (% Dry Weight) of wild-type and Cs98 mutant of *Camelina sativa*, excluding carbohydrate fractions. $N = 3$, significance: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. A) Crude protein, B) Ether extract of lipids, C) Ash, D) Moisture, E) Total digestible nutrients (TDN) for dairy cattle.

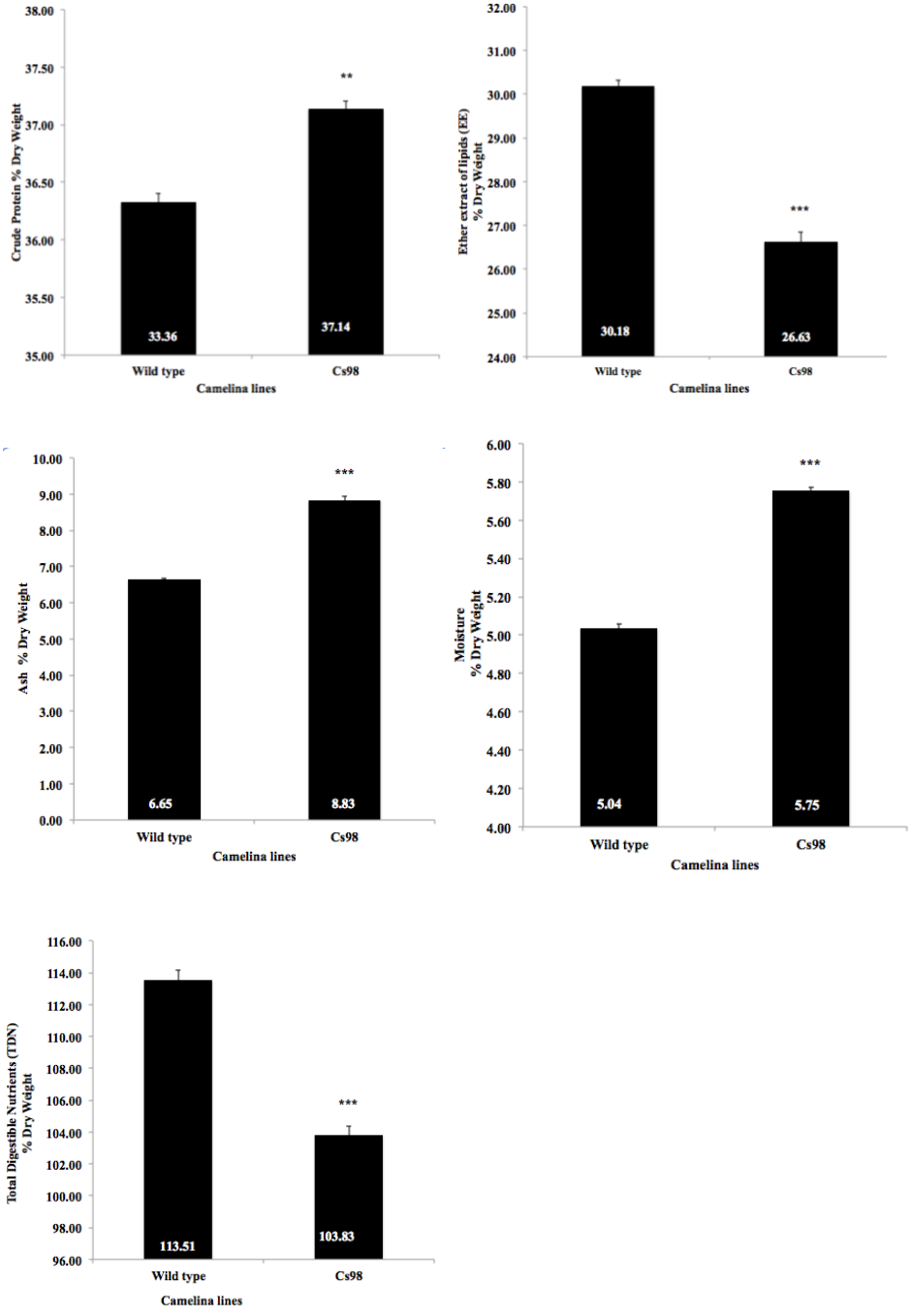


Figure 5: Analysis of seed carbohydrate fractions of wild-type and Cs98 mutant *Camelina sativa* seeds. ADF: acid detergent fiber; aNDF: amylase-treated neutral detergent fiber; NDSF: neutral detergent soluble fiber; NFC: non-fibrous carbohydrates; TNC: total nonstructural carbohydrates; WSC: water soluble carbohydrates. Modified from M. Atkinson (2018), Northwest Labs, Jerome, ID.

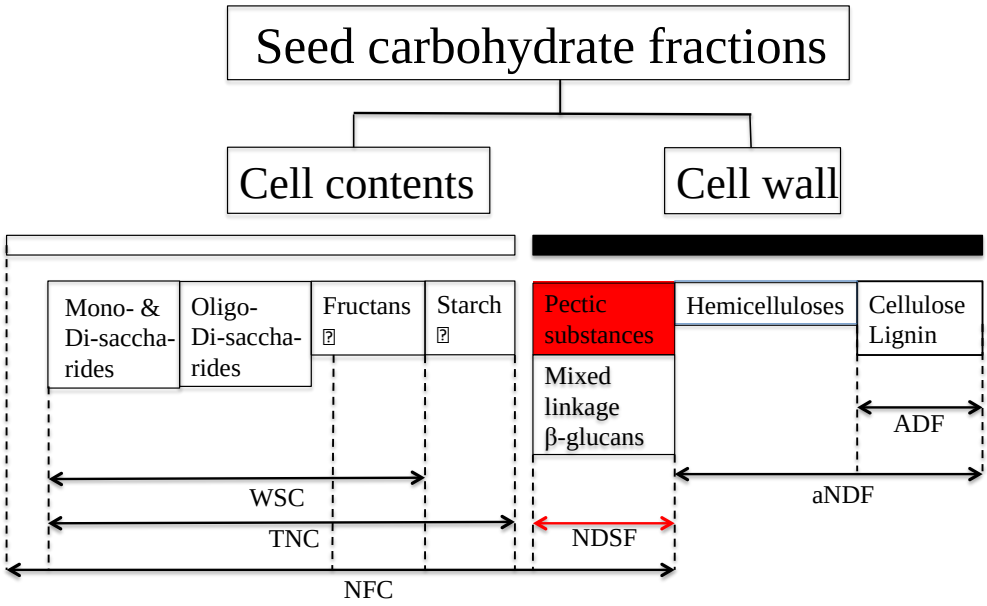


Figure 6: Compositional analysis of seed carbohydrates of wild-type and Cs98 mutant *Camelina sativa* seeds as % Dry Weight (DW), $n = 3$, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. Reported values are assayed percentages of total DW (after deducting moisture from sample weight). Calculated values are determined from DW based on subtracting other assayed fractions. A) ADF: acid detergent fiber (reported); B) NDICP: neutral detergent insoluble crude protein (calculated); C) aNDF: amylase-treated neutral detergent fiber (reported), D) WSC: water soluble carbohydrates (reported); E) Starch (reported); F) TNC: total nonstructural carbohydrates (calculated); G) NFC: non-fibrous carbohydrates (calculated); H) NDSF: neutral detergent soluble fiber, containing pectin (calculated).

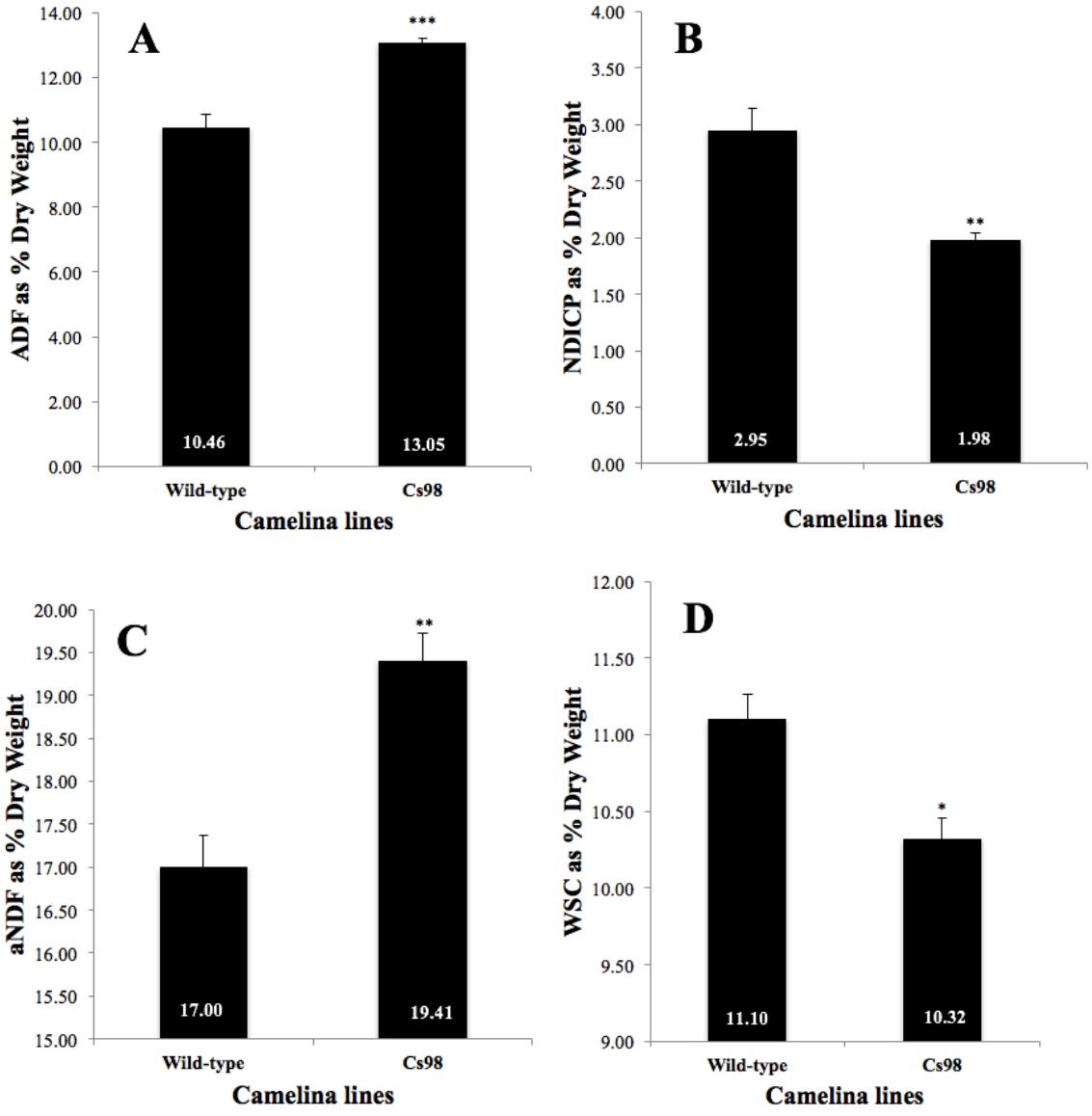


Figure 6: Compositional analysis of seed carbohydrates of wild-type and Cs98 mutant *Camelina sativa* seeds as % Dry Weight (DW), $n = 3$, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. Reported values are assayed percentages of total DW (after deducting moisture from sample weight). Calculated values are determined from DW based on subtracting other assayed fractions. A) ADF: acid detergent fiber (reported); B) NDICP: neutral detergent insoluble crude protein (calculated); C) aNDF: amylase-treated neutral detergent fiber (reported), D) WSC: water soluble carbohydrates (reported); E) Starch (reported); F) TNC: total nonstructural carbohydrates (calculated); G) NFC: non-fibrous carbohydrates (calculated); H) NDSF: neutral detergent soluble fiber, containing pectin (calculated).

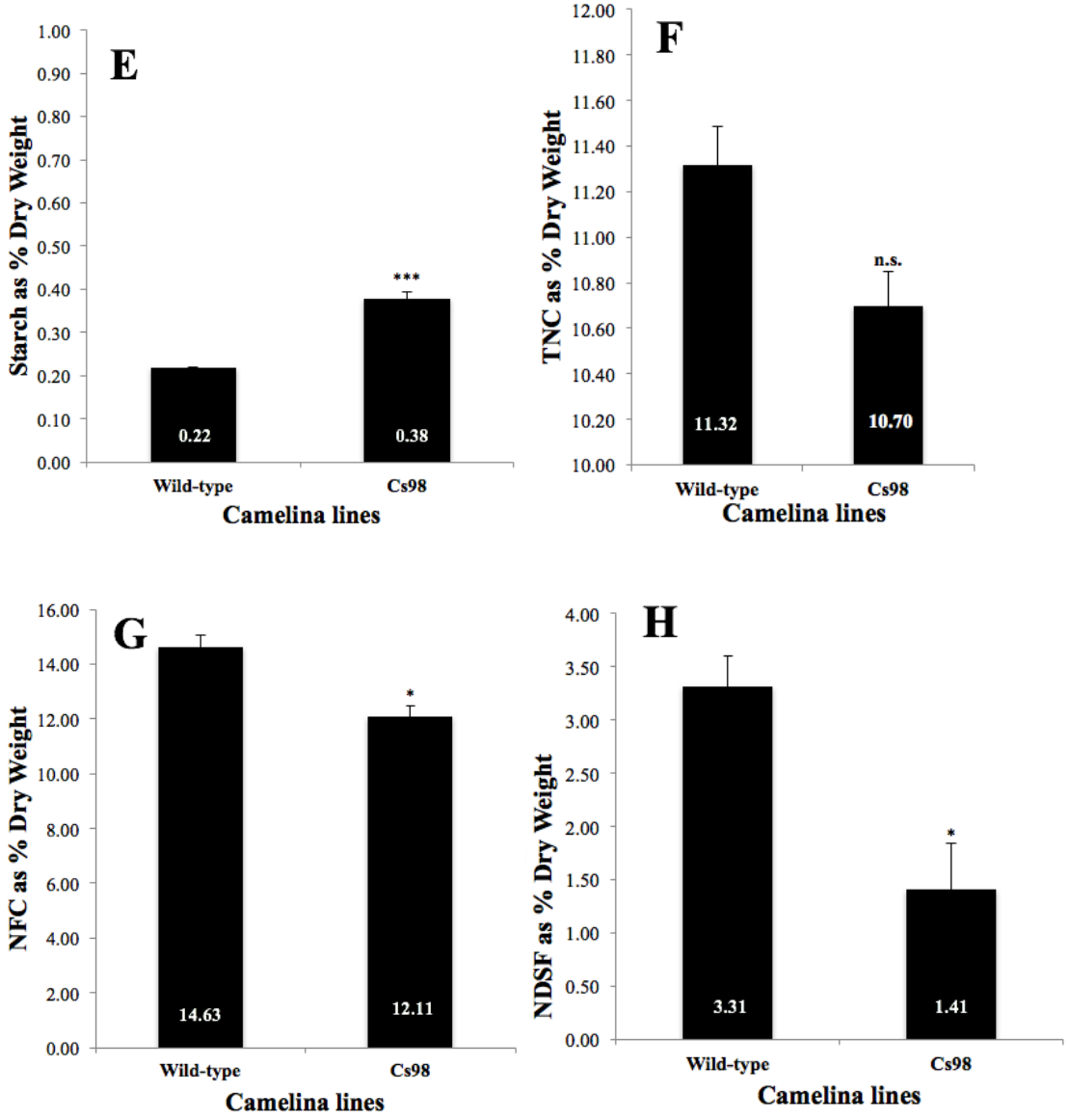


Table 1: Macro mineral content of ground seed, mean values ± SE.

Accession	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	S (mg kg^{-1})	P (mg kg^{-1})
Wild-type	976.7 ± 13.3	426.7 ± 3.3	346.7 ± 3.3	943.3 ± 14.5	896.7 ± 3.3
Cs98	940 ± 15.3	390 ± 5.8**	380 ± 0***	1016.7± 6.7*	873.3 ± 3.3**

$n = 3$
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Table 2: Micro minerals content of ground seed, mean values ± SE.

Accession	Al (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Wild-type	830.1 ± 30.0	7.37 ± 0.02	938.3 ± 24.2	64.6 ± 1.8	76.0 ± 0.7
Cs98	2005.4 ± 76.1***	10.21 ± 0.1***	2497.0 ± 131.6***	81.5 ± 2.2**	88.2 ± 0.5***

$n = 3$
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Table 3: Macro mineral content of oils and FAMEs, mean values ± SE.

Accession	Substrate	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	S (mg kg^{-1})	P (mg kg^{-1})	Comparison[1]
Wild-type	Oil	6.78 ± 0.06	22.04 ± 0.09	11.13 ± 0.04	93.92 ± 0.91	44.00 ± 0.31	
Cs98	Oil	8.88 ± 0.13**	31.83 ± 0.67***	16.95 ± 0.13***	71.58± 0.30***	62.65± 0.34***	Wild-type oil
Wild-type	FAMEs	0.04 ± 0.02	1.32 ± 0.04	0.0 ± 0.0	9.61 ± 0.03	0.71 ± 0.55	
Cs98	FAMEs	0.10 ± 0.05 n.s.	1.02 ± 0.05*	0.0 ± 0.0	9.05± 0.09**	0.71± 0.13 n.s.	Wild-type FAMEs
Wild-type	FAMEs from sorbent-treated oil[2]	0.0 ± 0.0 n.s.	1.03 ± 0.06*	0.11 ± 0.11 n.s.	7.15 ± 0.01***	0.45 ± 0.23 n.s.	Wild-type FAMEs
Cs98	FAMEs from sorbent-treated oil[2]	0.04 ± 0.04 n.s.	0.72 ± 0.02**	0.12 ± 0.12 n.s.	7.65± 0.02***	0.71± 0.30 n.s.	Cs98 FAMEs

$n = 3$

[1]T-tests compared mean values to listed substrate.

[2]Crude oils pretreated with 3% water and 1.5% adsorbent prior to transesterification.

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 4: Micro minerals from oils and FAMEs, mean values ± SE.

Accession	Substrate	Al (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Si (mg kg^{-1})	Mo (mg kg^{-1})	Sb (mg kg^{-1})	Comparison[1]
Wild-type	Oil	1.87 ± 0.05	0.00	4.74 ± 0.05	0.61 ± 0.01	0.92 ± 0.02	3.70 ± 0.06	0.32 ± 0.11	0.31 ± 0.16	
Cs98	Oil	2.34 ± 0.04**	0.00 n.s.	4.38 ± 0.04**	0.28 ± 0.02***	0.88 ± 0.01 n.s.	4.27 ± 0.07**	0.42 ± 0.19 n.s.	0.30 ± 0.12 n.s.	Wild-type oil
Wild-type	FAMEs	0.003 ± 0.003	0.00	0.003 ± 0.003	0.00	0.35 ± 0.05	11.21 ± 0.33	0.24 ± 0.08	0.11 ± 0.11	
Cs98	FAMEs	0.02 ± 0.02 n.s.	0.00 n.s.	0.0 ± 0.0 n.s.	0.00 n.s.	0.21 ± 0.04 n.s.	0.56 ± 0.03***	0.22 ± 0.10 n.s.	0.18 ± 0.18 n.s.	Wild-type FAMEs
Wild-type	FAMEs from sorbent-treated oil[2]	0.01 ± 0.01 n.s.	0.00 n.s.	0.0 ± 0.0 n.s.	0.00 n.s.	0.26 ± 0.19 n.s.	0.01 ± 0.1***	0.13 ± 0.10 n.s.	0.15 ± 0.11 n.s.	Wild-type FAMEs
Cs98	FAMEs from sorbent-treated oil[2]	0.0 ± 0.0 n.s.	0.00 n.s.	0.0 ± 0.0 n.s.	0.00 n.s.	0.13 ± 0.07 n.s.	0.0 ± 0.0***	0.26 ± 0.07 n.s.	0.08 ± 0.08 n.s.	Cs98 FAMEs

$n = 3$

[1]T-tests compared mean values to listed substrate.

[2]Crude oils pretreated with 3% water and 1.5% adsorbent prior to transesterification.

***** = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 5: Viscosity, total acid number, and pHLip assays of oils and FAMEs, mean values ± SE.

Accession	Substrate	V40 viscosity Mean cSt[3]	Comparison[1]	Total Acid Number (TAN) mg KOH g^{-1}	Comparison[1]	pHLip Bright/ Turbid	pHLip Mirror/ Contaminants	pHLip Red = Neutral pH	pHLip Pass/Fail[2]
Wild-type	Oil	32.04 ± 0.03							
Cs98	Oil*	30.87 ± 0.13***	Wild-type oil						
Wild-type	FAMEs	4.37 ± 0.01		0.17 ± 0.003		Bright	Mirror	Neutral	Pass
Cs98	FAMEs	4.40 ± 0.01 n.s.	Wild-type FAMEs	0.14 ± 0.0***	Wild-type FAMEs	Bright	Mirror	Neutral	Pass
Wild-type	FAMEs from sorbent-treated oil[4]	4.36 ± 0.02 n.s.	Wild-type FAMEs	0.18 ± 0.003 n.s.	Wild-type FAMEs	Bright	Mirror	Neutral	Pass
Cs98	FAMEs from sorbent-treated oil[4]	4.36 ± 0.01 n.s.	Cs98 FAMEs	0.12 ± 0.0***	Cs98 FAMEs	Bright	Mirror	Neutral	Pass

n = 3
[1]T-tests compared means to listed substrate.
[2]See Supplemental Figure 1 for photos of pHLip assays of FAMEs.
[3]Kinematic viscosity at 40° C measured in centi-Stokes (cSt); 1 cSt = 1 mm2 s-1.
[4]Crude oils pretreated with 3% water and 1.5% adsorbent prior to transesterification.
***** = p<0.05
** = p<0.01
*** = p<0.001

Figure 7: Consolidated residues from Wild-type and Cs98 washes: WT (n = 21), Cs98 (n = 42), p = 0.4242.

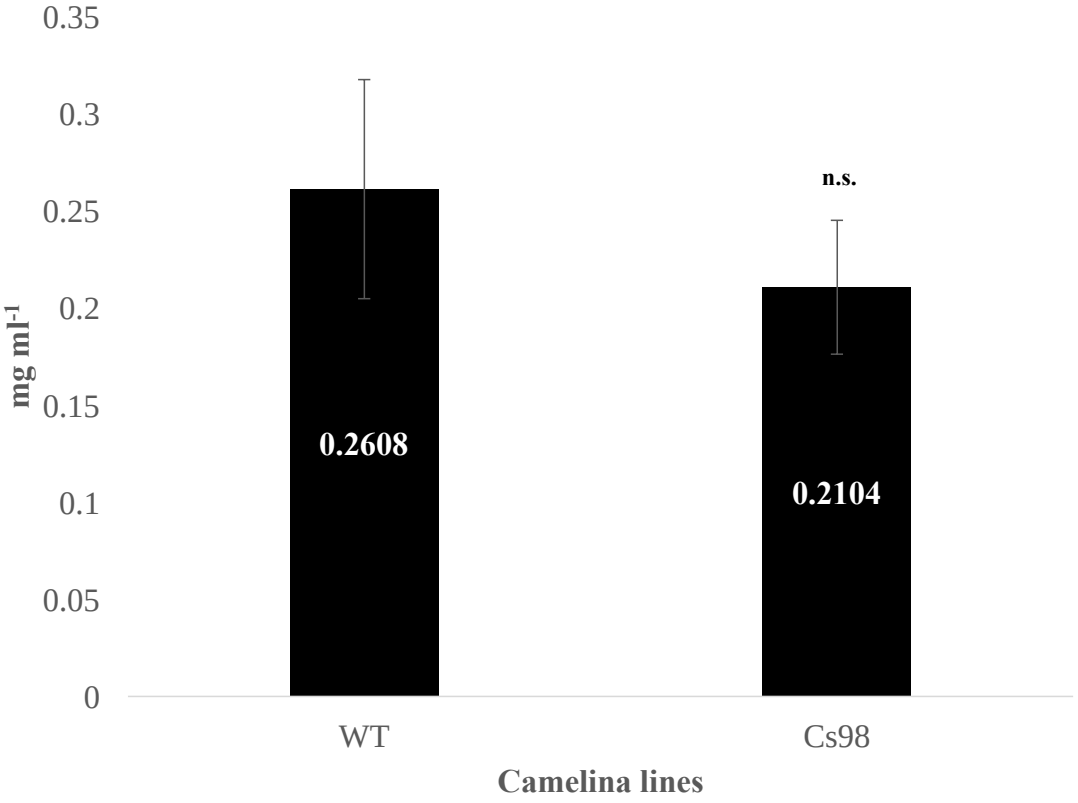
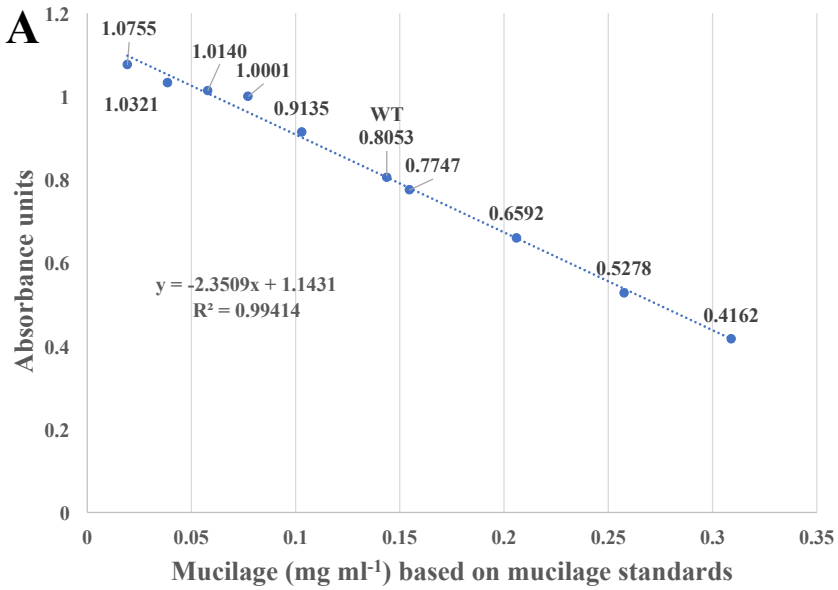
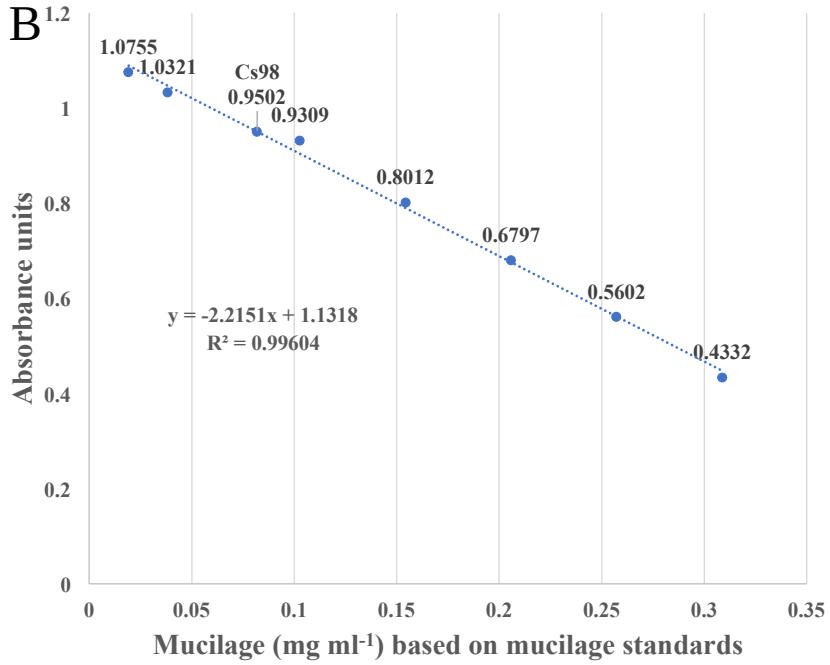
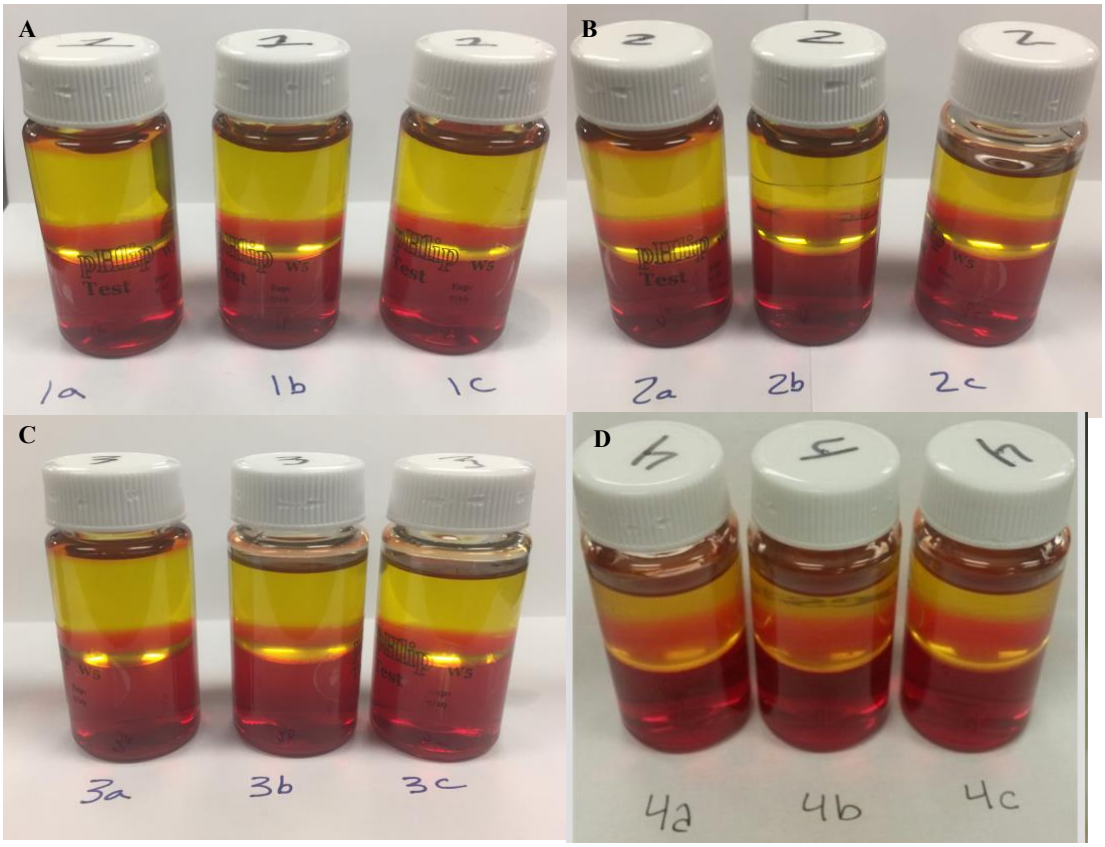


Figure 8: Consolidated washes from A) Wild-type and B) Cs98 oils: A) Colorimetric assay of consolidated WT A, B, and C washes (n = 160) with mucilage standards with WT having 0.1437 mg ml^{-1} mucilage; B) Colorimetric assay of consolidated Cs98 A, B, and C washes (n = 132) with mucilage standards with Cs98 having 0.0820 mg ml^{-1} mucilage;





Supplemental Figure 1: pHLip assay of triplicate samples of four FAMEs. All FAMEs passed assay, exhibiting Brightness (*vs.* turbidity), Mirror interface (with no contaminants noted), and Red color (neutral pH). A) Wild-type FAMEs, B) Cs98 FAMEs, C) Wild-type from sorbent-treated oil; D) Cs98 FAMEs from sorbent treated oil.



Supplemental Table 1: Mean values ± SE of Wild-type and Cs98 FAME substrates compared to selected U.S. ASTM specifications

Specification	ASTM D6751 limits[1]	Wild-type FAMEs	Cs98 FAMEs	ASTM limits: Pass/Fail
Kinematic Viscosity @ 40° C, $mm^2\ s^{-1}$	1.9 - 6.0	4.37 ± 0.01	4.40 ± 0.01	**Pass**
Gp I metals Na + K ($mg\ kg^{-1}$) max	5.0	0.04 ± 0.02	0.10 ± 0.05	**Pass**
Gp II metals Ca + Mg ($mg\ kg^{-1}$) max	5.0	1.32 ± 0.04	1.02 ± 0.05	**Pass**
Total Sulfur ($mg\ kg^{-1}$, max)	15.0	9.61 ± 0.03	9.05 ± 0.09	**Pass**
Phosphorus ($mg\ kg^{-1}$, max)	10.0	0.71 ± 0.55	0.71 ± 0.13	**Pass**
Total Acid Number (TAN) (mg KOH g^{-1} max)	0.5	0.17 ± 0.003	0.14 ± 0.0	**Pass**

$n = 3$

[1]Hoekman et al., 2012

[2]Oils pretreated with 3% water and 1.5% adsorbent prior to transesterification.

Supplemental Table 2: Primers to probe mucilage-defect genes

Primer Name	**Sequence from 5' to 3'**
CsTT2_16_33F1	CAACCACAGCCACAACCA
CsTT2_800_817R1	TTTCCGAGCCAGTCTTCA
CsTT2_23_40F2	AGCCACAACCACAAGAGA
CsTT2_806_823R2	TTGAAGTTTCCGAGCCAG
CsTT2_13_30F3	TACCAACCACAGCCACAA
CsTT2_799_816R3	TTCCGAGCCAGTCTTCAT
CsRSW3_36_53F1	CATGAACGAGGAGCCAAA
CsRSW3_2958_2977R1	AAGATAACCAACAATCCGAG
CsRSW3_38_55F2	TGAACGAGGAGCCAAAAT
CsRSW3_2958_2976R2	AGATAACCAACAATCCGAG
CsRSW3_38_55F4	GAACGAGGAGCCAAAATGA
CsRSW3_2958_2977R4	AAGATAACCAACAATCCGAG
CsTTG2_211890_211907_F1	CCAGAATCCGAAGTTCCA
CsTTG2_212360_212377_R1	GTAGTCCAGAAAGTTCCA
CsTTG2_211864_211882_F2	CTTCATCACATTGGCTACT
CsTTG2_212361_212379_R2	ATGTAGTCCAGAAAGTTCC
CsTTG2_211885_211902_F3	TGCAACCAGAATCCGAAG
CsTTG2_212360_212377_R3	GTAGTCCAGAAAGTTCCA
CsGL2_486_503_F1	ACACATGGAAGCGCTATT
CsGL2_1973_1990_R1	CTCTGTCTTGTCCCTTGG
CsGL2_498_515_F2	GCTATTCAAAGAGACACC
CsGL2_1974_1991_R2	CCTCTGTCTTGTCCCTTG
CsGL2_485_502_F3	GACACATGGAAGCGCTAT
CsGL2_1972_1989_R3	TCTGTCTTGTCCCTTGGA
CsBGAL6_273_290_F1	GTCTTGGGGTTGTGTTTG
CsBGAL6_2395_2412_R1	AGGATAGTTGAGACTGAG
CsBGAL6_275_293_F2	CTTGGGGTTGTGTTTGATT
CsBGAL6_2337_2354_R2	CCCAAGAGGATTACCACC
CsBGAL6_286_305_F3	GTTTGATTCTTCTGGTTACG
CsBGAL6_2346_2363_R3	CAAAGATATCCCAAGAGG
CsTTG2_253_270_F1	AGGGTTTCCTCTCAAATC
CsTTG2_1747_1765_R1	TTGCTTAGAAAGTTGTGGG
CsTTG2_292_309_F2	TTTTTGGCATTCTCACCG
CsTTG2_1741_1759_R2	AGAAAGTTGTGGGAAGCTA
CsTTG2_290_309_F3	TTTTTTTGGCATTCTCACCG
CsTTG2_1743_1761_R3	TTAGAAAGTTGTGGGAAGC

Primer Name	Sequence from 5' to 3'
CsPMEI6_741_758_R1	CATACACTTGGTAGCCTT
CsPMEI6_172_189_F1	AGTAGCGTTTGGATCGAA
CsPMEI6_170_187_F2	AAAGTAGCGTTTGGATCG
CsPMEI6_728_745_R2	GCCTTCCTCCTAACCATC
CsPMEI6_170_189_F3	AAAGTAGCGTTTGGATCGAA
CsPMEI6_740_757_R3	ATACACTTGGTAGCCTTC
CsSBT1.7_214_233_F1	CTTTCTTTCTCATCCTCTGT
CsSBT1.7_2444_2461_R1	TATGTCCAGCTAATCGCC
CsSBT1.7_217_235_F2	TCTTTCTCATCCTCTGTCT
CsSBT1.7_2448_2465_R2	CGACTATGTCCAGCTAAT
CsSBT1.7_213_230_F3	GCTTTCTTTCTCATCCTC
CsSBT1.7_2453_2470_R3	GTTTACGACTATGTCCAG
CsRHM2_248_265_F1	TTGGGTCTATCTGCTTCT
CsRHM2_2407_2424_R1	GGATCATAAGACGATGAG
CsRHM2_244_262_F2	TCTCTTGGGTCTATCTGCT
CsRHM2_2314_2331_R2	TTGTGGCGAAGAGTGTGA
CsRHM2_250_268_F3	GGGTCTATCTGCTTCTAAC
CsRHM2_2407_2426_R3	AAGGATCATAAGACGATGAG
CsIRX14_197_214_F1	AGCTCTCTGCTTTACATC
CsIRX14_1948_1965_R1	TCATTCCCCGAAAAACCT
CsIRX14_198_215_F2	GCTCTCTGCTTTACATCA
CsIRX14_1950_1967_R2	CATCATTCCCCGAAAAAC
CsIRX14_225_242_F3	AAATCGCCGGAGTAACAG
CsIRX14_1949_1967_R3	CATCATTCCCCGAAAAACC
CsGAUT11_48_65_F1	GACAGTGACGAAAGGATT
CsGAUT11_2173_2191_R1	AGTTTCTCTTCGGACCATA
CsGAUT11_73_90_F2	TCGATGCACTCAAATCCT
CsGAUT11_2175_2193_R2	TAAGTTTCTCTTCGGACCA
CsGAUT11_70_87_F3	CGATCGATGCACTCAAAT
CsGAUT11_2173_2191_R3	AGTTTCTCTTCGGACCATA
CsEXO70A1_157_174_F1	GAATGGATCTGCTAAGCG
CsEXO70A1_2263_2281_R1	AACCTCTGTCTCCAAGTAA
CsEXO70A1_176_193_F1	AAGAGCTGTGTTGATGAG
CsEXO70A1_2264_2282_R2	AAACCTCTGTCTCCAAGTA
CsEXO70A1_174_192_F3	GAAAGAGCTGTGTTGATGA
CsEXO70A1_2262_2279_R3	GAATGGATCTGCTAAGCG

Primer Name	**Sequence from 5' to 3'**
CsSEC8_161_179_F1	TGCGATTATTCAGTGAGTC
CsSEC8_3409_3427_R1	ATATGAACTCACTCTTGGG
CsSEC8_182_201_F2	CTGAAAAAATTGGCGACTTG
CsSEC8_3403_3420_R2	CACTGAAAAAATTGGCGAC
CsSEC8_180_198_F3	CACTGAAAAAATTGGCGAC
CsSEC8_3409_3428_R3	AATATGAACTCACTCTTGGG
CsPER36_58_76_F1	ATACCTGTCACACCTTCCT
CsPER36_1099_1116_R1	TCCTCCGGATCTCACCAT
CsPER36_55_72_F2	TTCATACCTGTCACACCT
CsPER36_1053_1070_R2	CATCTTCACGATCGACTT
CsPER36_59_76_F3	TACCTGTCACACCTTCCT
CsPER36_1060_1077_R3	TGTTCCCCATCTTCACGA

Chapter 5

Concluding Remarks

Summary

Camelina continues to be the focus of intense interest and ongoing research due to its strengths as a feedstock for edible oil and biodiesel applications. Due to high adaptability it can grow in marginal and low-fertility soils, and in semi-arid areas (Budin et al., 1995) in the western and southwestern United States (Hunsaker et al., 2013). Camelina requires modest irrigation and fertilizer inputs. As the five-year field trial of eight Camelina varieties (Chapters 2 and 3) demonstrated, Camelina can be successfully grown in semi-arid locales such as northern Nevada. With its modest input needs of 58.8 kg ha^{-1} N, and 338 mm water, Camelina can help diversify Nevada's agricultural sector, which is dominated by alfalfa, which requires higher mineral inputs and 20 to 25 fold the irrigation levels used for Camelina. Of the eight cultivars evaluated, Columbia, Cheyenne, and Calena demonstrated that they would be highly suited to northern Nevada growing conditions, with high seed mass yield and performance stability.

Until markets and infrastructure have been fully developed in the United States, the main incentive for farmers to plant Camelina is its use as a feedstock for biodiesel, classified as an advanced biodiesel, with a mandate for increasing production through 2022. Camelina and other promising feedstocks will continue to be studied and improved during this century, as fossil fuel resources diminish.

Camelina can be and should be improved, through genetic engineering (GE). Older GE technologies, such as EMS mutation, have produced mutants such as mucilage-deficient Cs98, having phenotypes that may answer an industrial or agricultural need. The study in Chapter 4 showed that Cs98 has significantly less mucilage and pectin then WT, and Cs98 oil can be successfully transesterified into biodiesel. However, the Cs98 FAMEs were shown to be substantially equivalent, and not superior to the WT FAMEs. However, other constitutive traits of Cs98, such as its ability to sequester aluminum and iron, could enable Cs98 to be used for phytoremediation, or as a feed ration supplement, respectively, after further engineering.

The most pressing reason for engineering Camelina is economic. If growers have prime agricultural land and ample irrigation, they currently choose canola over Camelina, as its unit revenue is 14% or greater than Camelina. Due to lower yields and lower price than other oilseeds, Camelina yields need to increase a minimum of 20%, if only to match revenue received for growing canola.

Future prospects of Camelina

Camelina's tractability to genetic modification through transgenesis has led to many exciting research efforts. To obtain EPA and DHA from Camelina (Ruiz-Lopez et al., 2014), rather than increasing consumption of overfished species, is on the horizon. To produce a sustainable source of jet fuel, reducing Camelina's TAG PUFA content and forming mid-length carbon chains is underway (Hu et al., 2017). Decreasing Camelina's

TAG PUFA content to produce greater 18:1 oleic acid (Kang et al., 2011) will make Camelina a more desirable biodiesel feedstock. Improving Camelina's salinity tolerance through transformation with a bacterial gene encoding ACC deaminase (acdS) has been demonstrated (Heydarian et al., 2018; Heydarian et al., 2016).

Camelina biodiesel currently does not met specifications of ASTM D6751 for cetane number, oxidative stability and distillation temperature, due to its TAG 90% PUFA content. Although the first two specifications can be corrected through additives, genetic modification of the feedstock to reduce PUFA levels was recommended to correct the distillation temperature issue (Ciubota-Rosie et al., 2013).

The 'omics' technologies have provided several useful resources to improve Camelina. With the publication of the Camelina leaf transcriptome (Liang et al., 2013) the developing Camelina seed transcriptome (Nguyen et al., 2013), the Camelina genome (Kagale et al., 2014), and the developmental Camelina transcriptome atlas (Kagale et al., 2016), researchers can locate genes of interest and design strategies for modification. Due to the high similarity with Arabidopsis, Arabidopsis constructs can be used directly or with slight modification for primer design and transgenic applications.

With the development of recent gene-editing technologies, TALEN, ZFN, and CRISPR/Cas9, Camelina's future as an edible or biodiesel feedstock looks bright. The gene editing technologies may be able to reduce glucosinolate and erucic acid levels in Camelina by knocking out or knocking down the genes regulating those phenotypes. In *B. napus*, high erucic acid rapeseed (HEAR) varieties with > 40% 22:1 have been bred as feedstocks for plastics, nylon13-13, and high temperature lubricants (Li et al., 2012).

Canola, which has <2% erucic acid, was developed as an edible oil. Using gene editing, researchers may be able to develop an improved Camelina with reduced erucic acid content for edible oil use.

An improved edible Camelina with ability to grow on marginal lands, with drought and cold resistance, would be a welcome addition to diversify the agricultural economies in semi-arid and arid regions. Nations such as the United States and Canada, which have embraced genome-editing technologies without imposing additional regulatory burdens, can move ahead of the rest of the world in developing more diverse foods for their citizens and feedstocks for animal rations. Improving crops and concomitant food security through technologies such as genome editing can gradually shift public opinion from fearful or ideological distrust of technology to a more pragmatic stance.

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