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Investigating the effects of temperature on oil quality of *Camelina sativa* for bio-based applications

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Thesis plan

LIST OF ABBREVIATION

Abbreviation	**Description**
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1n9	Oleic acid
C18:2n6	Linoleic acid
C18:3n3	Linolenic acid
C20:1n9	Eicosenoic acid
C22:1n9	Erucic acid
CE	Controlled Environment
Chol	Cholesterol
DAF	Day after flowering
DG or DAG	Diglyceride or diacylglycerol
ER	Endoplasmic Reticulum
FA	Fatty acid
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acids
GDD	Growing Degree Days
GDD-AF	Growing Degree Days after flowering
GDD-GS	Growing Degree Days growing season
GMO	Genetically modified organism

GS	Growing Season
Hp	Heptane
LOC	Location
MCFA	Medium Chain Fatty Acid
MUFA	Mono Unsaturated Fatty Acid
OF	Open Field
PUFA	Poly Unsaturated Fatty Acid
P8	Plastochromanol8
R^2	coefficient of determination
RR	Rate of reaction
r	Pearson's coefficient
ST	Sitosterol
α, γ, δT	α, γ, δTocopherol
TG or TAG	Triglyceride or Triacylglycerol
TKW	Thousand Kernel Weight
TLC	Thin Layer Chromatography
VLCFA	Very Long Chain Fatty Acid

Enzyme abbreviation	**Description**
ACCase	acetyl-CoA carboxylase
ACS	long-acyl-CoA-synthetases
DGAT	Diacylglycerol acyltransferase
DHLAT	dihydrolipoyl acyltransferase
ECR	enoyl-CoA reductase
ELO	Elongase
FAD2	Δ12 oleate desaturase
FAD3	Δ15 linoleate desaturase
FAE	Fatty Acid Elongase
FAS	Fatty Synthase Condensing Enzymes
FATA	acyl-ACP thioesterase A
FATB	acyl-ACP thioesterase B
GPAT	Glycerol-3-phosphate acyltransferase
HCD	β-hydroxyacyl-CoA dehydratase
KASI	3-ketoacyl-ACP synthases I
KASII	3-ketoacyl-ACP synthases II
KASIII	3-ketoacyl-ACP synthases III
KCS	β- ketoacyl-CoA synthase
KCR	β-ketoacyl-CoA reductase
LACS	Long-acyl-CoA-synthetases
LB	Luria Bertani

LPAAT	2-lysophosphatidic acid acyltransferase
LPCAT	lysophosphatidylcholine acyltransferase
LPD	Dihydrolipoamide dehydrogenase
MCMP	Malonyl-CoA:acyl carrier protein malonyltransferase
PDCT	phosphatidylcholine:diacylglycerol cholinephosphotransferase
PDHC	Plastidial pyruvate dehydrogenase complex
α,β - PDH	Pyruvate dehydrogenase α, β
PP	Phospatide phosphatase
SAD	Stearoyl–ACP Δ9-desaturase

ABSTRACT

In Europe, the growing interest of the bio-based industry for camelina (*Camelina sativa* L. Crantz) is largely due to its unique fatty acid (FA) profile, characterized by a very high content of polyunsaturated fatty acids (PUFAs, linoleic, C18:2n6 and linolenic, C18:3n3 >50%) and significant amounts of eicosenoic acid (C20:1n9 ~15%). This latter fatty acid could be used as source of medium-chain FAs, which in Europe are currently derived totally from imported coconut and palm kernel oils. The possible substitution of the above-mentioned imported oils with domestic sourced oil is the main focus of the European Project COSMOS (Camelina and crambe Oil crops as Sources of Medium-chain Oils for Specialty), in which this thesis was partially involved. With the general aim of introducing the cultivation of camelina in Europe, the specific objective of this study was to address the effects of temperature on FA composition in camelina oil. The thesis consists of three chapters: the first aims to understand the impact of growing conditions on oil content and FA kinetics in developing camelina seeds; in the second, the expression levels of the genes involved in the FA metabolism of camelina developing seeds are

analyzed in response to different temperatures and with regards to differences in alleles of three camelina varieties; in the third chapter, the effect of "location" and "sowing date" on the composition of final camelina oil are thoroughly investigated.

In the first study, camelina was grown in parallel in open field (OF) and controlled environment (CE) conditions under three different ranges of post-anthesis temperature. Developing seeds were collected at fixed intervals and analyzed to determine the total oil content and the complete FA profile. The results showed a significantly higher content of oil in camelina seeds developed at higher temperatures in OF than that found in CE experiments, which is probably related to limiting conditions occurring inside the growth chamber (e.g. high air humidity, artificial lightening, absence of pollinators). Temperature strongly affected FA dynamics in camelina seeds; in particular, low temperatures decreased oleic (C18:1n9) and C18:2n6 contents, while increasing the amounts of C18:3n3 and C20:1n9. Moreover, a significant and positive correlation was found between C18:3n3 and C20:1n9. Since the effect of temperature on PUFAs is widely documented, while that on C20:1n9 still remains poorly investigated, a study on gene expression analyses was carried out on the principal genes involved

in its metabolism. The analysis was performed on developing camelina seeds grown under two different temperature ranges in a growth chamber. The results showed increased expression levels of *CsFAD2* and *CsFAD3* (the genes responsible for desaturation of C18:1n9 to C18:2n6 and C18:3n3, respectively) under lower temperatures, but differences in *CsFAE1* (genes responsible for elongation from C18:1n9 to C20:1n9) expression were not revealed. Moreover, in the DNA sequencing study, when comparing the DNA fragments of three camelina varieties grown in the same conditions, but characterized by different contents of C20:1n9, no significant differences in *CsFAE1* alleles were detected. The simultaneous increase of C18:3n3 and C20:1n9 under low temperatures might be related to a compensatory mechanism able to maintain stable the total amounts of monounsaturated FAs, by increasing the content of C20:1n9, when PUFAs are overproduced (i.e., C18:2n6 and C18:3n3). The definition of the "time frame" in which the main variations in FA kinetics occurred, permitted in the third study, to develop empirical relationships based on the final camelina oil quality in a multi-year and multi-location study (11 experimental sites in Europe and Canada) in relation to the mean T_{min} occurred during the "critical period". The identified "critical period" was

defined as the time of main variation for each of the principal FA. The adoption of this empirical model permitted early evaluations of the final camelina oil quality up to 40 days before harvest, with relevant implications for bio-based industry. These empirical relationships, between temperature and oil quality, when applied to camelina plants grown in the same environment (Bologna) across different sowing dates (ranging from early autumn to spring) in two growing seasons, resulted stronger (higher R^2) than those observed in the multi-location study. Furthermore, in the sowing date study the elevation of the linear regression found for C20:1n9 was higher than that found in the multi-location study, thus possibly confirming that the increased C20:1n9 content in camelina seeds might be related to anticipation of sowing in autumn. In fact, camelina sown in autumn was characterized by a prolonged crop cycle, determining increased accumulation of carbon in camelina seeds.

The improved knowledge of the relations between temperature and FA metabolism in camelina can increase understanding of the biochemical and physiological processes involved in their regulation, thus optimizing the agronomic management for this emerging and promising new oil crop for Europe.

Keywords: Fatty acid dynamics, enzymatic activity, gene expression, sequencing, seed filling phase, multi-location study, sowing date, eicosenoic acid, linoleic acid, linolenic acid

GENERAL INTRODUCTION

1 Roles of vegetable oils in a bio-based economy

Nowadays, our society is highly dependent on petroleum for the production of energy, fuels and raw materials required by the chemical industry. Since fossil-based products are finite sources, worldwide policy aims at replacing them with vegetable oils and other bio-based products. As new branch of the economy, the "bioeconomy", explores the uses of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and energy. In this evolving scenario, vegetable oils play an essential role in the production of energy (e.g. high-energy biofuels) and industrial feedstocks, as well as in human and animal diets. In fact, global vegetable fat production increased from 132 Mt in 2008/2009 (Metzger, 2009) to 202 Mt in 2016/2017 (USDA, September 12, 2017). In 2016/2017, soybean and palm-palm kernel oils represented respectively 35% and 31% of global oil production, followed by rapeseed (14%) and

sunflower (9%) (*fig. 1.1*). The growing interest in oil-crops is mainly due to their worldwide availability, adequate prices (Monteiro de Espinosa and Meier, 2011) and the possibility to be used for the production of surfactants, cosmetics, synthetic rubber, lubricants, plastics and other products. About 75% of global vegetable oil production is derived from common oil crops with a FA profile characterized by molecules having a length of 16 or 18 carbon atoms, containing from one to three *cis* double bonds (Ohlrogge and Browse, 1995). Common profiles mainly contain five fatty acids: C16:0, C18:0, C18:1, C18:2 and C18:3 (Carlsson et al., 2011). The remaining 25% of vegetable oils are derived from minor oilseeds with unusual FA profiles that are characterized by C-C double bonds in uncommon positions, functional groups and/or carbon chain length >20.

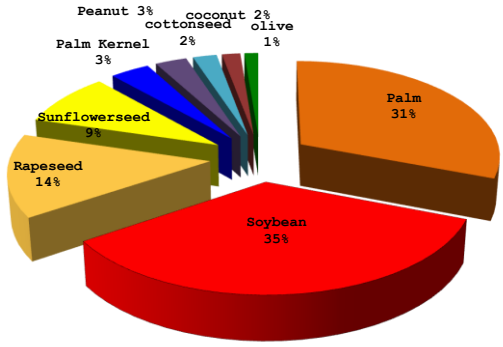


Figure 1.1 Global vegetable oil production in 2016-2017 (USDA, September 12, 2017)

2 The European Project COSMOS

The European Project COSMOS (Camelina and crambe Oil crops as Sources of Medium-chain Oils for Specialty oleochemicals, http://cosmos-h2020.eu/) started on March 2015 and will end on September 2019, and involves 18 partners from different European countries. The general aim of COSMOS is to limit the European dependence on imported coconut and palm kernel oils, as their cost is high and extremely volatile, by introducing *Camelina sativa* and *Crambe abyssinica* in European domestic cultivations. The interest in camelina and crambe is due to their high content of C20:1 (≈ 15%) and C22:1 (≈ 60%), respectively, which are long chain fatty acids with the double bond in an unusual position, suited for the chemical conversion to the MCFAs: C12:0 and C14:0. Given the low European acceptance for GMO products, the challenge of this project is to develop a value chain based on non-GMO oils. Oil extracted can be used as feedstock for flavor and fragrance precursors, high value polyamides and lubricants. Moreover, COSMOS foresees the valorization of the whole camelina and crambe biomass (pods, stems, leaves etc.) using an innovative "insect biorefinery", in order to produce valuable fats and proteins for animal and human consumption. The University

of Bologna collaborates with the Project by testing sustainable cultivation strategies for camelina and crambe in Italy. In particular, the effect of agricultural practices (e.g. crop rotation, density and date of sowing options) was tested on the yields and product qualities of selected varieties of crambe and camelina. The growing interest for camelina is due to its unique FA profile, good agronomic performance and wide environmental adaptability (Righini et al., 2016). The biological, morphological, agronomic and productive characteristics of camelina will be summarized in the next chapter.

3 *Camelina sativa*

Camelina (*Camelina sativa* L. Crantz), also known as Gold of Pleasure, False Flax, or linseed dodder, is native to the Mediterranean area. Camelina has been cultivated since the Early Bronze Age in Scandinavia (Karg, 2012) and became a fundamental part of human diet during the Iron Age (Zubr, 1997). In the Middle Ages, the importance of camelina declined as a food crop (Knorzer, 1978; Karg, 2012), even though its cultivation never disappeared in Eastern Europe. The renewed interest in *Camelina sativa* is highlighted by the increasing number of papers

published in the last years: 335 publications between 2013 and 2016 of which 149 since 2015 (Berti et al., 2016).

3.1 Biology and morphology

Camelina belongs to the *Brassicaceae* family (ex. *Cruciferae*). It is characterized by a short life cycle depending on environmental conditions, location, date of sowing (autumn or spring sowing) and spring or winter varieties. The reduction of growing cycle length can be due to dry and hot conditions. Camelina spring cultivars are able to complete their cycle in about 90 days (1200–1300 GDD, with a base temperature of 4 °C, Gesch and Cermak, 2011), whereas winter types need about 215 days (*Fig. 1.2*). The existence of both winter and spring varieties together with camelina high phenotypic plasticity allow camelina to grow in several environments.

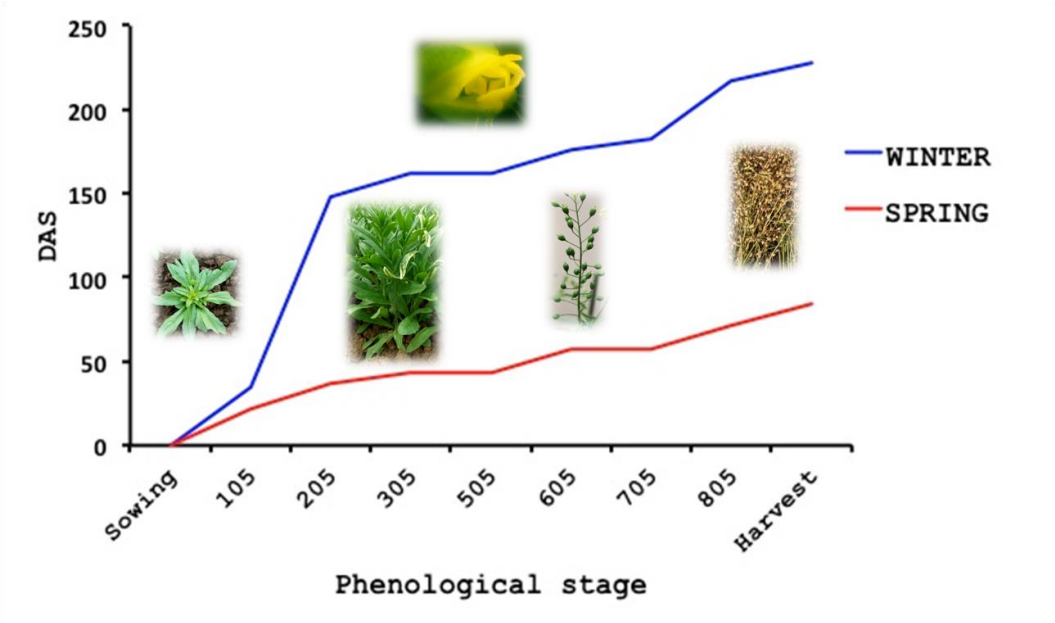


Figure 1.2 Principal growth stages of two Camelina sativa varieties, spring (Midas) and winter (Joelle), cultivated in Bologna during 2016-2017 growing season: 105, leaf development; 205, formation of side shoots; 305, stem elongation; 505, inflorescence emergence; 605, full flowering; 705, fruit development; 805, seed ripening

In fact, it is considered a crop that is tolerant to drought and low rainfall conditions (Hunsaker et al., 2011, Rodríguez-Rodríguez et al., 2013). Plants appear erect, with the main stem of about 90 cm height at full maturity and numerous lateral branches, which usually reach the same height. Height and total number of branches mainly depend on genotype, sowing date, plant density and environmental conditions during the growing cycle (*Fig. 1.3*).

Figure 1.3 Camelina different morphological traits: Winter varieties Luna (left) and Maczuca (right) grown in Bologna during the GS 2015-2016.

According to Masella et al. (2014), plant height and total number of branches diminish when delaying the date of sowing. Oblanceolate and short-stalked leaves are located alternate on subsequent nodes on the base of the main stem, whereas upper leaves are lanceolate and unstalked, *fig. 1.4a* (Martinelli and Galasso, 2011). Inflorescence, mainly autogamous, owns four pale yellow, spatulate petals of 4 to 5 mm, 4 erected sepals and six stamens divided in two groups of unequal length (Francis

and Warwick, 2009) (*Fig. 1.4b*). At each stem a variable number of silicles, also called seed capsules or pods, are attached (*Fig. 1.4c, 1.4d*).

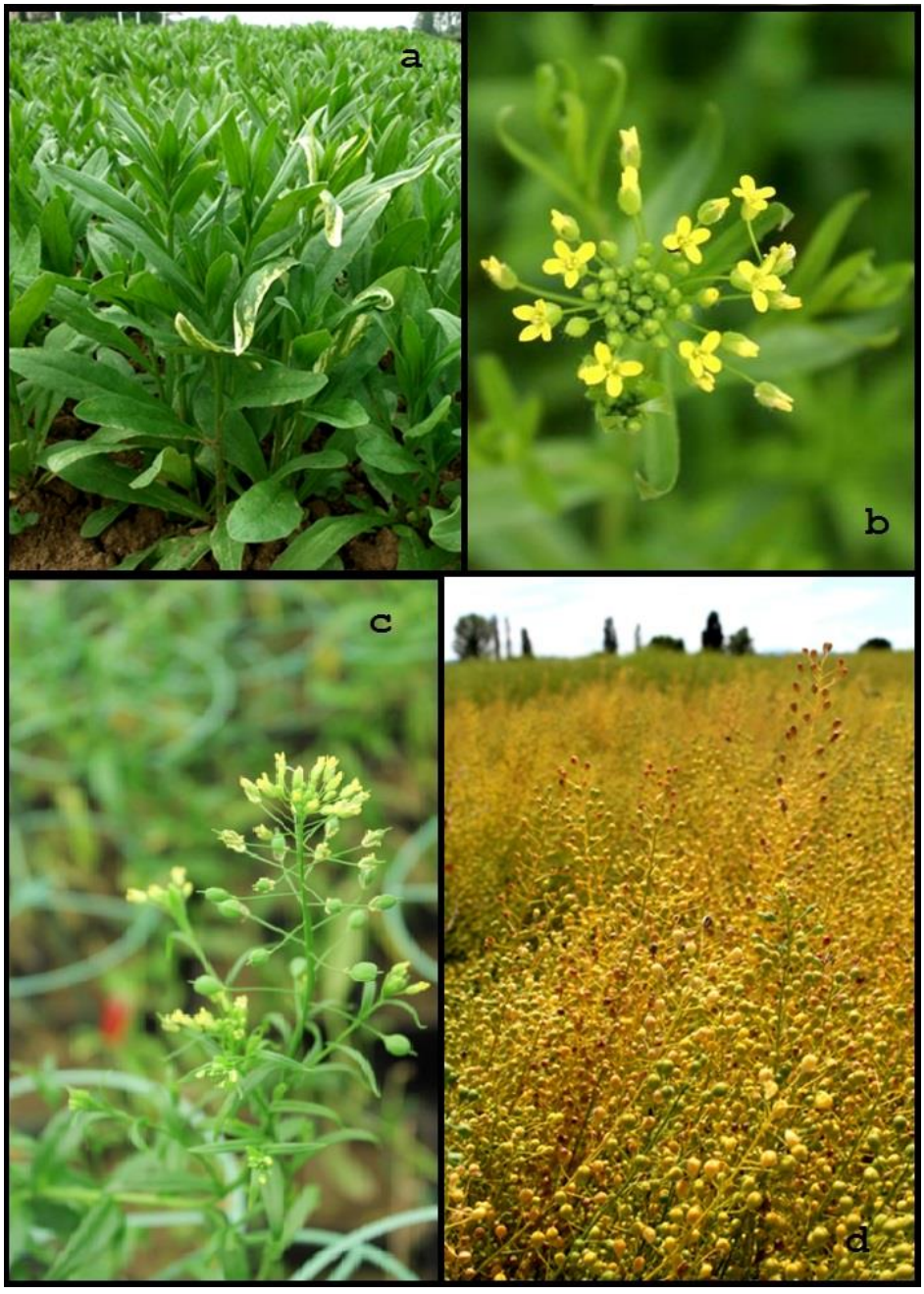


Figure 1.4 Camelina stem elongation, formation of leaves and side shoots (a), camelina yellow flowers during the flowering phase (b), developed of the first pods (c), camelina ripening phase in which pods turn from green to yellow-brown

Camelina pods are membrane partitioned and contain about 15-20 small, brown seeds of about 0.7 x 1.5 mm at maturity (*Fig. 1.5*).

Figure 1.5 Camelina mature seeds.

3.2 Agronomic management

3.2.1 Sowing

Optimal sowing dates of both spring and winter camelina biotypes depend on the location, environmental characteristics and oil uses. Gesch and Cermak (2011) and Berti et al. (2011) found, respectively in USA and Chile, that anticipation of the date of sowing in autumn leads to a significant increase in camelina seed yield due to the positive effects of milder temperatures during the ripening phase. Early sowing dates also lead to higher seed oil contents possibly due to the anticipation of flowering and, consequently, extension of the seed filling period (Gesch and Cermak, 2011). Delayed planting dates cause an increase in C18:1n9 and C18:2n6 contents and a parallel decrease in C18:3n3 (Pavlista et al., 2011). Commonly, camelina seeding rate varies between 4 and 6 kg ha^{-1}; seeding rates lower than 4 kg ha^{-1} resulted in reduced yields, while those higher than 6 kg ha^{-1} have not shown increased yields (Dobre et al., 2014). High levels of yield compensation due to the reduction of the plant stand at the rosette stage were demonstrated by McVay and Khan (2011). This high ability of camelina to maintain seed yield is due to increased branching, producing more pods and seeds per plant and increased

individual seed weight (Urbaniak et al., 2008; Berti et al., 2011). Among the sowing methods evaluated for camelina, there are broadcast and shallow drill seeding, which do not, however, show differences in seed yield. Currently, the recommended sowing depth is between 6 and 13 mm (McVay and Lamb, 2008). Nonetheless, the small seed size together with seed vigor can allow camelina to be sown deeper than expected.

3.2.2 Crop rotations and intercropping

The existence of winter and spring varieties together with the wide environmental adaptability and short growth cycle allow camelina integration in crop rotation (e.g. double cropping) and intercropping (relay cropping) systems. Double-cropping is defined as two crops grown on the same field within a year or as the seeding of a second crop, once the winter annual crop has been harvested (Berti et al., 2016). Differently, the relay cropping or relay intercropping is a method of multiple cropping, where a crop is planted into an already established crop. Recently, the high potential of camelina as a rotational crop has been documented in several studies mainly performed in North America (Lenssen et al., 2012; Gesch and Archer, 2013; Gesch et al., 2014; Berti et al., 2015). Gesch and Archer (2013)

found that yields of double-cropped soybean and sunflower with winter camelina are, respectively, 82% and 72% of their equivalent mono-crops, with higher economic returns when double-cropping was adopted. The agronomic viability of relay-cropping, a temporal crop intensification system of soybean with winter camelina, was reported by Gesch et al., 2014. Gesch and Johnson (2015) also highlighted the positive effects of camelina on subsequent crop seed yield.

3.2.3 Plant nutrition, disease and weed control

Although camelina is considered a low input crop, several studies have been conducted on its fertilization requirements. According to Berti et al. (2016), genotype, location and soil characteristics are the main factors influencing nitrogen (N) response. N fertilization has a positive effect on seed protein content, while it is negatively correlated with seed oil content. The optimal N dose ranges between 44 and 185 kg N ha^{-1}, resulting mainly in increasing number of branches and pods m^{-2} (Solis et al., 2013; Urbaniak et al., 2008; Wysocki et al., 2013). According to Solis et al. (2013), N rates exceeding 75 kg N ha^{-1} increase plant lodging and seed shattering. Different from other members of *Brassicaceae* family, camelina seed yield does not show response to

phosphorous and sulfur fertilization. Otherwise, camelina showed higher seed yield, seed protein and PUFAs content when 25 kg S ha^{-1} was applied (Jiang et al., 2014). Camelina is considered naturally resistant to several plant pathogens such as *Alternaria spp.* and *Leptosphaeria maculans*, probably due to the production of antimicrobial phytoalexins in leaves (Browne et al., 1991; Vollmann and Eynck, 2015). On the other hand, camelina was found to be sensitive to *Plasmodiophora brassicae woronin*, *Albugo candida* and *candidatus Phytoplasma asteris* (Vollmann and Eynck, 2015). Since camelina production of secondary metabolites constricts weed development (Lovett and Jackson, 1980), early planting and good stand establishment play a key role in the final production of camelina (Lenssen et al., 2012). In fact, camelina is not really competitive with some cool-season weeds such as *Bromus tectorum*, *Setaria viridis* and *Salsola kali* (Lenssen et al., 2012; Davis et al., 2013).

3.2.4 Harvesting

Uneven plant maturity together with seed shattering could allow considerable harvesting problems with high seed losses. Harvesting problems can be limited choosing optimal harvesting times. According to Berti et al.

(2016), early harvest (50% ripe silicles) resulted in 9.5% to 23.6% greater seed yield than mid harvest (70-80% ripe silicles) and late harvest (>90% ripe silicles). Camelina should be harvested when 75% of silicles are mature in order to achieve a balance between seed yield, seed oil content and acceptable loss due to shattering (Sintim et al., 2016). According to Gesch et al. (2014), similar seed yields are obtained if camelina is harvested by directly combining or swathing and then combining. After harvest, camelina seeds should be dried to about 80–100 g kg^{-1} moisture for good conservation, and then cleaned to obtain high quality seed (McVay and Lamb, 2008; Berti et al., 2016).

3.3 Productive performances and uses

Camelina seed yield, oil content and fatty acid composition are mainly affected by genotype and environmental conditions occurring among different locations and sowing times (Vollmann et al., 2007; Zubr, 1997). According to Gugel and Folk (2006) and Pavlista et al. (2016), the seed yield of camelina grown in good conditions can be up to 2.5-3.2 Mg ha^{-1}. According to the values reported in *tab. 1.1*, camelina oil content varies between 26% in the south to 43% in the north of Europe.

According to Zubr (2003), environmental factors such as temperature, solar radiation and precipitation play a key role on FA composition mainly due to the high content of polyunsaturated FAs (C18:2n6 and C18:3n3 > 50%), which are particularly affected by temperature. As for seed oil content, principal FA content also varies among different locations (*tab. 1.1*). In particular, C18:2n6 varies from a maximum of 20.3% in northern-central areas to 25.9 in southern Europe. Conversely, C18:3n3 varies from a maximum of 40.3 in colder conditions to 34.7 in warmer climates. A clear yellow color and a typical Broccoli-like aroma characterize Camelina oil. Despite its high PUFA content, the shelf-life of camelina oil is of 12-24 months due to its great content in αT (Berti et al., 2016). Chemical derivation of camelina oil has been mainly limited to biofuel (e.g. biodiesel, jet fuel), albeit monomers, resins and sensitive adhesives have also been produced. Unlike the other members of the *Brassicaceae* family, camelina seed meal (cold-pressed cake or solvent extracted meal) is characterized by a low level of glucosinolates (10-40 μmol g^{-1}, Gugel and Falk, 2006), a high level of high-quality proteins (45%), soluble carbohydrates (10%) and fiber, allowing its use as animal feed.

Table 1.1 Seed yield (Mg ha^{-1}), oil content (%), principal FAs content (%) of camelina grown in different localities of northern, central and southern Europe. [1]Kirkhus et al. (2013); [2]Zubr, 1997; [3]Vollmann et al. (2007), [4] Šípalová et al. (2011); [5]Angelini et al. (1997)

Location	Seed yield	Oil content	FAs							Ref
	Mg ha^{-1}	%	C16:0 %	C18:0 %	C18:1n-9 %	C18:2n-6 %	C18:3n-3 %	C20:1n-9 %	C22:1n-9 %	
Norway	1.5-2.0	40-42	5.1-5.4	2.4-2.5	12.5-13.7	15.3-16.5	36.4-38.6	14.7-15.1	2.6-3.0	[1]
Denmark	1.27-2.36	42-45	5.3-5.6	2.2-2.7	14.7-16.5	12.9-16.3	40.3-33.7	14.7-15.1	2.5-3.0	[2]
Austria	1.85	43.7	6.29	2.73	16.50	17.73	32.55	15.60	3.15	[3]
Czech Republic	/	37.0-41.2	6.9-11.0	2.8-3.3	12.8-16.3	18.2-20.3	32.9-37.5	15.2-16.4	/	[4]
Italy	/	23.6 - 27.5	/	/	12.8-15.1	18.7-25.9	25.5-34.7	11.2-15.6	/	[5]

Unfortunately, camelina meal contains high amounts of sinapine, an alkaloidal amine responsible for bitter taste, which causes a disagreeable taste in milk and meat from cows and calves fed on it (Colombini et al., 2014). However, additional non-food uses have been investigated for camelina seed meal (e.g. nanocomposite materials, monomers, alkyd resins and pressure sensitive adhesives).

4 Lipid metabolism and FA kinetics regulation

Lipids are mainly found as phospholipids and triglycerides in all plant cells, representing 5-10% of total dry weight (Ohlrogge and Browse, 1995). In particular, phospholipids are the major constituents of cell membranes, exploiting functions of protection and regulation. Otherwise, triglycerides are accumulated in the endoplasmic reticulum (ER) with the main function of energy reserve. Moreover, lipids can be found outside the cells in the cuticular wax on the surface of all terrestrial plants. FA biosynthesis involves all plant cells and is essential to determine final the FA composition. Lipid biosynthesis, as described by Li-Beisson et al. (2013), is a complex mechanism that requires a large number of enzymatic reactions, occurring at the same time, and more than 600 genes that encode proteins and regulatory factors (*Figure 1.7*). The entire process of lipid biosynthesis can be divided in three main stages (Li-Beisson et al., 2013):

I. Plastid de novo FA synthesis
II. Membrane desaturases and elongases
III. Kennedy pathway: TAG biosynthesis

Considering all environmental factors, temperature has the greatest effects on FA kinetics. However, other environmental (e.g. solar radiation and precipitation) and genetic factors play a role on FA biosynthesis.

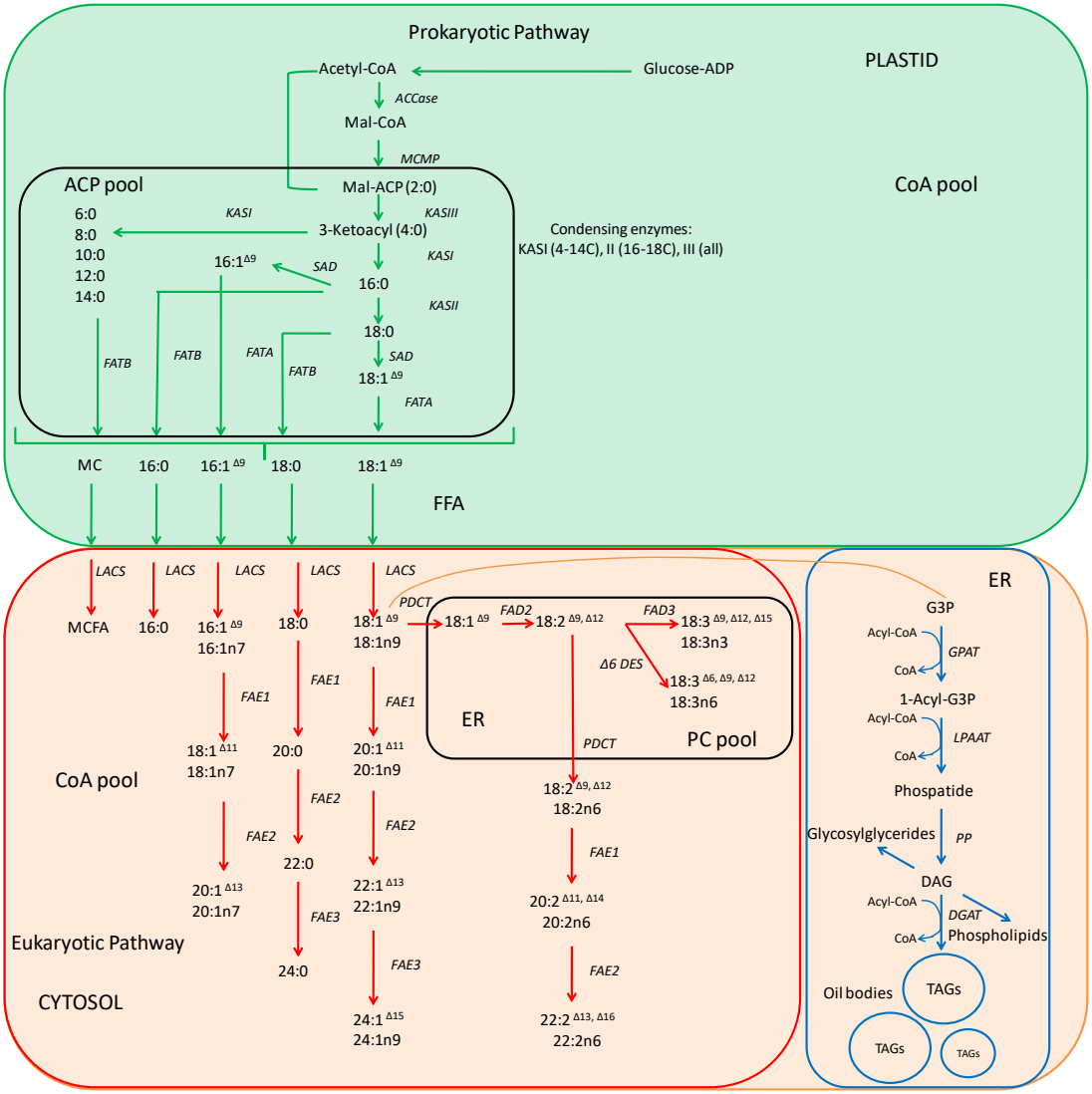


Figure 1.6. Camelina FA kinetics in cell plastids, prokaryotic pathway (green upper part), in cytosol and ER, eukaryotic pathway (orange)

4.1 Plastid de novo FA synthesis

FA synthesis in plants, different from other eukaryotes, takes place in plastids, determining the level of saturated FAs and carbon chain length. The metabolic pathway is different among plant species and genotypes. Acetyl-coenzyme A (CoA) stored into plastids represents the "carbon source" of FA metabolism; its origin depends on plant species, variety and plastids (e.g. chloroplasts or protoplasts). In most seeds, carbon is delivered to FA synthesis via glycolysis with hexose and/or triose as the predominant carbohydrate entering the plastid (Bates et al., 2013). An alternative pathway takes place in plants with green seeds (e.g. *Brassicaceae* family), which can also use light to produce NADPH and ATP, bypassing glycolysis. The second case is more efficient, with 20% more acetyl-CoA available for oil synthesis (Bates et al., 2013). Acetyl-CoA is used as starting point with CO_2 to produce malonyl-CoA in plastid de novo FA synthesis. This reaction is catalyzed by acetyl-CoA carboxylase (ACCase) through two steps using the CoA pool as a cofactor. Many studies have shown that ACCase activity is strictly light-dependent. In particular, ACCase carboxylation, and consequently de novo FA synthesis, occur only during light hours. Before fatty acid

elongation, the malonyl group is transferred from CoA to acyl carrier protein pool (ACP) by malonyl-CoA:acyl carrier protein malonyltransferase (MCMP), on which all FA assembly reactions take place. Fatty acid synthase (FAS), a complex of monofunctional enzymes, is responsible for the production of 16:0 and 18:0-ACP, starting from acetyl-CoA and malonyl-ACP, and the latter provides two-carbon units at each step. Seven cycles are needed for production of 16:0-ACP, including four reactions at each step: condensation, reduction, dehydration and reduction. Three condensing enzymes of the FAS complex, also known as 3-ketoacyl-ACP synthases (KASI, KASII and KASIII), are needed for FA synthesis. KASIII catalyzes the first reaction of acetyl-CoA and malonyl-ACP to form 3-ketoacyl-ACP with 4-carbon units. In the next six cycles, condensation is catalyzed by KAS isoform I, which form 16:0-ACP. At the end, KASII is responsible for elongation of 16:0-ACP to 18:0-ACP. A double bond is introduced on 16:0 and 18:0-ACP by stearoyl–ACP Δ9-desaturase (SAD), a soluble acyl-acyl carrier protein desaturase found exclusively in the stroma of the plastids, forming, respectively, $16:1^{\Delta 9}$-ACP and $18:1^{\Delta 9}$-ACP. The activity of acyl-ACP thioesterases A and B, FATA and FATB, leads to the formation of FFA in the plastids. Monounsaturated fatty acids $16:1^{\Delta 9}$-ACP and

18:1$^{\Delta 9}$-ACP are mainly hydrolyzed by FATA, whereas all saturated ACP FAs by FATB. Final products are exported from the plastid to the cytoplasm and immediately esterified with CoA by the activity of long-acyl-CoA-synthetases (LACS). FAs exported take part in the "eukaryotic" pathway of lipid synthesis whereas FAs retained in the plastid are used for the "prokaryotic" pathway.

4.2 Membrane desaturases and elongases

The next step of FA biosynthesis is set into the ER and the cytoplasm, and involves a series of desaturation and elongation reactions. In the ER, fatty acyl-CoAs are incorporated into the phosphatidylcholine (PC) pool by LPCAT, on which desaturation activities take place. Unlike SAD, membrane desaturases are associated with the ER and chloroplasts, leading to the production of C18:2n6 and C18:3n3 from C18:1n9. Oleate desaturases retain the fundamental role of wall fluidity regulation and interchange between cells and external environment. Moreover, it has been demonstrated that at low temperatures, polyunsaturated membranes are indispensable for maintaining plant viability and cellular function (Ohlrogge and Browse, 1995). The most active membrane

desaturases in plants are Δ12 oleate desaturase (FAD2) and Δ15 linoleate desaturase (FAD3), which form, respectively, C18:2n6 and C18:3n3. FAD2 and FAD3 desaturation occurs via aerobic mechanisms, in which molecular oxygen is reduced to water by 4 H^+. Hydrogen atoms can be derived from FA substrates or some reductants as NADH, NADH-cytochrome-b5 and cytochrome-b5 (Rodríguez-Rodríguez et al., 2016). Temperature is considered the main factor that influences desaturase activity in ER; in particular, it affects the unsaturation ratio (linoleic/oleic ratio), which is always higher at low temperatures (Martínez-Force et al., 1998). Although the molecular mechanisms on which temperature affects FA kinetics are not well documented, many hypotheses have been made over the years. Harris and James (1969) suggested that at low temperatures the solubility of the oxygen, acting as a co-substrate, increases, with a consequent growth of the total desaturase activity. According to Brenner (1984), the level of unsaturation is controlled by mechanisms of adjustment of membrane fluidity in plant cells. It has long been known that at low temperatures membrane fluidity declines, increasing instead at growing temperatures (Los and Murata, 2004). At low temperatures, plant cells activate desaturase enzymes in order to

increase the amount of unsaturated FAs and consequently membrane fluidity, leading to cell survival at colder temperatures. According to Zhang et al., 2005, drought and salt stresses also decrease the amount of linolenic acid contained within plant leaves. As for de novo FA synthesis in plastids, fatty acyl-CoAs are elongated by reiteratively adding two atoms of carbon, derived from the condensation of malonyl-CoA with acyl-CoAs, in the ER. Elongation of C16 and C18 Acyl-CoAs is catalyzed by fatty acid elongation complex (FAE), which produces C20-C24 molecules. Very Long Chain FAs (VLCFAs) can be synthesized in both leaves as precursors of cuticular waxes and in seeds, in which acyl-CoAs are the precursors of substances of reserve. Although elongase activity is highly influenced by ACCase, the activity occurs only in light conditions, although VLCFAs can be produced with low intensity in the dark (Bao et al., 1998).

4.3 Kennedy pathway: TAG biosynthesis

Triglycerides or triacylglycerols (TAGs) are compact molecules composed of tri-esters of glycerol with fatty acids. TAGs are about 95% of total lipids contained in seed oils, representing the principal reserve of energy and carbon storage. The assembly of TAGs, also named the

Kennedy pathway or *glycerol phosphate pathway*, take place in the ER. The *Kennedy pathway* involves the glycerol-3-phosphate (G3P) and acyl-CoA pool, derived by glycolysis and FA synthesis, respectively, into four main steps:

I. G3P is acylated on the *sn*-1 position by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT)
II. A second acylation is catalyzed by the enzyme 2-lysophosphatidic acid acyltransferase (LPAAT), which adds an acyl-CoA on the *sn*-2 position to form phosphatidic acid (PA);
III. PA is then phosphorylated by phosphatidate phosphatase (PP), producing diglycerides or diacylglycerols (DAGs). DAGs are fundamental molecules, since they represent the precursors for production of both TAGs and membrane lipids.
IV. The third acylation involves the enzyme diacylglycerol acyltransferase (DGAT), which adds an acyl-CoA at the *sn*-3 position to form TAGs.

The selectivity of GPAT, LPAAT and DGAT for the different fatty acids in the acyl-CoA pool determines diversification in TAG composition and, consequently, their different properties. Once synthesized, TAGs are accumulated in many organelles named “oil bodies” or “lipid droplets” (Li-Beisson et al., 2013). Since oil bodies are surrounded by phospholipid monolayer membranes

with the hydrophilic part facing outwards, they never combine with each other. Kennedy pathway represents the last process of FA biosynthesis. According to Izquierdo et al. (2016), as for membrane desaturases, temperature affects the distribution of FAs on TAGs molecules. In particular, growing temperatures determine a more symmetrical distribution of saturated fatty acids, increasing the content of saturated-unsaturated-saturated oil species.

Below the major steps of FA kinetics within the environmental factor involved in the regulation are summarized in *tab. 1.2.*

Table 1.2 List of enzymes involved in lipid metabolism and relative effects of environmental factors as documented by [a]Ohlrogge and Browse, 1995, [b]Rodríguez-Rodríguez et al., 2016, [c]Zhang et al., 2005, [d]Izquierdo et al., 2016

Multi-enzyme complex	**Enzymes**	**Role**	**Environmental regulation**
PDHC	α, β - PDH DHLAT LPD	Oxydative decarboxylation of pyruvate to produce Acetyl Co-A, CO_2 and NADH	
FAS	ACCase	Formation of malonyl-CoA from acetyl-CoA	light-dark cycles[a]
	MCMP	Transfer of the malonyl group from CoA to ACP	
	KASI	2-14C acyl-ACPs for condensation with malonyl-ACP for making 6-16 C FAs	
	KASII	Chain lengthening of 16C to 18C and control their final ratio	
	KASIII	Initial condensation reaction using acetyl-CoA and malonyl-ACP	
	SAD	Introduction of a double bond in 18:0-ACP to produce 18:1-ACP	

	FATA	Hydrolysis of 18:1-ACP to 18:1 FFA	
	FATB	Hydrolysis of 16:0 and 18:0 ACP to 16:0 and 18:0 FFA	
	ACS	Esterification of FFA with CoA pool	
	PDCT	Transfer of 18:1 into PC pool and from PC to diacylglicerol	
FAD	FAD2	Introduction of a double bond in 18:1-CP to produce 18:2-CP	Light-dark cycles[a], positively effects of low temperatures on FAD2 and FAD3 activity[b]. High drought and salt stress negatively affect the final amount of of C18:3[c]
	FAD3	Introduction of a double bond in 18:2-CP to produce 18:3-CP	
FAE	KCS	Condensation of malonyl-CoA with fatty acyl-CoA to produce β-ketoacyl-CoA	
	KCR	Reduction of β-ketoacyl-CoA to β-hydroxyacyl-CoA	
	HCD	Dehydration of β-hydroxyacyl-CoA to enoyl-CoA	
	ECR	Reduction of enoyl-CoA to acyl-CoA	
	GPAT	Acylation of G3P at the *sn*-1 position	Cold temperature induces differences in fatty acyl-CoA attached to *sn*-2 position, more linoleate than oleate. More symmetrical distribution of saturated FA with increasing temperature (sat-unsat-sat)[d]

5 Effect of temperature on FA kinetics

Among all environmental factors, temperature during seed ripening phase holds the main role in determining the final FA composition. Since the final oil value is strictly dependent on its qualitative characteristics, understanding variations in FA composition in relation with temperature becomes extremely important. The relationship between seed yield, seed oil content and/or FA composition with temperature in different oil crops has been extensively studied (*Tab. 1.3*).

Many studies took place in field trials (about 60%), in wide geographical areas worldwide, with different environmental conditions. About 30% of these studies were performed in controlled environment (CE) conditions, so that only the effect of temperature was taken into account. Since the effect of temperature is higher in plants with PUFA profiles, more than three-quarters of authors have studied sunflower (39 studies), rapeseed (23), camelina (22) and soybean (12), which are characterized by a high content of PUFAs.

Table 1.3 Literature reporting the effect of mean, minimum, maximum, day and night temperature on oil yield, oil content and FA composition in the seed oil from different crops.

Temperature	Experiment	Plant	Author (year)
day, (Tmax-Tmin)	OF	soy	Song et al. (2016)
day, mean	OF	rapeseed	Walton et al. (1999)
day, night	OF, CE	sunflower	Harris et al. (1978)
heat treatments	CE	arabidopsis	Kaplan et al. (2004)
heat treatments	CE	rapeseed	Aksouh et al. (2001)
heat treatments	CE	rapeseed	Aksouh-Harradj et al. (2006)
max	Model	camelina, rapeseed, soy, sunflower	Schulte et al. (2013)
mean	CE	flax	Dybing and Zimmerman (1966)
mean	CE	rapeseed	Iqbal et al. (2011)
mean	CE	rapeseed	Wilmer et al. (1996)
mean	CE	rapeseed	Wilmer et al. (1997)
mean	CE	rapeseed	Namazkar et al. (2016)
mean	CE	soy	Wolf et al. (1982)
mean	CE	soy	Dornbos and Mullen (1992)
mean	CE	soy	Heppard et al. (1996)
mean	CE	soy	Gibson and Mullen (1996)
mean	CE	soy	Pipolo et al. (2004)
mean	CE	soy	Byfield and Upchurch (2007)
mean	CE	sunflower	Garcés et al. (1992)
mean	CE	sunflower	Garcés et al. (1994)
mean	CE	sunflower	Martínez-Force (1998)
mean	CE	sunflower	García-Diaz et al. (2002)
mean	CE	sunflower	Fernández-Moya et al. (2002)
mean	CE	sunflower	Sánchez-García et al. (2004)
mean	CE	sunflower	Serrano-Vega et al. (2005)
mean	CE	sunflower	Rolletschek et al. (2007)
mean	CE	sunflower, castor, flax	Harris and James (1969)
mean	CE	sunflower, rape	Trémolières et al. (1982)
mean	CE	rapeseed, safflower, sunflower, flax, castor	Canvin (1965)
mean	CE	rape, white mustard	Yaniv et al. (1995)
mean	OF	camelina	Gugel and Folk (2006)
mean	OF	camelina	Vollmann et al. (2007)
mean	OF	camelina	Gesch and Cermak (2011)
mean	OF	camelina	Berti et al. (2011)
mean	OF	camelina	Kirkhus et al. (2013)
mean	OF	camelina	Gesch and Archer (2013)
mean	OF	camelina	Pecchia et al. (2014)
mean	OF	camelina	Gesch (2014)
mean	OF	camelina	Sintim et al. (2016)
mean	OF	crambe	Vollmann and Ruckenbauer (1993)
mean	OF	crambe	Fontana et al. (1998)
mean	OF	crambe	Zanetti et al. (2016)
mean	OF	flax	Mirshekari et al. (2012)

mean	OF	olive tree	Fuentes de Mendoza et al. (2013)
mean	OF	olive tree	García-Inza et al. (2014)
mean	OF	olive tree	García-Inza et al. (2016)
mean	OF	rapeseed	Omidi et al. (2010)
mean	OF	rapeseed	Weymann et al. (2015)
mean	OF	soy	Piper and Boote (1999)
mean	OF	sunflower	Nagao and Yamazaki (1984)
mean	OF	sunflower	Lajara et al. (1990)
mean	OF	sunflower	Flagella et al. (2002)
mean	OF	sunflower	Barros et al. (2004)
mean	OF	sunflower	Roche et al. (2006)
mean	OF	sunflower	Zlatanov et al. (2009)
mean	OF	sunflower	Turhan et al (2010)
mean	OF	sunflower	Gesch and Johnson (2013)
mean	OF	sunflower	Piao et al. (2014)
mean	OF	camelina, rapeseed, brown mustard	Pavlista et al. (2011)
mean	OF	camelina, soy	Gesch et al. (2014)
mean	OF	rapeseed, soy, sunflow	Werteker et al. (2010)
mean	OF	camelina, rapeseed, brown mustard	Pavlista et al. (2012)
mean	OF, CE	camelina	Jiang (2013)
mean	CE	curly cale	Steindal et al. (2015)
min	Model	sunflower	Pereyra-Irujo and Aguirrezábal (2007)
min	Model	rape	Gilardelli et al. (2016)
min	OF	sunflower	Seiler (1986)
min	OF	sunflower	Grunvald et al. (2013)
min	OF	crambe, ethiopian and brown mustard, rapeseed	Zanetti et al. (2006)
min	Model	rapeseed	Baux et al. (2013)
min	Model	sunflower	Pereyra-Irujo et al. (2009)
min	OF	rapeseed	Baux et al. (2008)
min night	Model	sunflower	Izquierdo et al. (2006)
min night	OF	sunflower	Echarte et al. (2010)
min night	OF, CE	sunflower	Izquierdo and Aguirrezabal (2008)
min, max	OF	camelina	Zanetti et al. (2017)
min, max	OF	olive tree	Rondanini et al. (2014)
min, max	OF	camelina, cress, coriandrum	Angelini et al. (1997)
min, max	OF	crambe, rapeseed, field mustard	Adamsen and Coffelt (2005)
min, mean, max	OF	borage	Gilbertson et al. (2014)
min, mean, max	Model	rapeseed	Habekotté (1997)
min, mean, max	OF	sunflower	Qadir et al. (2006)
min, mean, max	OF	sunflower	Neto et al. (2016)
night	CE	sunflower	Pleite et al. (2008)
night	CE	sunflower	Izquierdo et al. (2013)
night	CE	sunflower	Izquierdo et al. (2016)
night	OF	sunflower	Izquierdo et al. (2002)
not specified	OF	camelina	Zubr and Matthäus (2002)
not specified	OF	camelina	Zubr (2003)
not specified	OF	camelina	Schillinger et al. (2012)
not specified	OF	camelina	Guy et al. (2014)
not specified	OF	camelina	Pavlista et al. (2016)
not specified	OF	crambe	Laghetti et al. (1995)
not specified	OF	crambe	Wang et al. (2000)
not specified	OF	soy	Hemingway et al. (2015)
not specified	OF	sunflower	Zheljazkov et al. (2011)
not specified	OF	sunflower	Zheljazkov et al. (2012)
not specified	OF	camelina, rape, brown mustard	Aiken et al. (2015)
thermal time	Model	sunflower	Durruty et al. (2016)

Most studies (about 60%) were conducted by considering the mean daily temperature. Interestingly, during the past 14 years, the effects of minimum and night temperatures on FA composition have been taken into account (Baux et al., 2013; Echarte et al., 2010; Izquierdo et al., 2016). Baux et al. (2008) and Grunvald et al. (2013) reported that the minimum temperature, occurring at the seed ripening phase, was the best indicator of final FA composition in rapeseed and sunflower oil, respectively. According to Izquierdo et al. (2002), light or metabolite associated with the day-night cycle can affect the activity of enzymes involved in FA synthesis in sunflower seeds. Moreover, night temperature affects sunflower oil properties through its effect on FA synthesis and on the distribution of FAs on TAGs (Izquierdo et al., 2016). Recently, on the basis of the relation between night or minimum temperature and fatty acid biosynthesis, models have been developed to predict the final oil composition of sunflower and rapeseed (Izquierdo et al., 2006; Pereyra-Irujo and Aguirrezábal, 2007: Pereyra-Irujo et al., 2009; Baux et al., 2013; Gilardelli et al., 2016).

OBJECTIVE

The major objective of the present study is to investigate thoroughly the effects of temperature occurring at seed filling phase on FA composition of camelina seed oil. Temperature has often been considered one of the main factors influencing PUFA biosynthesis in oil crops, but little is still known about its effects on eicosenoic acid (C20:1n9). For the first time, C20:1n9 accumulation dynamics at different temperature ranges have been investigated in depth, bringing new knowledge not only to the complex metabolism of FA but also in defining proper agronomic crop management for camelina. The effects of temperature on camelina oil quality have been studied at different levels, covering agronomic studies as well as biochemical investigations on the mechanisms of FA dynamics.

The present study falls within the European Project COSMOS (Grant Agreement No. 635405) aimed at introducing the cultivation of *camelina and crambe* in Europe.

CHAPTER 1: Impact of growing conditions on oil content and fatty acid metabolism in developing camelina seeds

1.1 Preliminary concepts

FA composition varies highly during camelina seed filling phase, leading to significant modifications in final oil quality. Although at this stage the enzymatic reactions related to biosynthesis of FAs and lipids occur together in plant cells from plastids to the ER, the rate of accumulation of each FA is higher in certain moments because of competition between the activity of different enzymes and substrate availability. With the general aim of improving the understanding of the effects of temperature on camelina oil quality, this chapter is focused on identifying the main changes in FA composition on camelina immature seeds developed under different ranges of temperature. In all experiments, Camelina var. Midas (Linnaeus Plant Sciences, Saskatoon, Canada) was used. Interestingly, although Midas is a spring variety, in Italy (Bologna) it can be successfully grown as winter

crop. Before starting all experiments, in either open field (OF) or controlled environment (CE), an appropriate oil extraction method was established in terms of time (duration of analysis), reagents and seed consumption. Thereafter, a preliminary study was established in order to determine the period, expressed in growing degree day after flowering GDD-AF (for GDD-AF calculation see par. 1.3.1), in which the most significant variations in FA composition in camelina developing seeds occurred. Camelina plants were grown in OF and CE, aiming at distinguishing the effects of temperature from that of the overall growing environment.

1.2 Establishing of an appropriate oil extraction method on immature camelina seeds

Lipid extractions were performed using 1 g of camelina seeds (n=3). Three different extraction methods were tested: Folch modified Boselli (Boselli et al., 2001), Folch modified Christie (Christie, 1989) and Hara and Radin (Hara and Radin, 1978). They were selected considering the small amount of available immature seeds. Extracted camelina oil was then characterized with quantitative (lipid chromatograms) and qualitative (TLC)

analyses. Laboratory analyses were performed at the Department of Agricultural and Food Sciences, University of Bologna. The description of the statistical analysis performed and results obtained on these preliminary studies are reported in the appendix 1 and 2, respectively.

1.2.1 Oil characterization

1.2.1.1 Thin Layer Chromatography (TLC)

TLC analyses were performed to qualitatively evaluate the oil composition of camelina seeds. Oil, extracted with the three methods, was dried, added to chloroform and loaded on a TLC plate covered with silica gel (highly polar, stationary phase). Standard solutions: αT, tristearin (TG), βST, 1,3 dipalmitin (DG) and cholesterol (Chol) were also added. The Plate was moved into a glass container, in which hexane/ethylene mixture (non-polar mobile phase, 65:35, v.v.) was added. The container was left at 4 °C, where the mobile phase drawn up the plate via capillary action, moving the different components depending on their polarity. As soon as the solvent mixture finished the run, the plate was removed, dried and splashed with phosphomolybdic acid to detect dark spots at different layers. Oil components were determined

by comparing the dark spots of the oil samples with those of standard solutions.

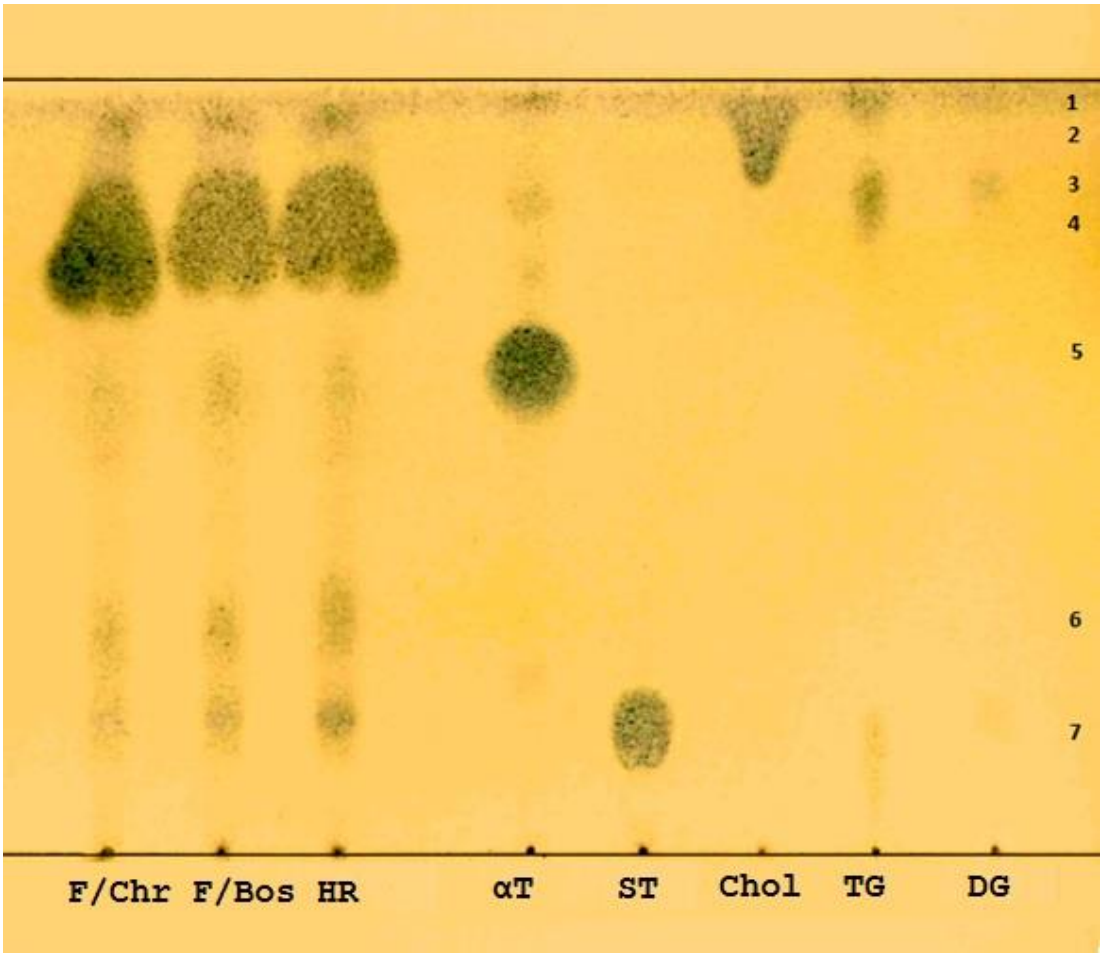


Fig. 1.1 TLC of camelina seed oil extracted with the methods: Folch modified Christie (F/Chr), Folch modified Boselli (F/Bos) and Hara and Radin (HR). Layers: 1, hydrocarbons; 2, Chol; 3, DGs; 4, TG; 5, αTs; 6, FFAs; 7, βSTs.

The principal components of camelina oil were: TGs, DGs, αTs and STs (fig. 1.1). Tocopherols content in camelina oil was also investigated by Zubr and Matthäus (2002),

who found that γT was predominant (651-922 ppm) followed by αT, δT and P8 (15-20 ppm).

1.2.1.2 Lipid chromatograms

To quantify camelina oil components, an appropriate quantity of lipids extracted with the three methods was added to hexane, obtaining a solution of about 20 mg/ml. Lipids were then injected in a gas-chromatography apparatus with a fused silica low-polarity capillary column SE52 (10 m length, 0.25 mm I.D., 0.1 µm film thickness). The stationary phase was 5% difenil-95% dimethyl-polysiloxane with a starting temperature of 100 °C and an increase of 5 °C/min until reaching 350 °C; the latter temperature was maintained for 30 min. The different oil components were revealed with a flame ionization detector (FID). In the resulting lipid chromatograms (*fig. 1.2*) FFAs, STs, DGs and TGs were discriminated by their different retention times.

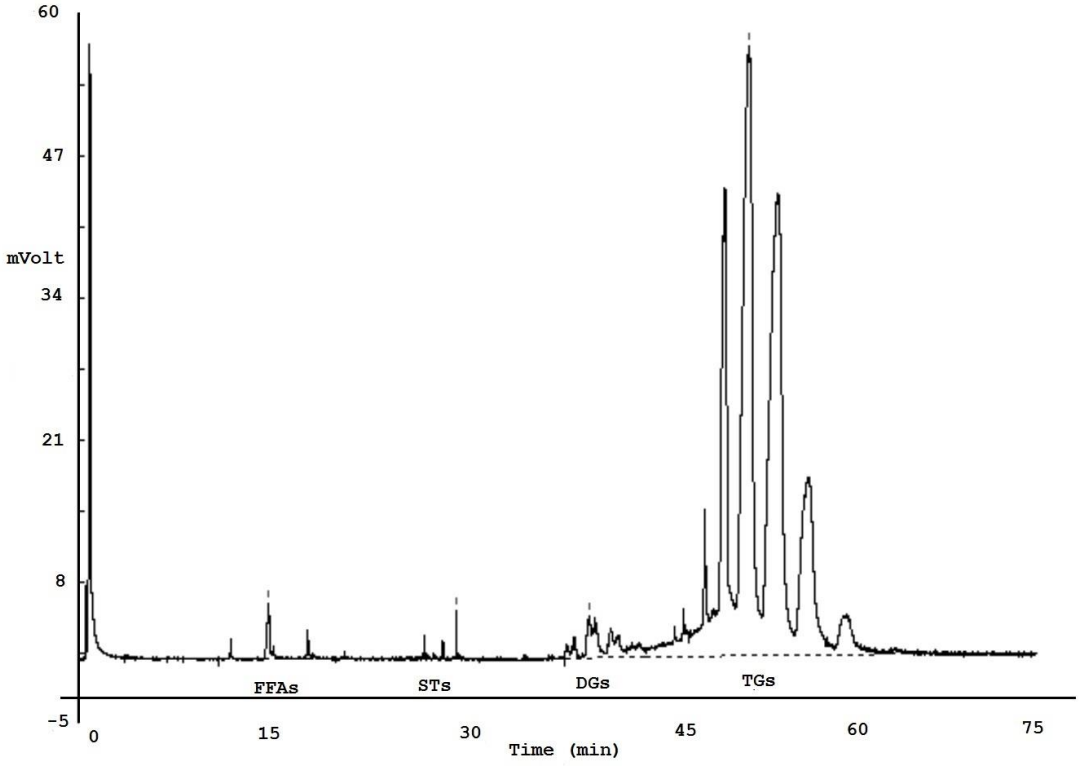


Figure 1.2 Example of lipid chromatogram of camelina oil extracted with Boselli (2001) method. Starting from left: the FFA, ST, DG and TG groups of peaks.

1.3 Materials and methods

1.3.1 Open Field trials

OF trials took place during the 2015/16 growing season at the experimental farm of the University of Bologna, set in Cadriano Italy (44°33'N, 11°23'E). This site is characterized by silty loam soil (*table 1.1*). Midas was sown in 2015 at three different sowing dates: in spring (04.01.2015, OF1) and in autumn (10.26.2015, OF2; 10.09.2015, OF3). The same sowing density (500 seeds/m^2), plot surface (10 m^2) and row distance (0.15 m) were adopted in each trial. The experimental design was complete randomized blocks with three replications.

Table 1.1 Soil characteristics (0.5 m soil layer).

Soil characteristics	Value
Loam (%)	46
Sand (%)	22
Clay (%)	32
Organic matter (%)	1.56
pH	7.4
N (g kg^{-1})	1.17
P_2O_5 (mg kg^{-1})	116
K_2O (mg kg^{-1})	122

Both irrigation and pesticide applications were not necessary, while weed control and N fertilization (50 kg of N/ha at stem elongation phase) were manually performed. Daily minimum (T_{min}), maximum (T_{max}) and mean (T_{mean}) temperatures were collected by a meteorological station located near the experimental fields. The GDD were used to obviate difficulties due to differences in the time of development among the different conditions (OF1, OF2 and OF3) considered. For each trial, the GDD were calculated, as follows:

$GDD = \Sigma[(T_{max}-T_{min})/2-T_{base}]$

Where T_{max} and T_{min} are daily maximum and minimum air temperature, respectively, and T_{base} is the base temperature for which a value of 5°C was adopted, as reported by Gesch, 2014. All immature seeds contained in the first six basal pods of the main stem of 15 plants were randomly sampled in each plot at the same GDD-AF (154, 210, 290, 350, 448, 546) in each experiment. Seeds (*fig. 1.3*) were collected into vials, weighed and stored at -80 °C until laboratory analysis.

Figure 1.3 Midas immature seeds during camelina ripening phase.

1.3.2 Weather conditions

The experimental site is characterized by a mean annual cumulative precipitation of 613 mm and mean annual temperature of 13.4 °C. Daily mean temperatures occurred in OF1 growing season, GS (*fig. 1.4*) were in line with the average long-term values (2003-2014) for the site, even though lower temperatures were registered between May 20 and 31, while higher temperatures occurred until June 15 (post-anthesis period). Mean daily temperatures occurred during OF2 and OF3 GSs (*fig. 1.4*) were higher in the first half of April compared to mean historical values. Precipitation during OF2 and OF3 GSs was similar to the average values of the reference period, reaching 491 and 426 mm, respectively (*figure 1.4*). However, Midas sown in autumn (OF2 and OF3) was affected by an uncommonly dry period between December 2015 and February 2016.

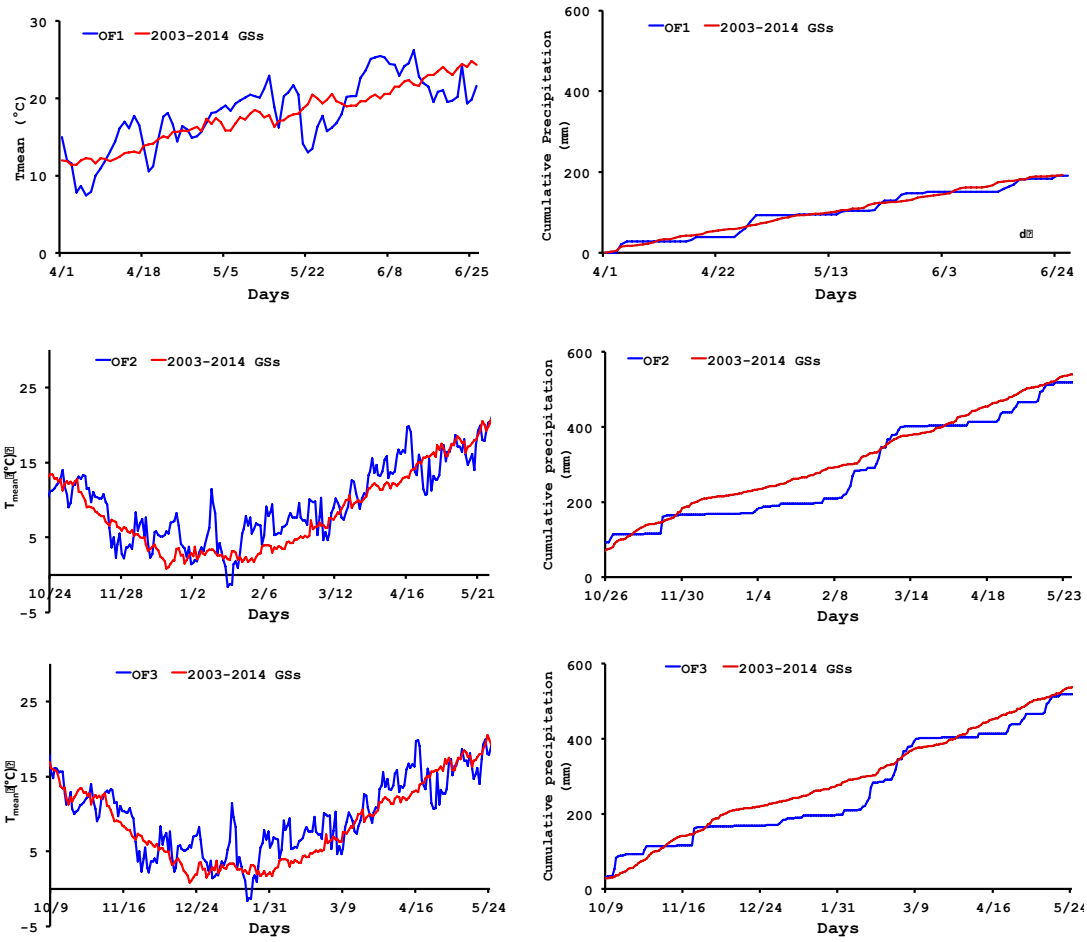


Figure 1.4 Daily mean temperature (left) and daily cumulative precipitation (right) for OF1, OF2 and OF3 (blue lines) compared with the mean daily values for Cadriano between 2003 and 2014 (red lines).

1.3.3 Controlled environment experiments

Three different CE experiments were carried out from August 2015 to June 2016. Midas plants were grown in square-shaped pots (0.11 x 0.11 m) filled with sandy soil (sand, 71%, loam, 19%, clay, 10%). Five seeds were sown

Figure 1.5 Midas plants grown in the growth chamber after the start of flowering.

at 0.005 m depth in the center of each pot. Seedling emergence was surveyed at 7 (60%) and 12 days after sowing (98%). Seventeen and 30 days after sowing, young plants were thinned, respectively, at two and finally at one plant per pot. Pots were placed in a greenhouse until the start of flowering; during this period, NPK fertilization (20-20-20 NPK) was applied every two weeks, while phytosanitary treatments were not performed. As soon as the first flower opened (defined as the start of flowering), pots were moved into a growth chamber (*fig. 1.5*) of 4 m^2 set with three different day-night temperature ranges in each of the three experimental conditions: 24-14 °C (CE1), 20-9 °C (CE2) and 14-4 °C (CE3). Temperature ranges were chosen to mimic the mean day-night temperatures registered in the last 10 years at Cadriano during the ripening phase of the corresponding OF trials (OF1, OF2 and OF3). Pots were moved and irrigated manually three times a week to maintain camelina plants under well-

watered conditions. Photoperiod (14/10 hours day/night), humidity, and light intensity were maintained constant during the three experiments. A weather station T-monitor/ZT was used for monitoring the environmental conditions inside the growth chamber (T_{min}, T_{max}, T_{mean}, humidity). All seeds contained in the first 8 basal pods of 30 plants (10 plants for each replicate) were sampled at the same GDD-AF of the OF trials and then stored at -80 °C until analyses.

1.3.4 Analytical methods

1.3.4.1 Seed water content

The residual moisture content of seeds was determined as reported by Rodríguez-Rodríguez et al. (2013).

1.3.4.2 Oil content

The lipid fraction was extracted with a modified version of Hara and Radin method by adding hexane/isopropanol (3:2, v.v.) to camelina seeds and homogenized with an ULTRA-TURRAX (mod. T25, IKA, Germany). The sample was centrifuged and the upper phase collected. This procedure was repeated three times by re-suspending seeds in hexane/isopropanol and collecting the suspension. Non-lipids were removed by mixing the suspension with aqueous

sodium sulfate for 10 minutes in a separating funnel; the aqueous solution was obtained from 1 g of the anhydrous salt and 15 ml of water. Lipid phase was added to sodium sulfate anhydrous and left at 4 °C. After three hours, the extracted was filtered, dried with the rotavapor and N flux and weighed to determine the total oil extracted. The oil was stored at -20 °C.

1.3.4.3 FA characterization

FAs were transmethylated to the corresponding FAMEs with potassium hydroxide:water:methanol (KOH-MeOH-Water), after the addition of nonadecanoic acid (C19:0) as an internal standard (IS). Total FAs were characterized with a Thermo-Finnigan, 8000 series (Thermoquest, Milano, Italy) with a FID and a fused silica Restek (Restek Corporation, U.S., Bellefonte, PA) capillary column (105 m length; 0,25 mm I.D.; 0,20 µm film thickness) using as stationary phase 88-Cyanopropil-Aryl-Xyloxan. Helium (He) was used as carrier gas at a constant flow of 0.75 mL min^{-1}. The injector and detector temperatures were 250 °C, the starting oven temperature was 100 °C, increasing at 3 °C min^{-1} until 180 °C. The oven was maintained at 180 °C for 10 minutes, after which the temperature was increased by 3 °C min^{-1} until it reached 240 °C, which was maintained for 30 minutes. The injection split ratio

was 1:67. The reference standard 463 (NU-CHEK, MN, U.S.A.) was injected three times in order to recognize the different peaks corresponding to each FA (*fig. 1.6*) and calculate the correction factor (K), which allowed to align the response of the detector for each FA.

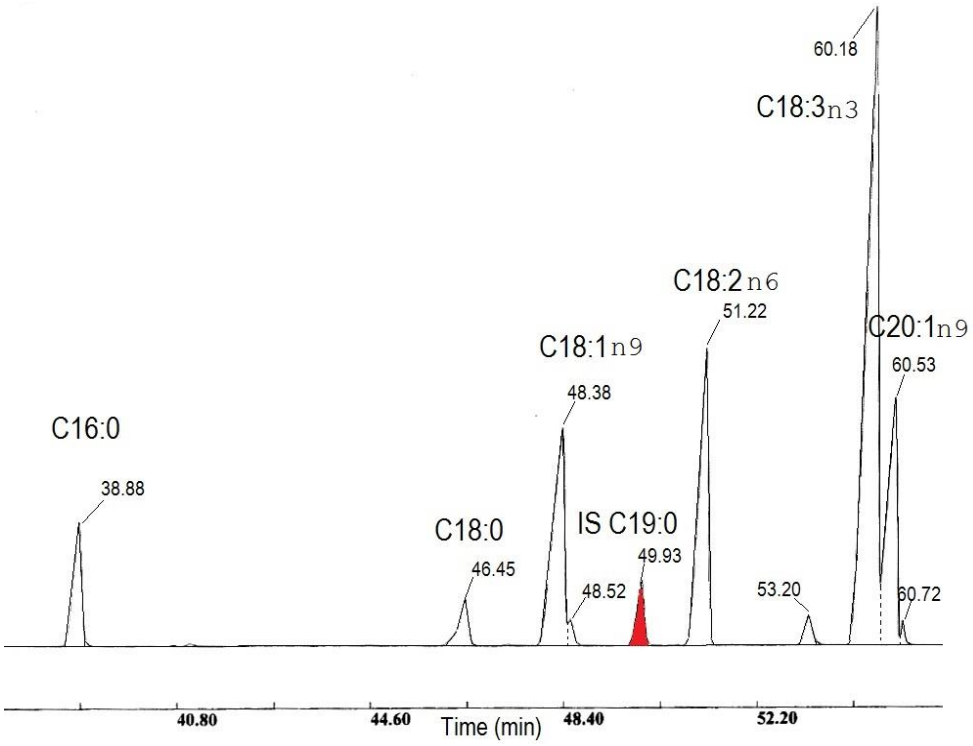


Figure 1.6 Example of FA chromatogram of Midas developing seeds in which peaks of the principal FAs (C16:0, C18:0, C18:1n9, C18:2n6, C18:3n3, C20:1n9) and of the internal standard (C19:0, red) are reported with the corresponding retention times.

1.3.5 Rate of reaction

The rate of reaction, defined as the concentration of substrate consumed (or product produced) per unit of time by enzyme activity, was calculated for each of the principal enzymes involved in camelina FA metabolism. This parameter was determined by adding all FAs (%) produced by enzyme activity at each developmental stage (*Fig 1.7*).

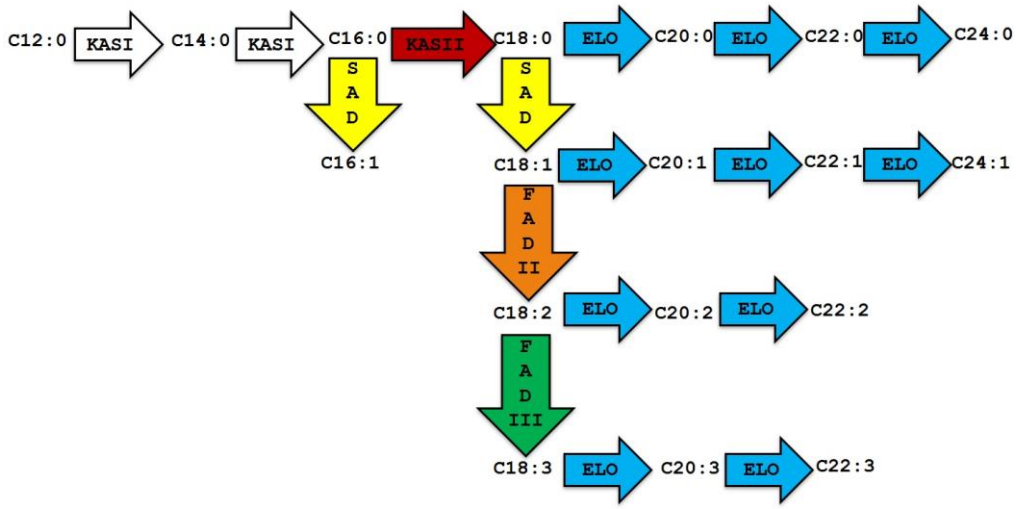


Figure 1.7 Simplified scheme of FA accumulation kinetics in camelina seeds, in which the enzymes are shown: KASI (Σ FAs > C12), KASII (Σ FAs > C16), SAD (Σ unsaturated FAs), FAD2 (Σ PUFAs), FAD3 (Σ ω3 FAs), ELO (VLCFAs)

Through differentiation of the rate of reaction functions, the derivative graphs for FAD3 and ELO

reaction rate (%) were calculated using Origin 9.0 software.

1.3.6 Statistical analysis

Prior to ANOVA, the homoscedasticity of variance was verified with Bartlett's Test. To analyze seed oil content, one-way ANOVA was performed by first comparing growing conditions separately (OF vs. CE) at each GDD-AF; a second one-way ANOVA was performed comparing the two growing environments (CE1 vs. OF1, CE2 vs. OF2, CE3 vs. OF3) within the same temperature range, and considering the sampling date (GDD-AF) as a random effect; finally, a further one-way ANOVA was performed to compare, within each sampling date (GDD-AF) and temperature, the growing conditions (at 154 GDD-AF CE1 vs. OF1, CE2 vs. OF2, CE3 vs. OF3, etc.). The results of principal FA contents were analyzed with one-way ANOVA for the OF and CE conditions separately, comparing within each environment the three temperature ranges at each GDD-AF. Finally, for MUFA/PUFA, C20-24/C16-18 and n3/n6 ratios, a two-way ANOVA was run separately for OF and CE experiments, considering as 1st factor the "temperature range" and as 2nd factor the "GDD-AF". LSD test was adopted to separate means when the analyses of variance revealed statistical

differences ($P \leq 0.05$). ANOVA analyses were performed with CoStat 6.3 Software (CoHort Software, Monterey, CA, USA).

Principal component analysis (PCA) was run on principal FAs (C18:1n9, C18:2n6, C18:3n3 and C20:1n9) and T_{min} occurred at the final sampling date (540 GDD-AF) in both CE and OF trials separately. The analysis was performed with the Software JMP (SAS Institute Inc.). Principal components were presented when their eigenvalue was ≥ 1.

1.4 Results

The first experiment (OF1) was used to identify the "temporal frame", expressed in GDD-AF, in which the main changes in principal FA composition occurred. FA accumulation dynamics in camelina developing seeds are represented in *fig. 1.8*. The main variations in FA composition started at about 154 GDD-AF, in which C16:0, C18:1n9 and C18:2n6 contents dropped, while C18:3n3 and C20:1n9 increased ($P \leq 0.05$). After 540 GDD-AF, the FA composition of camelina was almost stable without any significant variation; at that time only variations in the total fat content of the seed is normally occurring.

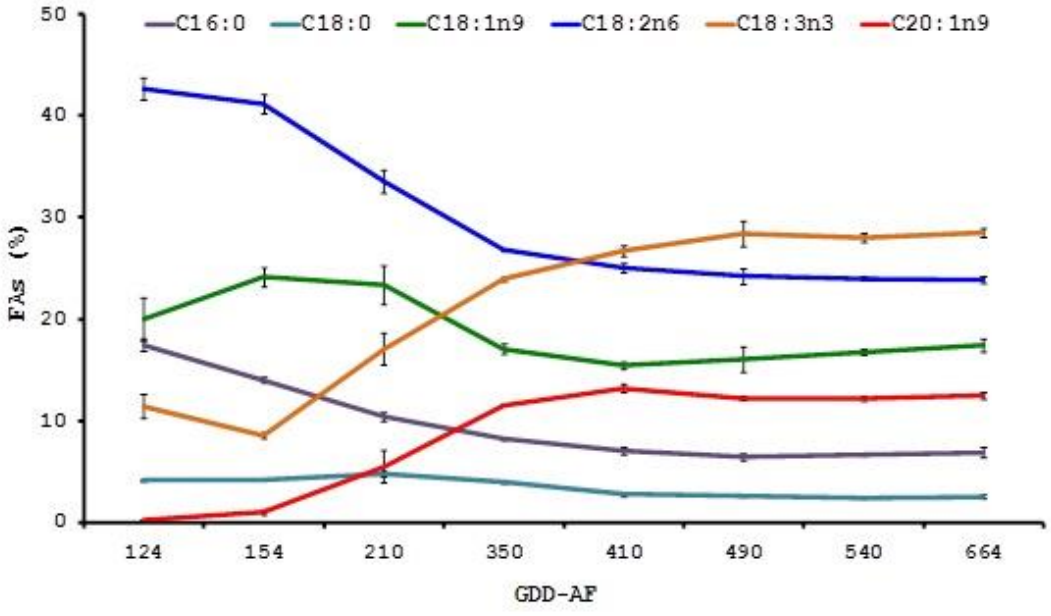


Figure 1.8 Accumulation kinetics of the principal FA contained in camelina var. Midas developing seeds in spring 2015 (OF1 trial)

at different GDD-AF: 124, 154, 210, 350, 410, 490, 540, 664. Vertical bars: standard deviation

In the "temporal frame", identified between 154 and 540 GDD-AF, all samples of both OF and CE experiments were performed at the same GDD.

When comparing oil accumulated (%) in CE conditions (*fig. 1.9a*), at 450 and 540 GDD-AF significant differences emerged between CE3 and the other two CE experiments, with the latter showing lower oil contents of about 50% compared to CE3 (*P≤0.001*). On the other hand, seed oil content in OF1 at 540 GDD was significantly higher (about 20%) than that of OF2 and OF3 conditions (*P≤0.01*), *fig. 1.9b*.

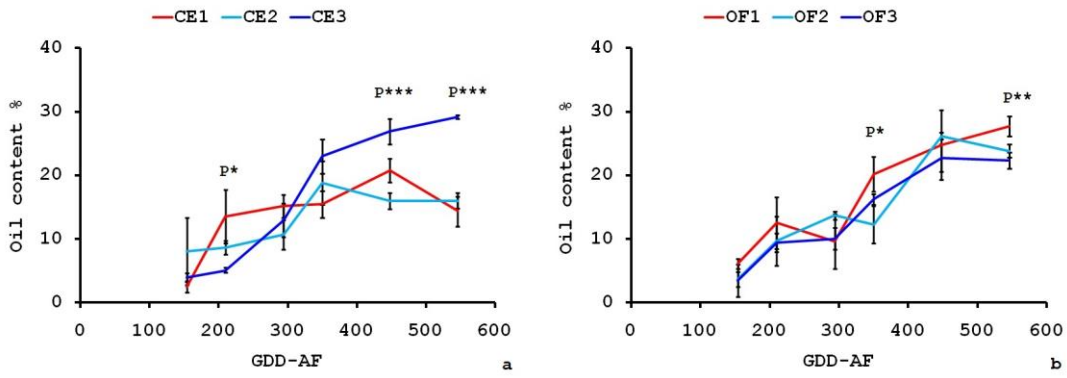


Figure 1.9 Oil content (%) in camelina developing seeds at different GDD-AF, in OF (a) and CE (b) conditions. P = P≤0.05, P** = P≤0.01, P*** = P≤0.001. Vertical bars: Standard Deviation.*

Oil accumulated (%) in camelina developing seeds significantly differed between CE1 and OF1 at 154 and 540 GDD-AF, in which CE1 oil was about 40% and 50% of OF1 oil, respectively ($P \leq 0.01$). Plants grown in OF2 were also able to produce significantly more oil (about 40%) than that produced in CE2 conditions, at 450 and 540 GDD-AF ($P \leq 0.01$). On the other hand, between CE3 and OF3 there was a significant higher amount of oil (%) at 154 (about 50%), 350 (about 30%) and 540 GDD-AF (about 30%) in controlled environment conditions. In both OF and CE trials, seed oil content was much lower than that normally found in camelina mature seeds, which usually varies between 26% to 43% (Righini et al., 2016).

C18:1n9, C18:2n6, C18:3n3 and C20:1n9 kinetics in developing camelina seeds was affected by the different growing conditions in both OF and CE experiments. At higher temperatures, after 350 GDD-AF, C18:1n9 (%) was significantly higher ($P \leq 0.05$) in OF1 than in OF2 and OF3 conditions. In CE conditions, C18:1n9 content was only slightly different between experiments and developmental stages. Otherwise, since 350 GDD-AF C18:2n6 (%) was significantly higher in seeds filled at higher temperatures in OF, OF1 ($P \leq 0.05$) and CE, CE1 and CE2, ($P \leq 0.05$). On the other hand, at 350 and 540 GDD-AF C18:3n3 and C20:1n9 (%) were significantly lower at

higher temperatures in OF, OF3, ($P \leq 0.05$) and at 458 and 540 GDD-AF in CE conditions, CE3 ($P \leq 0.05$).

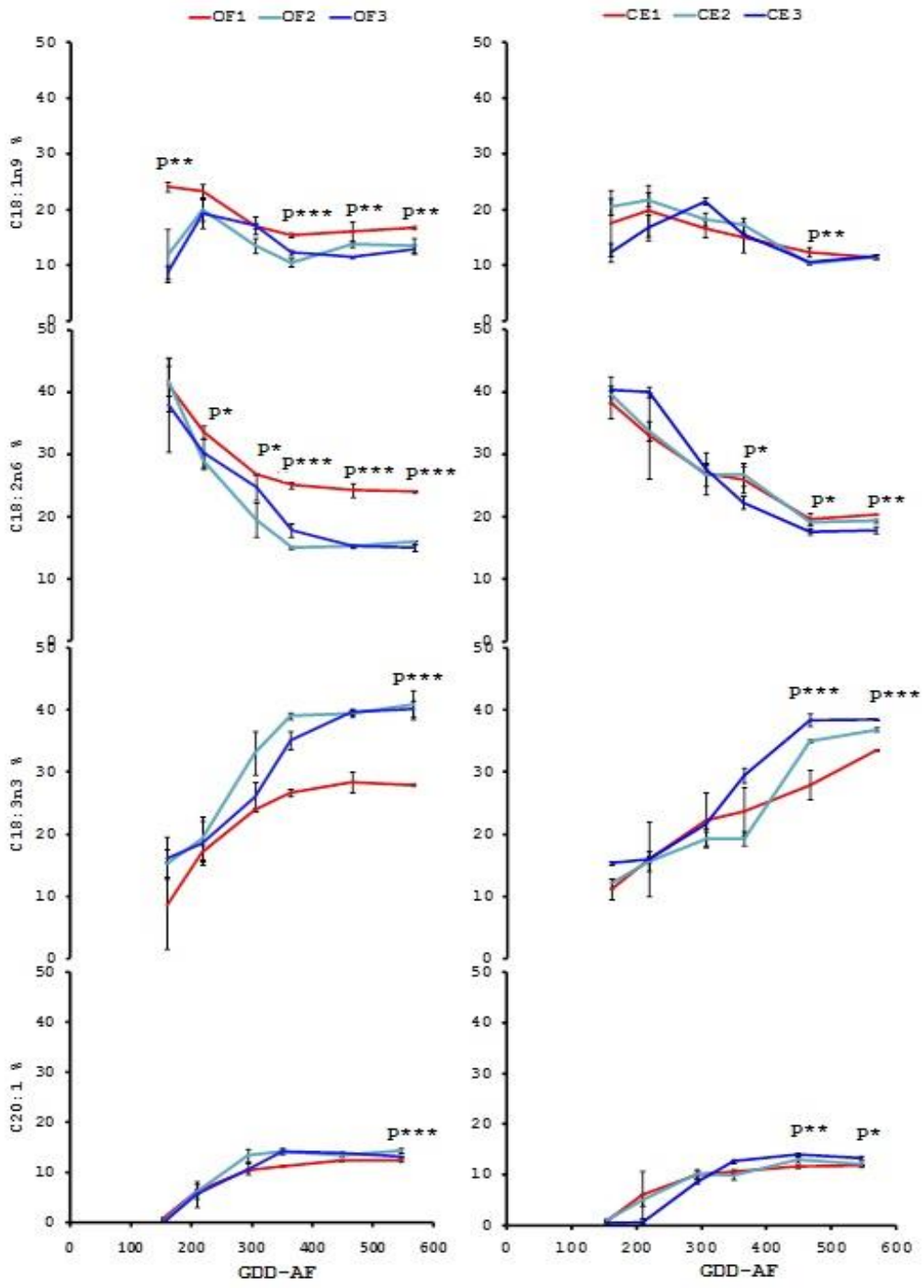
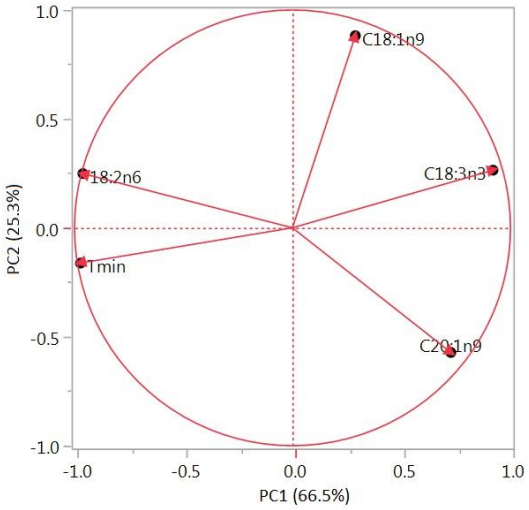


Figure 1.10 FA accumulation kinetics (%) of the four principal FAs (C18:1, C18:2n6, C18:3n3, C20:1n9) contained in developing

Midas seeds at different GDD-AF in OF and CE conditions. Vertical bars: standard deviation.

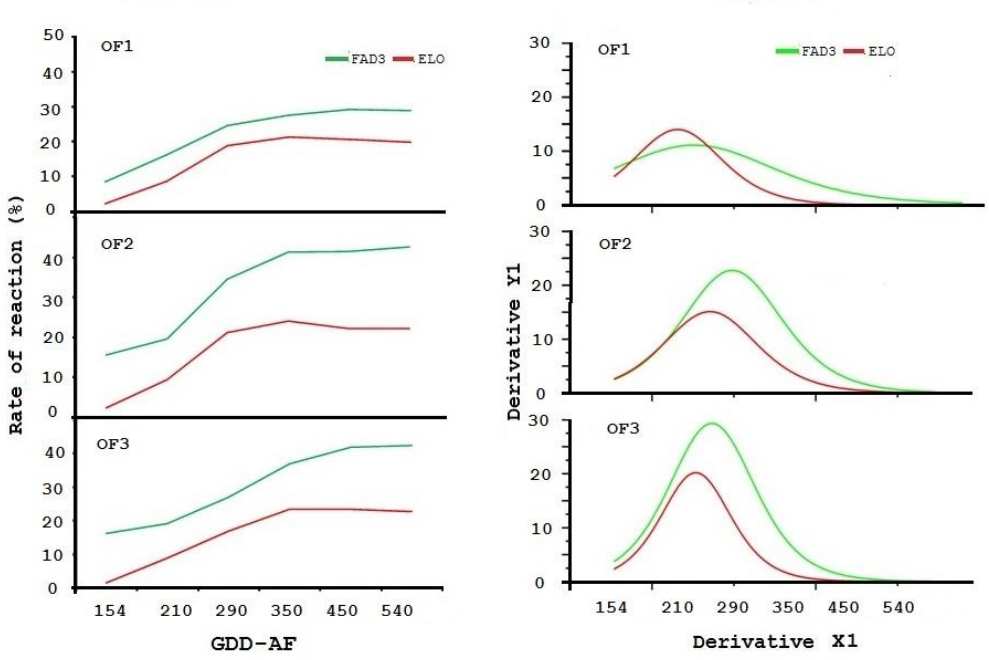
Multivariate analysis (PCA) was run on both OF and CE trials, unfortunately in the OF trials only one component (PC1) reported an eigenvalue ≥ 1, and thus plotting the results was not possible. In the CE trials, PCA covered the 91.8 % of the total variance, identifying two principal components: PC1 and PC2 (*fig. 1.11, tab. 1.2*). PC1 explained the majority of data variance (66.6%) and was correlated (correlation coefficient > 0.5) with C18:2n6, C18:3n3 and T_{min}. The PC2 accounted for 25.2% of variance and was correlated to C18:1n9 and C20:1n9 (*tab 1.2*). On the basis of the correlation vectors, positive correlations were found between C18:2n6 and T_{min} (PC1) and negative correlations were found between C18:3n3 and C18:2n6, C18:3n3 and T_{min} (PC1). Negative correlations were also found between C18:1n9 and C20:1n9.



	PC1	PC2
Tmin	-0.53305	-0.14398
C18:1n9	0.15817	0.78460
C18:2n6	-0.52704	0.22139
C18:3n3	0.50431	0.23537
C20:1n9	0.39843	-0.50918

Left: Figure 1.11 Variables factor map (PCA) for Midas sampled at 540 GDD-AF seeds in CE experiments. Right: Table 1.2 PCA factor loadings table

To further investigate the positive relationship occurred between C18:3n3 and C20:1n9, the reaction rates of FAD3 and ELO enzymes (%) in OF1, OF2 and OF3 conditions are compared in *fig. 1.12*. The reaction rates showed a plateau for both enzymes, starting between 350 (ELO) and 450 GDD-AF (FAD3). The derivatives shown in *fig. 1.13* represented both the velocity and peak of reaction of the FAD3 and ELO reaction rates in OF conditions.



Left: *figure 1.12 Rates of reaction (%)* of FAD3 (green lines) and *ELO (red lines) in OF1, OF2, OF3 trials. Right: figure 1.13* D*erivative* of the rates of reaction *of FAD3 (green lines) and ELO* (red lines) in *OF1, OF2 and OF3 trials*

The results in *fig 1.13* showed an earlier interruption in ELO activity with respect to the FAD3 activity in all the OF trials. The FAD3 peak of reaction was higher respect to that of ELO in seeds developed at lower temperatures (OF2 and OF3), but lower in seeds developed under milder conditions (OF1).

Two-way ANOVA results on n3/n6, PUFA/MUFA and C20-24/C16-18 are reported in *tab. 1.3* and *1.4*. ANOVA showed significant differences among conditions ($P \leq 0.05$) and

GDD-AF in OF and CE ($P \leq 0.05$). Interestingly, the interaction "COND x GDD-AF" was significant for the n3/n6 ratio in OF and CE experiments ($P \leq 0.05$), while for PUFA/MUFA and C20-24/C16-18 ratios only in CE experiments the interaction COND x GDD-AF was significant (PUFA/MUFA, $P \leq 0.05$; C20-24/C16-18, $P \leq 0.05$).

Table 1.3 Main effect: "condition" for n3/n6, PUFA/MUFA, C20-24/C16-18 values (%) for Midas seeds developed in CE and OF conditions. Standard Deviation < 10% in all conditions.

Conditions	n3/n6	PUFA/MUFA	C20-24/C16-18
CE1	0.88**c**	1.81**b**	0.22**a**
CE2	0.99**b**	1.87**b**	0.21**a**
CE3	1.11**a**	2.19**a**	0.17**b**
OF1	0.81**c**	1.74**b**	0.18**b**
OF2	1.58**a**	2.34**a**	0.22**a**
OF3	1.41**b**	2.27**a**	0.21**a**

Table 1.4 Main effect: "GDD-AF" for n3/n6, PUFA/MUFA, C20-24/C16-18 averaged values (%) for Midas seeds developed in CE and OF conditions. Standard Deviation < 10% in all GDD-AF.

GDD-AF	CE			OF		
	n3/n6	PUFA/MUFA	C20-24/C16-18	n3/n6	PUFA/MUFA	C20-24/C16-18
154	0.32**e**	2.46**a**	0.03**e**	0.34**e**	3.59**a**	0.02**e**
210	0.46**d**	2.04**bc**	0.08**d**	0.59**d**	1.75**b**	0.10**d**
294	0.76**c**	1.59**e**	0.20**c**	1.15**c**	1.71**b**	0.24**c**
350	1.08**b**	1.72**de**	0.27**b**	1.69**b**	1.83**b**	0.30**a**
448	1.63**a**	1.86**cd**	0.34**a**	1.94**a**	2.00**b**	0.26**bc**
540	1.72**a**	2.08**b**	0.28**b**	1.92**a**	1.81**b**	0.28**ab**

The n3/n6 ratio resulted significantly higher at latter developmental stage (high GDD-AF) in both OF and CE conditions, indicating an increase in n3 FA content (i.e., C18:3n3). Moreover, n3 FA content resulted significantly lower at higher temperatures (CE1 and OF1, *fig. 1.10*). As for n3/n6, PUFA/MUFA ratio was higher in plants grown under lower temperatures (CE3 and OF3), while C20-24/C16-18 ratio was higher at lower temperature in CE (CE3) and at higher temperature in OF (OF1). Moreover, C20-24/C16-18 ratio significantly increased with the GDD in both CE and OF conditions.

1.5 Discussion

The three tested extraction methods (Boselli et al., 2001; Christie, 1989; Hara and Radin, 1978) reported similar results for oil extracted (%), FA composition (%) and oil components (%). The Hara and Radin method was preferred to the others as it was faster, less expensive and because the solvent used (hexane-isopropanol) had lower levels of toxicity. Although no differences were found in TAGs and DAGs (%) among the tested methods, in general quicker extractions are preferred as they lead to lower levels of TAG hydrolysis and consequently to lower levels of DAG production (unwanted compounds). As expected, extracted oil (%) in developing Midas seeds, within the "temporal frame" in both OF and CE experiments, was lower than that found in camelina mature seeds. In fact, between 150 and 540 GDD-AF camelina seeds were not at their physiological maturity stage, which is recognized to be at about 43 days after flowering, DAF (i.e., corresponding at 645 GDD-AF) in camelina varieties grown at 25-15 °C day-night (Rodríguez-Rodríguez et al., 2013). The GDD-AF required for Midas plants to reach the final FA composition (%) was the same under different CE and OF conditions (540 GDD-AF), and as expected shorter periods (DAF) were observed for plants grown at higher

temperatures. In particular, 540 GDD-AF corresponded in OF1 at 33 DAF, compared to the 54 DAF (OF2) and 60 DAF (OF3). The increased GDD accumulation in shorter periods, under higher temperatures, leading to plants nearer to maturity, in terms of DAF, might explain the higher amount of oil accumulating (%) in OF1 vs. OF2 and OF3. The significant higher oil content (%) in Midas seeds grown in OF1 and OF2 than those grown in CE1 and CE2 conditions respectively, could be related to some limiting conditions possibly occurring in the growth chamber (e.g., root growth limitations due to pot size, elevated humidity, artificial illumination, absence of pollinators, etc.). According to Johnson and Gesch (2013), growing plants in a pot might limit root growth, which together with the absence of pollinators could cause lower yields in CE conditions. On the other hand, in field conditions the effects of temperature on seed oil content (%) could be mistaken with the effect of other environmental variables such as precipitations and diseases (Harris et al., 1978). On the contrary, in CE conditions lower oil content was found in seeds developed under higher temperatures (CE1 and CE2) that could be explained by the inverse relationship observed between protein and oil content (%) in camelina (Zanetti et al., 2017; Zubr, 2003). In fact, with increasing temperatures

the availability of nitrogen in soil contained in pots increases. Consequently, there would have been a higher absorption of nitrogen by the plants, which would have competed for carbon in the developing seeds, diverting the available carbon into proteins rather than into oil as explained by Canvin (1965). Gesch (2014) and Pecchia et al. (2014) reported that cooler seasons occurred at seed filling stage led to an increase in seed oil content (%). While the oil content (%) in Midas seeds might be related to many different environmental factors, temperature was the major factor influencing FA composition. The increasing of C18:1n9 and C18:2n6 contents, together with the decreasing of C18:3n3, (%) at elevated temperatures are in agreement with Berti et al. (2002) and Gilbertson et al. (2014). Differently, the effect of temperature on C20:1n9 still remains a controversial and little known aspect. However, a significantly higher amount of C20:1n9 (%) in the oil of autumn sown camelina compared with the spring one was documented by Pecchia et al. (2014). The positive correlation found in this study between C18:3n3 and C20:1n9 was confirmed by Zanetti et al. (2017). This correlation could be related to a compensatory effect: at lower temperatures, the production of PUFAs increases at expenses of their precursor (C18:1n9). Thus, in order to

compensate the total amount of MUFAs, C20:1n9 is produced. This assumption is supported by Canvin (1965) who found that in rapeseed oil, a decrease in C22:1n9 was compensated by an increase in C18:1n9 at higher temperatures, with a consequent variation in carbon chain length (C22 versus C18). The higher PUFA/MUFA ratio found in CE3, OF2 and OF3 conditions could be explained by the increased activation of the desaturase enzymes at lower temperatures, according to Garcés et al. (1992). At lower temperatures, the increased levels of PUFAs could make camelina oil susceptible to oxidation, reducing its quality for industrial biobased applications. However, the oxidative tendency of camelina oil due to the high PUFA content is fortunately counterbalanced by its high content of tocopherols (vitamin E), as extensively reported by Zubr and Matthäus (2002). The significant increase of C18:3n3 and decrease of C18:2n6 contents, thus increasing the n3/n6, under lower temperatures could be interesting from a nutritional point of view when considering camelina oil as a valuable source of OMEGA3 FAs (Zubr, 2003).

CHAPTER 2: Gene expression and DNA sequencing in camelina seeds

2.1 Preliminary concepts

Since variations in principal FA composition between seeds of the same camelina variety developed at different temperature ranges could be due to differences in gene expression levels, the aim of this chapter is to investigate the effects of temperature on the production mechanisms of FAs in developing camelina seeds using a molecular approach. Differences in FA composition of camelina seeds grown in the same environment could be linked to differences in gene expression, enzymes activity or encoding DNA sequence (alleles). First, expression of the genes *CsSAD*, *CsFAD2*, *CsFAD3* and *CsFAE1* encoding for the enzymes involved in C18:1n9, C18:2n6, C18:3n3 and VLCFAs production in Midas developing seeds, was investigated at different temperatures. While the effect of temperature on the expression level of *CsSAD*, *CsFAD2* and *CsFAD3* is documented, the effect on *CsFAE1* expression, which encode principally for C20:1n9 production in camelina is still a controversial and

little known aspect. Thereafter, differences in the DNA sequences were investigated through isolation, cloning and sequencing of specific genes in different camelina varieties (Midas, Omega and Calena).

2.2 Materials and methods

2.2.1 Experimental locations and plant materials

Gene expression and DNA sequencing were carried out at the laboratories of the Department of Biochemistry and Molecular Biology of Plant Products, Instituto de la Grasa, Spanish Council for Scientific Research (CSIC), Seville, Spain. DNA was sequenced at the SECUGEN laboratories (Madrid, Spain). Three camelina varieties were compared in these studies: Midas, Omega and Calena. In particular, in Midas both gene expression analysis and DNA sequencing were carried out, while in Omega and Calena only sequencing was done. Midas and Omega were chosen since they were significantly different in terms of C20:1n9 content (i.e., 13.75% vs. 14.46% in Midas and Omega, respectively) when grown in a multi-year multi-location trial, conducted within the COSMOS Project. The third camelina line, named Calena, was included in the study as a reference variety, since its high C20:1n9 content (about 17%) is reported in the literature (Vollmann et al., 2007). Camelina seeds var. Midas were provided by Linnaeus Plant Sciences, Saskatoon (Canada), while Omega is a commercial Polish variety (Poznan University, Poland). Calena seeds were provided by the

National Research Council of Italy – Institute of Agriculture Biology and Biotechnology, CNR-IBBA, Milan, Italy.

2.2.2 Gene expression

2.2.2.1 Growth chamber experiments

Midas plants were grown in a growth chamber after flowering initiation in order to mimic the conditions adopted in CE experiments carried out in Bologna. In particular, only two ranges of temperature were applied (25-15°C, CE1, and 20-10°C, CE2, day-night temperatures, as in CE1 and CE2 experiments in Bologna). Midas seeds were germinated into a plastic plateau filled with soil, and placed in a growth chamber, set at 25-15°C day-night. Emergence was completed (100%) two days after sowing. During the rosette phase, plants were transferred in round-shaped pots with a diameter of 0.10 m and then thinned, maintaining one individual plant in each pot. NPK fertilizer was regularly applied to the pots until flowering. After the start of flowering, plants were moved into two growth chambers: one set at 25-15°C (CE1) and the other at 20-10°C (CE2) day-night. The total number of plants was 36, with 18 plants transferred in each growth chamber. Other parameters such as photoperiod

(14 hours of light and 10 hours of dark) and humidity were maintained constant in the two growth chambers. All immature seeds contained in the first eight basal pods of three plants (*n*=3) were sampled at 154, 210, 290, 350, 448, 546 GDD-AF in both experiments. Unfortunately, the CE2 experiment was interrupted at about 400 GDD-AF due to technical problems. Consequently, only four samplings of Midas immature seeds were performed in CE2, corresponding to: 154, 210, 290 and 350 GDD-AF. For each replicate, seeds were collected into two separate probes: one for FAMEs determination and the other for RNA extraction. Sampled seeds were weighted and frozen immediately in liquid nitrogen. All seeds were then stored at -80 °C for analysis.

2.2.2.2 Lipid extraction, trans-methylation and total FA determination

Sampled seeds were weighed and added to 1 ml of methanol/toluene/sulfuric acid, 1 ml of heptane (Hp) and 1 mg/ml of heptadecanoic acid (C17:0) as internal standard in the tube. The mixture was placed in a water bath at 80°C for 90 minutes, verifying that there was no evaporation. During this process, the organic phase containing lipids and Hp was separated from the other. After cooling, samples were added with Hp and the upper

layer, containing just the lipid phase, was transferred in a new tube and added with 1 ml of sodium sulfate (Na_2SO_4). The sample was mixed until the solution in the upper layer bleached, then transferred in a new tube and dried under a nitrogen flux at 40 °C. Thus, FAMEs were added with Hp and analyzed on a gas chromatography apparatus, with a two fused silica capillary columns (Supelco SO2380) of 30 m length, 250 µm diameter and 0.20 µm film thickness. Hydrogen was used as gas carrier at a linear rate of 40 cm/s. Oven and detector temperatures were set at 240 °C and 250 °C respectively, with a pressure of 63 KPa. Fatty acids were identified through the comparison of their retention times with those of reference standards. Prior to calculate the enzymatic activity, the FA composition was adjusted to 100%. The enzyme activity, a measure of the quantity (µg) of active enzyme present, was determined in the interval among the different samples with the formula:

Enzymatic activity = (RRII – RRI)/(GDDII-GDDI)

Where RR is the rate of reaction (the RR calculation is explained in the chapter 1.3.5)

2.2.2.3 mRNA preparation and cDNA synthesis

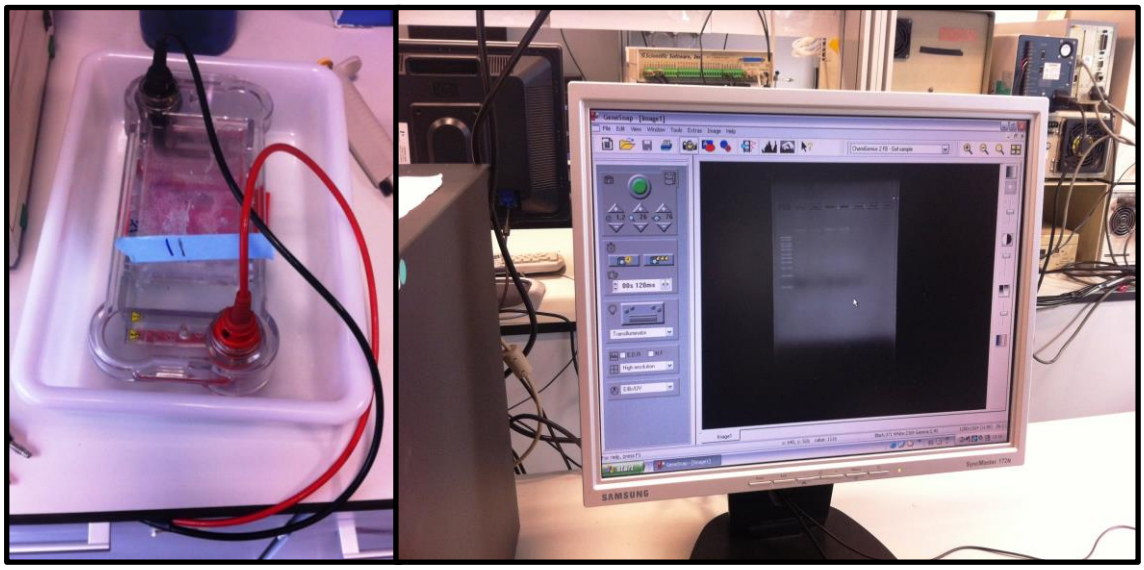
Additional samples of immature Midas seeds, obtained at each sampling date, were crushed using pre-cooled, sterile mortar and pestle. Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). RNA was then quantified with Nanodrop, annotating both concentration (ng/µl) and purity (A_{260}/A_{280}), and checked by gel electrophoresis.

Gel electrophoresis is a powerful method used in molecular biology, which allows the identification of DNA or RNA molecules, depending on their length. RNA and DNA molecules negative charged are moved to the positive electrode, by applying an electrical current through agarose gel (medium, *fig. 2.1)*. In this case, gel was prepared mixing agarose and Tris-Acetate-EDTA (catalyst) in a flask. Agarose gel concentration can vary from 0.7 to 2%, depending on the expected molecular size.

Higher concentration → lower pore size → higher separation of molecules of low size

The gel mixture was heated, RedSafe RS (Nucleic acid Staining Solution 20000X) was added, and transferred into an electrophoresis apparatus. Once solidified, total RNA mixed with a fluorescent dye (Loading Dye Purple 6X) and

diluted with water was placed in the gel into slots. A specific RNA ladder was used to identify the RNA fragments size expressed as base pairs (bp). After the electrophoretic run, the results were acquired with GeneSnap Software (*fig. 2.2*).



Left: Figure 2.1, gel electrophoresis run. Right: figure 2.2 Acquisition of the results with GeneSnap Software.

Messenger RNA (mRNA) was isolated from total RNA using the GenElute mRNA Miniprep Kit (Sigma-Aldrich). Then, reverse transcription of mRNA to cDNA was done using the Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Life Sciences, Uppsala, Sweden).

2.2.2.4 Quantitative real time PCR (RT-qPCR) and gene expression analyses

RT-qPCR was performed with CFX ConnectTM Real-Time PCR Detection System, BioRad. Each 20 µl reaction mixture contained 10 µl of SYBR Green I Master, 0.16 µl of specific pair of primers synthesized by Eurofins MWG Operon, Germany (0.8 µL primer-F + 0.8 µl primer-R) for each gene (*CsSAD*, *CsFAD2*, *CsFAD3* and *CsFAE1*), 1 µl of cDNA and 7.4 µl of sterilized H_2O. PCR conditions were 95 °C for 3 min (denaturation), followed by 39 cycles at 95 °C for 15 s, 55 °C for 30 s and 60 °C for 50 s, and monitoring the resulting fluorescence. In 96 well plates a template control was included, using 20 µl of sterilized water. Each sample was run twice in RT-qPCR (technical replicates). A standard serial dilution series (1/10, 1/100, 1/1000 and 1/10000) of cDNA from all samples were performed. The results were then used to generate a standard curve by plotting the log of the starting quantity of template against the C_T value obtained during the amplification of each dilution. The qPCR efficiency, between 90 and 105%, was determined by adopting a regression line, in which r was >0.990, R^2 was >0.980. The house-keeping gene was *CsActin*, while the target genes were *CsSAD*, *CsFAD2*, *CsFAD3* and *CsFAE1*, the latter (*CsFAE1*, CE1) was chosen as calibrator. Target

genes activity was expressed as an increase or decrease in relation to the expression of the house-keeping gene and of the calibrator.

The $2^{-\Delta\Delta C_T}$ or Livak Method (Livak and Schmittgen, 2001) was used to calculate the comparative expression, through the following three subsequent steps:

I. Normalization of the C_T of all target genes and calibrator gene to that of the reference gene:

$\Delta C_{T\ (test)} = C_{T\ (target,\ test)} - C_{T(reference,\ test)}$

$\Delta C_{T\ (calibrator)} = C_{T\ (target,\ calibrator)} - C_{T(reference,\ calibrator)}$

II. Normalization of the ΔC_T of all target genes with ΔC_T of the calibrator gene:

$\Delta\Delta C_T = \Delta C_{T\ (test)} - \Delta C_{T\ (calibrator)}$

III. Calculation of the normalized expression ratio:

$2^{-\Delta\Delta CT}$

2.2.3 DNA sequencing

2.2.3.1 DNA isolation

Fifty seeds for each of the three camelina varieties (Midas, Omega and Calena) were germinated at 30°C into a plate filled with wet vermiculite. After five days (cotyledons opened), 15 seedlings from each variety were

triturated together by mortar and pestle, previously sterilized in autoclave and cooled in liquid nitrogen. Genomic DNA (gDNA) was isolated from seedling tissues using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). Once extracted, gDNA was amplified with PCR, using VELOCITY DNA polymerase reaction and specific primers designed for the gene *CsFAE1* (primers CsFAE_Kpn_F3 and CsFAE_Kpn_R3). DNA fragments of about 1517 bp were amplified with the PCR and then checked with gel electrophoresis.

2.2.3.2 DNA cloning

Whole gDNA (40 µl) was amplified and the *CsFAE1* fragment separated by electrophoresis at 120 V for 40 min. The *CsFAE1* fragments in the agarose gel were viewed with UV light, cut and purified with isolate II PCR and Gel Kit (Bioline). The DNA cloning protocol consisted in four subsequent steps: ligation, transformation, isolation of high copy plasmid DNA and restriction. DNA ligation was performed to physically link a plasmid vector to the amplified gene *CsFAE1*. The *CsFAE1* gene was inserted into plasmid vectors, creating closed circular molecules, or "recombinant plasmids", which can be incorporated into a host bacterial DNA strain (transformation). Purified DNA (7 µl) was added with 1 µl T4 DNA ligase Buffer (10x), 1

µl pMBL T-Vector and 1 µl T4 DNA Ligase. The molar ratio of the vector to insert was calculated as follows:

Ratio vector = Vector (ng)* Insert length (bp)/Vector length (bp)* Insert (ng)

The mix was incubated at 22 °C for more than 2 hours. After ligation, recombinant plasmids were transferred into bacteria. Since bacteria divide rapidly, they can be used as "factories" to copy DNA fragments in large quantities. At the end, transformation allowed the production of many copies of the *CsFAE1* gene in bacteria. For each camelina variety, LB agar-plates were prepared by adding into each Petri dish about 25 mg of LB agar powder, 12.5 µl of Thermo Scientific IPTG Solution ready-to-use, 40 µl of Thermo Scientific X-Gal (X, blue colour; Gal, Galactosidase enzyme) solution ready-to-use and 25 µL of ampicillin (antibiotic). About 5 µl of the ligand mix was added with *Escherichia coli* (host bacteria), mixed and left on ice for 30 minutes. Then, it was kept at 42 °C for 90 minutes, causing a heat shock to the bacteria. The heat shock makes bacterial membranes more permeable, allowing the passage of the plasmids. After that, the ligand mix with *Escherichia coli* was held on ice for 3 minutes, added to 800 µL of sterilized LB agar

pre-warmed at 42 °C, and mixed. The solution was agitated at 220 rpm, 37 °C for 45 minutes and then centrifuged at 13xg for 30 s. After that, bacterial cells dropped down on the bottom of the tubes. Cells were pipetted on each plate and spread with a sterile spatula on the plates. Then, plates were closed and left at 37 °C for 24 hours. Plasmids used in DNA cloning contain an antibiotic resistance gene, enabling their selection once all bacteria were transferred into the antibiotic plate. Since not all *Escherichia coli* cells were transformed, the identification of successful products of cloning was performed with the blue-white screening method. In fact, transformed bacteria cells with vectors containing recombinant DNA produces white colonies, while empty vectors forms blue colonies. White colonies on each plate were transferred in sterilized tubes filled with 20 µL of autoclaved water. Half of the volume (10 µl) was amplified with PCR, to check the success of DNA cloning. Subsequently, white colonies were transferred into the "*inoculum*": a sterilized tube filled with 4 ml of LB agar and 4 µl of ampicillin and then shaken overnight at 220 rpm, and 37 °C. Recombinant plasmids were isolated using ISOLATE II – Plasmid Mini Kit (Bio-line). Mini Prep DNA (MPDNA) was added with 10X Buffer (1:1), dH_2O and KpnI (10v/µl) and incubated at 37 °C for 30 minutes. Then, it

was run in an agarose gel at 120 V for 25 minutes. At the end of this procedure, two bands were recognized: *CsFAE1* fragments of 1517 bp and the vector gene of 2946 bp. The MPDNA, diluted in dH_2O, was sent to SECUGEN for *CsFAE1* sequencing.

2.2.3.3 Sequence analysis

DNA sequences were analyzed with the Program Sequence Scanner, which aligned the forward and reverse DNA sequences. Three sequences of FAE1 genes from *Camelina sativa cv.* DH55, *XP_010432404.1, XP_010437603.1 and XP_010447066.1,* were downloaded from GenBank (https://www.ncbi.nlm.nih.gov) and compared with the obtained sequences.

2.2.4 Statistical analyses

Prior to ANOVA, the homoscedasticity of variance was verified with Bartlett's Test ($P \leq 0.05$). One-way ANOVA was performed on the relative expression of *CsFAD2* and *CsFAD3* genes considering the "conditions" (CE1 and CE2) as the main factor at each GDD-AF (154, 210, 290 and 350). The LSD test was used to separate means when ANOVA showed significant differences among conditions ($P \leq 0.05$).

2.3 Results

Due to differences in mRNA translation rates, mRNA and protein half-life, and protein activation/inactivation mechanisms, in Midas the mRNA expression levels were not directly related with enzymatic activities. The enzyme SAD, which processed about 90% of total FAs to C18:1n9 inside the plastids, compared with the others, (*fig 2.3a*) showed very low expression levels, but compensated by increased activity peaking after 300 GDD. Likewise, ELO showed low expression levels, associated with high enzymatic activity, which was enough to elongate around 14% of the synthesized FAs. On the other hand, FAD2 and FAD3 enzymes working in the ER membranes showed higher expression levels in camelina developing seeds than the other enzymes, increasing up to 450 GDD-AF when *CsFAD2* expression was more than double than that of *CsFAD3* (*fig. 2.3a*). The *CsFAD2* and *CsFAD3* genes are encoding for the desaturases responsible of C18:2n6 and C18:3n3 synthesis, respectively, the latter representing the most abundant FA in camelina developing seeds (*fig. 2.3b*).

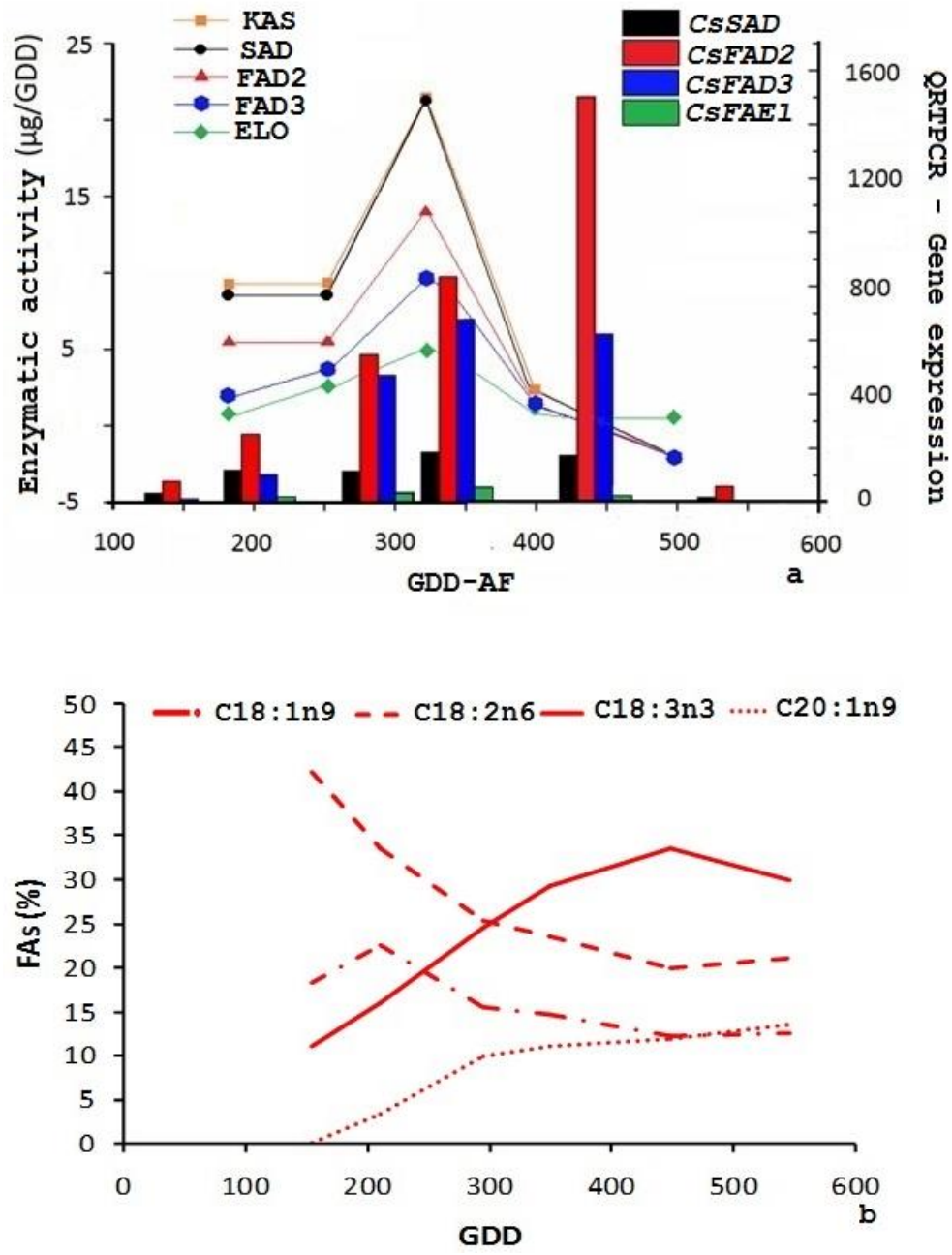


Figure 2.3 Representation of the relative expression of CsSAD, CsFAD2, CsFAD3 and CsFAE1 genes and the activity of KASII, SAD, FAD2, FAD3 and ELO enzymes in Midas developing seeds (µg/GDD) (a). Accumulation kinetics of C18:1n9, C18:2n6, C18:3n3 and C20:1n9 (%) at 25-15 °C day/night temperatures in Midas developing seeds (b).

Under lower temperatures (CE2), in Midas developing seeds the relative gene expression of FAD2 at 290 ($P \leq 0.05$) and 350 ($P \leq 0.05$) GDD-AF and FAD3 at 350 ($P \leq 0.05$) GDD-AF (*fig. 2.4*) was significantly higher than under high temperature (CE1).

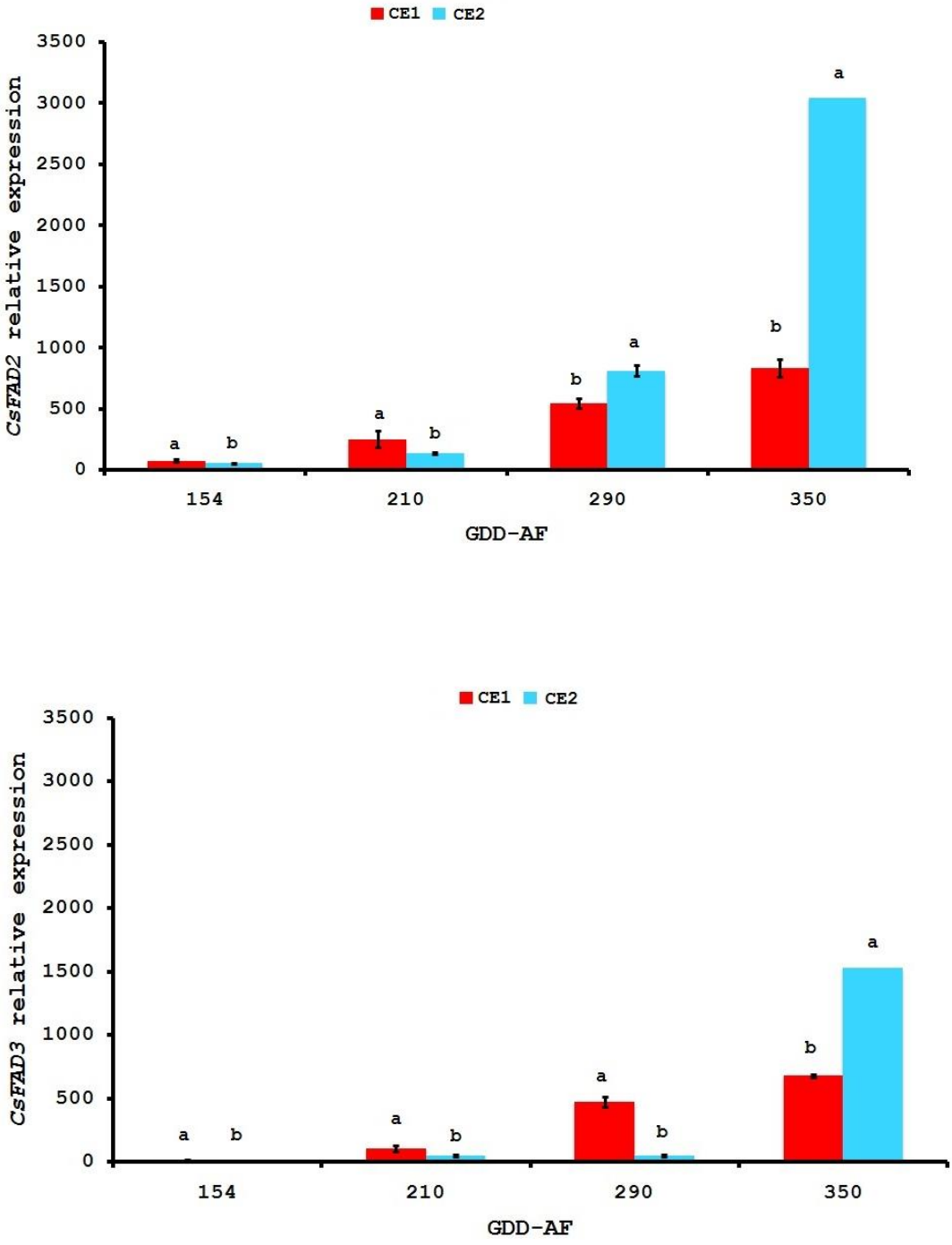


Figure 2.4. Main effect: "condition" (CE1 and CE2). top: CsFAD2 relative expression in developing Midas seeds under two temperature ranges 25-15 °C (CE1) and 20-10 °C day-night (CE2); Bottom: CsFAD3 relative expression in developing Midas seeds

under two temperature ranges 25-15 °C (CE1) and 20-10 °C day-night (CE2). Vertical bars: standard deviation. Different letters at each GDD-AF (154, 210, 290, 350): significant different values (P ≤ 0.05, LSD test).

The sequencing results are reported in *fig. 2.5.* The alignment of the amino acid sequences of *FAE1* proteins showed that residues, directly involved in the enzyme activity, were not different among the three tested camelina varieties (*fig. 2.5*). Only some conservative changes in Omega (T15S) and Calena (N241H) sequences were observed compared with the deposited sequences. Further semi-conservative changes were observed just as in the Omega variety (H480D).


XP_010447066.1
Calena
Omega
MIDAS
XP_010437603.1
XP_010432404.1

Figure 2.5 Multiple alignment of the amino acid sequences of the FAE1 gene for tested camelina varieties (Calena, Omega and Midas) and the three deposited sequences XP_010432404.1, XP_010437603.1, XP_010447066.1 of the cultivar DH55. In the sequences, different colors correspond to: yellow - differences among residues of the three deposited sequences; purple - differences among the sequences of the tested camelina varieties and deposited ones; black - active sites, blue - Malonyl Co-A binding site; dark green - product binding sites.

2.4 Discussion

The parallel analysis of FA composition and gene expression enabled deeper knowledge on FA kinetics, considering together with the expression of the genes, even FA composition and enzyme activity at different seed developing stages of Midas. Results on gene expression showed, as expected, that mRNA expression levels were not directly related to protein activities. In fact, mRNAs had different half-lives before being hydrolyzed. The rate of protein synthesis is highly dependent on the availability of tRNA and the reaction substrate. For example, SAD and ELO genes showed reduced expression, associated with increased enzyme activity, which might be related to the amount of available substrate. Low temperatures significantly affected the relative expression levels of *CsFAD2* at 290 and 350 GDD-AF and *CsFAD3* at 350 GDD-AF. However, since the experiment under CE2 conditions (20-10 °C day-night) was interrupted, further investigations to precisely define the "time frame" in which the main changes in FA composition occur in Midas seeds might be worth. Sequencing results indicated that differences in the final C20:1n9 content in the selected camelina varieties were not due to differences in FAE1 alleles. If differences in C20:1n9

content were observed in plants grown under the same conditions, this could be related to the promoter region of FAE1 genes, where the interaction of transcription factors modulated their expression. Another factor which might have contributed to the observed variation in 20:1n9 content among Midas, Omega and Calena could be the expression of LPCAT, the enzyme responsible for the incorporation of oleic acid to the PC pool (see chapter 4.2 of the introduction) before being desaturated by FAD2. LPCAT was not considered in this study, although it holds important roles in FA synthesis. Lower LPCAT activity would produce a relative accumulation of oleoyl-CoA, increasing the substrate for FAE1, and leading to a possible increase of the 20:1n9 content in camelina oil.

CHAPTER 3 Influence of location and sowing date on camelina oil composition at harvest

3.1 Preliminary concepts

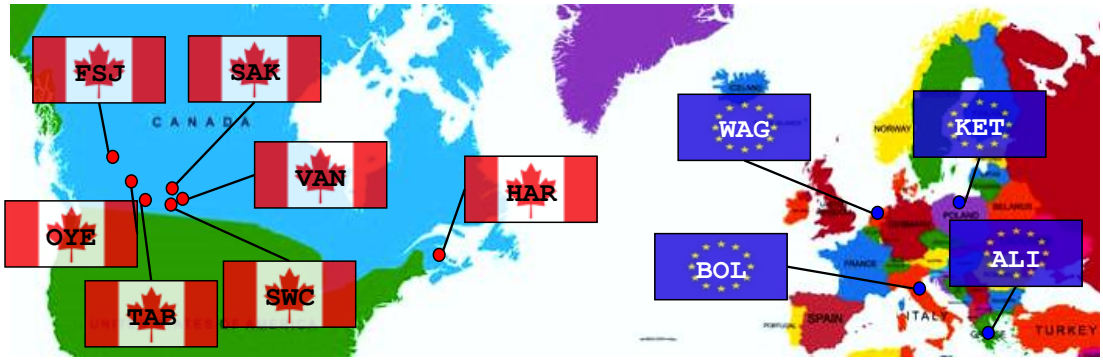
As elucidated by the previous experiments (chapters I and II), temperature was demonstrated to significantly influence the kinetics of FA in developing camelina seeds, affecting mRNA expression levels and consequently the activity of the enzymes involved in FA accumulation. Starting from this assumption, the relation between the final FA composition of camelina oil, at harvest, and temperatures occurred during the seed filling stage was thoroughly analyzed. For this scope, two different studies were carried out in order to test the wider possible range of temperatures and environmental conditions. In the first, Midas was grown in a variety of different European and Canadian locations (LOC study). In the second, Midas was tested in Bologna against different sowing times, in autumn and spring (SD study).

3.2 Multi-location study

3.2.1 Materials and methods

3.2.1.1 Experimental set up

Midas plants were grown in Europe and Canada in a multi-location study (11 sites, *fig. 3.1*) in the spring of 2015 and 2016.



	Province (country)	Site ID	Coordinates
Canada	Fort St. John (British Columbia)	FSJ	56° 15' N, 120° 50' W
	Hartland (New Brunswick)	HAR	46° 17' N, 67° 30' W
	Oyen (Alberta)	OYE	51° 21' N, 120° 28' W
	Saskatoon (Saskatchewan)	SAK	52° 7' N, 106° 40' W
	Swift Current (Saskatchewan)	SWC	50° 17' N, 107° 47' W
	Taber (Alberta)	TAB	49° 47' N, 112° 8' W
	Vanguard (Saskatchewan)	VAN	49° 54' N, 107° 18' W
Europe	Aliartos (Greece)	ALI	38° 22' N, 23° 6' E
	Bologna (Italy)	BOL	44° 33' N, 11° 23' E
	Ketrzyn (Poland)	KET	53° 58' N, 21° 8' E
	Wageningen (The Netherland)	WAG	51° 57' N, 5° 38' E

Figure 3.1 Canadian and European locations in which Midas was grown in the spring of 2015 and 2016.

Similar experimental design (i.e., randomized complete block with three or four replicates) and crop management were adopted in all tested locations. In Europe, Midas

was sown between mid-march and mid-April, while in Canada the sowing period was between early May and early June. The growing cycle lasted three to four months, depending on locations (*Tab. 3.1*). Daily air temperatures (T_{min} and T_{max}) and precipitation were collected by weather stations located near each experiment. GDD were calculated as described in chapter 1.3.1. Laboratory analyses on harvested seeds from each location (i.e., seed oil content and FA profile) were all performed at the laboratories of Agriculture and Agri-Food Canada (AAFC), Saskatoon, (Canada), according to the methods reported by Zanetti et al. (2017).

Table 3.1 Midas sowing, flowering and harvesting dates in the multi-location and multi-year trial in Europe and Canada. GDD-GS and cumulate precipitations GS (mm) refer to data from the whole camelina growing season. GDD-F refers to GDD accumulated until flowering start. T_{min}-AF and T_{max}-AF refer to minimum and maximum temperatures surveyed after flowering start. NA: data not available.

GS	Site ID	Sowing date	Flowering date	Harvest date	GDD-GS °d	Cumulate precipitation GS mm	GDD-F °d	Mean Tmin-AF °C	Mean Tmax-AF °C
2015	ALI	Apr-15	May-15	Jul-15	1473	87.1	468	16.2	28.3
	BOL	Apr-15	May-15	Jun-15	1117	190.5	462	14.5	26.7
	KET	Apr-15	Jun-15	Jul-15	959	207.6	464	11.2	22.5
	WAG	Apr-15	Jun-15	Jul-15	970	160.5	458	12.3	22.7
	FSJ	May-15	Jul-15	Sep-15	1183	177.8	465	8.1	21.0
	HAR	Jun-15	Jul-15	Oct-15	1446	NA	466	11.6	24.9
	OYE	May-15	Jul-15	Oct-15	1520	112.5	462	7.7	21.7
	SAK	May-15	Jun-15	Aug-15	1294	196.2	469	12.2	24.6
	SWC	May-17	Jun-15	Aug-15	1350	127.6	465	7.7	26.4
	VAN	May-15	Jun-15	Aug-15	1211	247.9	499	12.0	26.0
2016	ALI	Mar-16	May-16	Jun-16	1251	25.5	459	14.4	28.3
	BOL	Mar-16	May-16	Jun-16	1227	225.8	434	13.8	25.3
	KET	Apr-16	Jun-16	Aug-16	1412	427.9	461	13.0	23.1
	OYE	Jun-16	Jul-16	Sep-16	1190	194.3	463	8.4	21.1
	SAK	May-16	Jun-16	Aug-16	1212	210.6	467	12.7	23.6
	SWC	May-16	Jun-16	Aug-16	1085	207.9	465	11.6	23.6
	TAB	May-16	Jul-16	Sep-16	1141	NA	466	9.7	22.9
	VAN	May-16	Jun-16	Sep-16	1364	308.6	469	10.6	22.4

3.2.1.2 Statistical analysis

The empirical relations among principal FAs contained in Midas seeds developed at different locations and the mean T_{min} were prior tested with a correlation analysis, using the Software Costat. Thereafter, the Leave one Out Cross Validation (LOOCV) analysis was performed in order to estimate the accuracy of the predictive model (R Software 3.4). In the Cross Validation analysis data were partitioned into complementary subsets; the analysis was performed on one subset (training set) and validated on the other subset (validation set). Multiple rounds of cross-validation were performed to reduce variability and the results were averaged to derive a more accurate estimation of model predictive performances.

3.2.2 Results

The main changes in camelina FA composition were identified in chapter I as occurring in the "time frame" between 154 and 540 GDD-AF. A set of linear regression lines was tested to relate each of the principal camelina FAs (i.e., C18:1n9, C18:2n6, C18:3n3 and C20:1n9) with minimum, maximum, or mean temperatures occurring in the above mentioned post-anthesis period. These tests aimed at determining the best fitting option (which temperature

and which period) according to R^2 values for each principal FA. Once these parameters were obtained, the final FA composition in camelina oil was related with temperature. The best fitting period within the "time frame", named "critical period", was different among the principal FAs contained in Midas seeds harvested across different European and Canadian locations, but for all FAs considered the mean T_{min} showed the highest relation with final content. The correlation matrix (different temperature x different "critical period", expressed as GDD-AF) for C18:1n9, C18:2n6, C18:3n3 and C20:1n9 is presented in *tab 3.2*.

Table 3.2 R^2 of the correlations between the averaged T_{min}, T_{mean}, T_{max} and different critical periods after flowering (GDD-AF, 170-490 GDD-AF, 218-305 GDD-AF, 150-350 GDD-AF, 100-300 GDD-AF and 350-550 GDD-AF) for C18:1n9, C18:2n6, C18:3n3, C20:1n9. ns = not significant.

Critical periods	C18:1n9			C18:2n6			C18:3n3			C20:1n9		
	T_{min}	T_{mean}	T_{max}	T_{min}	T_{mean}	T_{max}	T_{min}	T_{mean}	T_{max}	T_{min}	T_{mean}	T_{max}
GDD-AF	0.27	0.29	0.20	0.49	0.54	0.44	0.64	0.65	0.52	ns	ns	ns
170-490 GDD-AF	0.21	0.17	0.11	**0.67**	0.66	0.53	**0.72**	0.67	0.51	**0.09**	0.07	0.06
218-305 GDD-AF	0.24	0.13	ns	0.34	0.31	0.27	0.44	0.34	0.26	ns	ns	0.08
150-350 GDD-AF	**0.40**	0.14	ns	0.51	0.27	0.14	0.67	0.36	0.16	ns	ns	ns
100-300 GDD-AF	ns	ns	ns	0.13	0.14	ns	0.16	0.20	0.06	ns	ns	ns
350-550 GDD-AF	ns	0.08	0.08	0.62	0.66	0.59	0.60	0.60	0.48	0.08	0.06	ns

In detail, the best fitting period for C18:1n9 was found between 150 and 350 GDD-AF, whilst for C18:2n6, C18:3n3 and C20:1n9 it was between 170 and 490 GDD-AF (*fig. 3.2, bold*).

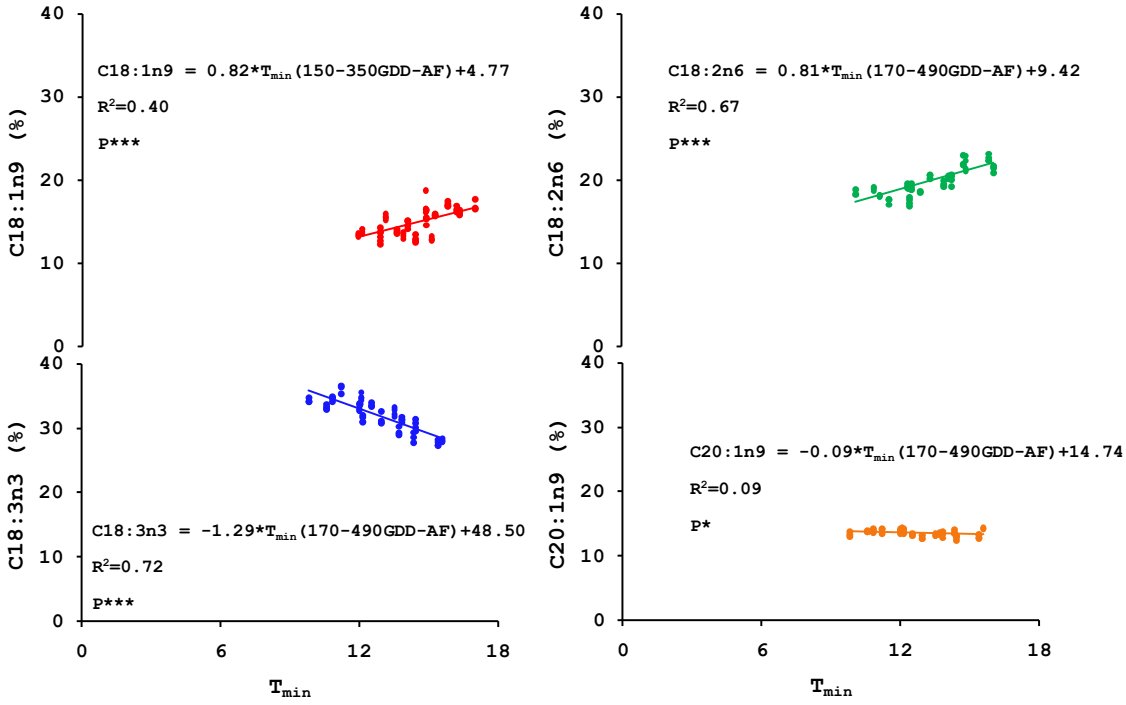


*Figure 3.2 Relation between the principal FA (%) contained in Midas seeds harvested across different locations and the mean T_{min} in the identified critical periods between 150-350 GDD-AF, for C18:1n9, and 170-490 GDD-AF, for C18:2n6, C18:3n3 and C20:1n9. P*** = P≤0.001, P* = P≤0.05*

In particular, C18:1n9 and C18:2n6(%) significantly increased ($P \leq 0.05$) with increasing T_{min}, while C18:3n3 (%) presented an opposite behavior ($P \leq 0.05$). The C20:1n9 content was significantly negative correlated

with increasing T_{min} , but R^2 was very low (*Fig. 3.2*). The results of the LOOCV analysis, presented in *tab. 3.3*, validated the relationships among C18:1n9, C18:2n6, C18:3n3 C20:1n9 and T_{min}, showing R^2 values very close to those presented in *fig. 3.2*.

Table 3.3 Cross-validation analyses results.

FAs	**RMSE (%)**	**R^2**
C18:1n9	1.26	0.35
C18:2n6	0.93	0.65
C18:3n3	1.32	0.70
C20:1n9	0.46	0.04

The adoption of this empirical relationship among the principal FAs and the mean T_{min} during the "critical period" allowed satisfactory prediction of the final camelina oil quality several days before harvesting, ranging from 12 to almost 40 days, depending on location and year.

3.3 Sowing date study

3.3.1 Materials and methods

3.3.1.1 Experimental set up

Midas plants were grown across two autumn (SD1 and SD2) and four spring (SD3, SD4, SD5, and SD6) sowing dates during two consecutive growing seasons 2015-2016 and 2016-2017 at the experimental farm of the University of Bologna in Cadriano, Italy (*fig. 3.3*). Sowing took place at the beginning and end of October in the autumn dates, while in the spring dates it ranged between mid-February and mid-April (*tab. 3.4*). The main meteorological data (T_{min}, T_{max} and precipitation) for the trials were collected by a weather station near the fields.

Figure 3.3 Camelina var. Midas grown during the season 2016-2017 in Cadriano experimental farm (Bologna, Italy) at different sowing dates.

Table 3.4 Sowing, flowering and harvest dates in the SD study set in Cadriano during 2015-17. GDD-GS and cumulate precipitations GS (mm) refer to data from the whole camelina growing season. GDD-F refers to GDD accumulated until flowering start. T_{min}-AF and T_{max}-AF refer to minimum and maximum temperatures surveyed after flowering start.

GS	ID	Sowing date	Flowering date	Harvest date	Cumulate precipitation GS mm	GDD-GS °d	GDD-F °d	Mean Tmin-AF °C	Mean Tmax-AF °C
2015-2016	1	09/10/2015	14/03/2016	24/05/2016	491.2	1123	469	8.9	19.9
	2	26/10/2015	24/03/2016	26/05/2016	426.2	1013	380	9.6	20.8
	3	12/02/2016	29/04/2016	20/06/2016	383.2	1158	465	12.8	23.7
	4	15/03/2016	07/05/2016	28/06/2016	226.6	1232	462	14.0	25.5
	5	30/03/2016	14/05/2016	07/07/2016	247.4	1332	468	14.9	26.9
	6	12/04/2016	24/05/2016	13/07/2016	237.4	1345	466	16.3	28.5
2016-2017	1	13/10/2016	20/03/2017	22/05/2017	327.2	1045	435	8.6	21.2
	2	25/10/2016	24/03/2017	24/05/2017	271.8	982	372	8.8	21.6
	3	17/02/2017	28/04/2017	07/06/2017	100.0	1023	484	12.2	24.8
	4	01/03/2017	28/04/2017	07/06/2017	98.8	1000	461	12.3	24.9
	5	15/03/2017	11/05/2017	14/06/2017	95.2	1056	506	14.4	28.1
	6	29/03/2017	18/05/2017	21/06/2017	97.6	1095	506	15.4	29.4

Midas was seeded in 10.5 m^2 plots, in which row distance was set at 0.13 m and sowing density at 500 seeds m^{-2}. The experimental design was a randomized complete block with *n*=4 in the autumn SDs, and *n*=3 in the spring SDs. All trials were rain fed. At harvest, all Midas plants in the central 10 rows of each plot were manually cut and the total biomass (TB) weighed. Cut plants were then threshed using a plot combine harvester (Wintersteiger, Austria); seeds and straws were collected and individually weighed. Residual moisture in each component of the yield (seed and straw) was determined by oven drying a representative sample at 105°C for 24 h until constant weight was reached. On representative seed samples for each replicate, TKW (g) was determined with an automatic seed counter (DataCount S25, Data Technologies, Israel) at LaRAS laboratories, University of Bologna. Analyses on harvested seeds from each SDs (i.e., seed oil content and FA characterization) were all performed at the laboratories of Agriculture and Agri-Food Canada (AAFC), Saskatoon (Canada), according to the methods reported by Zanetti et al. (2017).

3.3.1.2 Statistical analysis

As in chapter 3.2.1.2, the relations among C18:1n9, C18:2n6, C18:3n3 and C20:1n9 contained in Midas seeds from the different SDs, and mean T_{min} during the critical periods detected in the LOC study (150-350 GDD-AF, 170-490 GDD-AF) were tested with correlation analysis, using the Software Costat. Thereafter, the elevations and slopes of the regression lines obtained in the SD study were compared to those obtained in the LOC study using the Software Statistix 8.0.

The correlation analysis was also performed between each of the principal FAs (C18:1n9, C18:2n6, C18:3n3, C20:1n9, %) and TKW (g) considering all SDs in both GSs, with the Software Costat.

Prior to ANOVA, the homoscedasticity of data related at the TKW (g), seed oil content and principal FA (%) at harvest was verified with Bartlett's test. Thereafter, if variance was homogeneous, a one way ANOVA was performed comparing the different SDs, while year was considered as a random effect. Finally, the Newman-Keuls test was used to separate means resulted different at a significant level $P \leq 0.05$.

3.3.2 Results

Unfortunately, in spring 2016 a strong hailstorm occurred on the 26 June highly reduced seed yield and also TB (Mg ha^{-1}) in the majority of spring sown plots (i.e., SD3, SD4, SD5 and SD6). For this reason, the quantitative parameters are not presented.

The linear regressions between C18:1n9 and T_{min} occurred at 150-350 GDD-AF and between C18:2n6, C18:3n3, C20:1n9 and T_{min} occurred at 170-490 GDD-AF are presented in *fig. 3.4*. These results confirmed the strong positive correlation between C18:1n9, C18:2n6 and T_{min} ($P \leq 0.05$). Otherwise, C18:3n3 and C20:1n9 significantly decreased with increasing T_{min} ($P \leq 0.05$). Interestingly, the R^2 values of the linear regression between C20:1n9 and T_{min} in the SD study was much higher than that of the LOC study (0.58 vs. 0.09, in the SD study and LOC study respectively). Additionally, the R^2 value for C18:1n9 was higher in the SD study (R^2 = 0.64) than in the LOC study (R^2 = 0.40). Otherwise, restrained differences occurred between the R^2 values of C18:2n6 (R^2 = 0.80, SD study vs. R^2 = 0.67, LOC study) and C18:3n3 (R^2 = 0.80, SD study vs. R^2 = 0.72, LOC study) regressions.

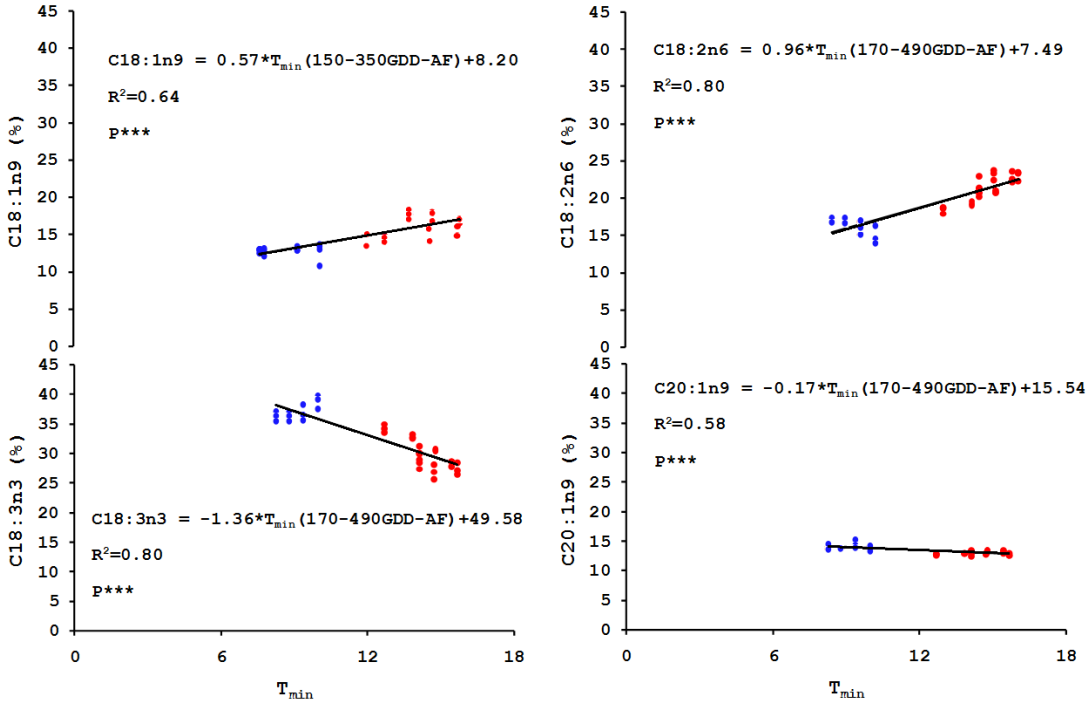


*Figure 3.4 Correlations between the principal FAs (%) contained in seeds of Midas plants in the SD study and mean T_{min} (°C) in the identified critical periods. The critical periods were 150-350 GDD-AF for C18:1n9, and 170-490 GDD-AF for C18:2n6, C18:3n3 and C20:1n9. Blue points: Midas autumn sowing; Red points: Midas spring sowing. P*** = P ≤ 0.001*

The comparison analysis of the linear regressions obtained in the SD and LOC studies (chap. 3.2.2) showed differences close to significance (*P = 0.05*) in the elevations for C18:1n9 and C20:1n9 (*tab. 3.5*). The slopes of the linear regressions for C20:1n9 were nearly different (*P = 0.05*) in SD and LOC studies. The slope of C18:2n6 linear regression in the SD study was also

significantly different from that of the LOC study (*P ≤ 0.05*). On the other hand, no differences were detected for C18:3n3.

Table 3.5 P values (P≤0.05) from the comparison of slopes and elevations of the regression lines fitted between C18:1n9, C18:2n6, C18:3n3 and C20:1n9 and T_{min} in the SD and LOC study.

Principal camelina FAs	C18:1n9	C18:2n6	C18:3n3	C20:1n9
Comparison of Slopes	0.28	0.02	0.12	0.05
Comparison of Elevations	**0.05**	0.78	0.81	**0.05**

Interestingly, all the principal FAs in Midas seeds were strongly correlated with the TKW (g) (*P ≤ 0.05, fig. 3.5*). In particular, with increasing TKW (g) there was a significant decrease in C18:1n9 and C18:2n6 (%). Conversely, TKW (g) was positively correlated with C18:3n3 and C20:1n9 (%).

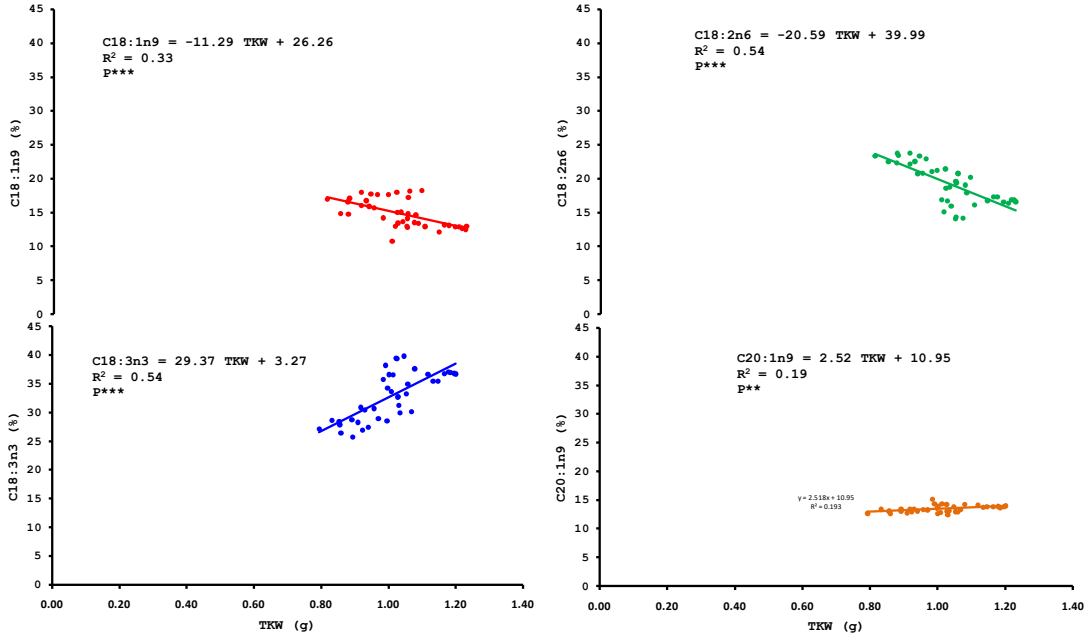


*Figure 3.5 Relation between the principal FAs (%) contained in seeds of Midas plants sowed at different times in Bologna in the GS 2015-2016 and 2016-2017 and the TKW (g) at harvesting. P*** = P≤0.001, P** = P≤0.01*

Midas seed quality (TKW, oil content and FAs) resulted significantly affected by SD (*tab 3.6*). TKW (g) and seed oil content (%) were significantly reduced when SD was delayed. The seeds of Midas plants sown in autumn (SD1 and SD2) contained significant higher amounts of C18:3n3 and C20:1n9. Differently, higher contents of C18:1n9 and C18:2n6 were found in Midas seeds of plants sown in late spring (SD5 and SD6).

Table 3.6 TKW (g), seed oil content (%), C18:1n9, C18:2n6, C18:3n3 and C20:1n9 contents (%) in the SD study. Mean value ± SD. Different letters: significant different values (P ≤ 0.05).

SD	TKW	Seed Oil Content	C18:1n9	C18:2n6	C18:3n3	C20:1n9
	g			*%*		
1	1.08±0.09**ab**	41.47±0.68**a**	12.79±1.04**b**	16.78±0.45**c**	36.38±0.59**a**	14.15±0.57**a**
2	1.10±0.08**a**	40.14±0.92**a**	13.07±0.22**b**	15.52±1.42**c**	38.01±1.80**a**	14.02±0.20**a**
3	1.03±0.03**bc**	40.36±1.54**a**	16.23±1.99**a**	19.54±1.22**b**	32.41±2.20**b**	12.94±0.33**b**
4	1.00±0.04**c**	40.50±0.62**a**	16.18±1.83**a**	20.62±1.53**b**	30.64±2.56**bc**	13.15±0.22**b**
5	0.92±0.02**d**	39.03±1.79**b**	16.40±1.45**a**	22.07±1.36**a**	28.83±2.16**cd**	13.20±0.31**b**
6	0.85±0.03**e**	36.62±0.33**c**	16.08±1.00**a**	22.96±0.65**a**	27.92±0.92**d**	13.04±0.33**b**

3.4 Discussion

The results of the first part of the study highlighted the strong relationship between T_{min} during the "critical period" and final FA composition of Midas, grown across different locations in Europe and Canada. The relationships among principal FAs and temperature agreed with the results found in the previous chapters (chapter I and II) of this thesis, showing an increase in C18:1n9 (%) and C18:2n6 (%) and a decrease in C18:3n3 (%) and C20:1n9 (%) in response to increasing T_{min} during the identified critical periods (150-350 GDD-AF for C18:1n9, and 170-490 GDD-AF for the other principal FAs). A sensitive period (i.e., "critical period") between 680 and 930 GDD-AF, adopting a T_{base} = 0 °C, was also determined by Baux et al. (2013), who build an empirical model to predict the final C18:3n3 content in different rapeseed varieties simply based on the mean T_{min} at that period. Considering that in the present study GDD-AF were calculated adopting a T_{base} of 5 °C, the critical periods considered in both of the studies were very similar.

The relationship between the final FA composition in different oil crops (e.g., camelina, rapeseed, sunflower) and locations has been widely studied in several multi-location trials. Echarte et al. (2010) and Grunvald et

al. (2013) found an increase in C18:1n9 (%) in sunflower seeds with increasing minimum temperatures in different locations in Argentina (Balcarce, Sáenz Peña and Paraná) and Brazil (Ceára, Goiás, Maranhão, Mato Grosso, Minas Gerais, Piauí ,Rondônia, São Paulo). Baux et al. (2013) found that higher contents of C18:3n3 (%) were associated with lower temperatures during the "critical period" in rapeseed seeds grown at different locations in Switzerland. The effect of "location" on final FA composition in camelina seed oil has also been reported by Zubr and Matthäus (2002), Gugel and Folk (2006), and more recently by Zanetti et al., (2017). Zubr and Matthäus (2002), who tested camelina in different locations in Central, Northern Europe and Scandinavia, reported a strong effect of location on C18:1n9, C18:2n6 and C18:3n3 contents, while the effect on C20:1n9 amount was limited. Differently, Gugel and Folk (2006), in a multi-location and multi-variety camelina study in western Canada, reported higher variation in the principal FAs related to "variety" than to "location". Finally, Zanetti et al. (2017) reported the final oil composition of different camelina varieties across Europe and Canada, showing a significant effect of location on PUFAs and C18:1n9 (%) and lower effects on C20:1n9 (%).

Schulte et al. (2013), when comparing the effect of T_{max} at the seed filling stage on the final FA composition of different oil crops (i.e., sunflower, soybean, camelina and canola), found that camelina oil composition was nearly independent of maximum temperatures compared to the other species tested. The authors suggested that this low correlation might be related to the limited temperature range reviewed in available camelina studies (19-28 °C) compared to those found in the literature for the other oil crops (about 10-40 °C).

The broad data-set used in the present thesis, including 11 locations covering latitudes between 38°22'N and 56°15'N and longitudes between 120°50'W and 23°6'E, allowed investigation for the first time of a significant range of T_{max} (21-28 °C) and T_{min} (8-16 °C) after flowering in camelina. Moreover, the adoption of the same camelina variety, Midas, completely excluded the "genotype" effect on the results, thus permitting, for the first time for camelina, to the best of the author's knowledge, a thorough analysis of the climate effect alone.

When comparing the relations among principal camelina FAs and T_{min} in the LOC and SD studies, the results showed higher R^2 values in the latter, most probably because the growth of camelina in one site across different sowing dates permitted exclusion of all the other environmental

(soil, pest and disease, etc.) and climatic (precipitation, day length, etc.) factors that the LOC study actually included. Interestingly, elevation of regression lines between C18:1n9, C20:1n9 and T_{min} were higher in the SD study compared to the LOC one, demonstrating that it would be possible to significantly increase C20:1n9 content in Midas seeds by growing it as winter crop than spring sowing it in cold environments. Presumably, since autumn sowing leads to a significant increase of the growing cycle length (+130 days SD1 vs. SD6) of Midas, the longer permanence of plants in the soil permitted to significantly increment plant biomass and consequently the carbon stock available. The effect of the higher amount of carbon stocked in Midas sown in autumn was tested putting in correlation each of the principal FAs with the TKW (g), considered as a "carbon sink" at harvest. In particular, C18:1n9 and C18:2n6 were negatively correlated with TKW, while C18:3n3 and C20:1n9 were positively correlated with TKW. A few published studies (i.e. Echarte et al., 2012; Ruiz and Maddonni, 2006) have considered the effects of carbon availability in the seed (i.e. TKW) on oil FA composition. In the literature, it has been reported that the sowing date effect on final FA composition of different oil crops is mainly related to difference in temperatures at the seed

filling stage. To the best of the author knowledge, previous studies have never considered the effects of temperature together with the carbon availability on final oil quality of camelina. From the results presented, it should be possible to give the following interpretation to FA metabolism in camelina grown under different SDs:

- Low TKW (g) corresponded to low assimilates available for FA metabolism allocated in the camelina carbon sinks;
- In conditions of low assimilate availability (low TKW) and high temperatures during seed filling (spring sowings), de novo FA synthesis (chapter 4.1 of the introduction) and membrane desaturases and elongases (chapter 4.2 of the introduction) in plastids were subsaturated, resulting in increased contents of C18:1n9 and C18:2n6;
- In conditions of high assimilate availability (high TKW) and low temperatures (autumn sowing), *CsFAD2* and *CsFAD3* genes showed higher expression levels until "saturation" of oleic desaturation, reaching the maximum production of C18:2n6 and C18:3n3; at this point, the accumulation of C18:1n9, which is the ELO substrate for producing C20:1n9, led to a final increase in the C20:1n9;

This interpretation is supported by Echarte et al. (2012), who found that sunflower oil composition was directly mediated by the assimilate availability in the seed. The authors analyzed oil and FA contents as a function of carbohydrate equivalents for grain biomass synthesis, which estimated the carbon allocated to the seed. Echarte et al. (2012) found that with increased assimilates allocated to sunflower seeds, oleic desaturation became saturated with the consequent increase of C18:1n9 content. The typical biosynthetic pathway of *Brassicaceae* species, which includes long chain MUFAs (C>20), different from *Compositae,* might explain the final differences in oil composition in camelina (increased C20:1n9) compared to sunflower (increased C18:1n9), when substrate availability increases.

GENERAL CONCLUSIONS

The growing interest of the European bio-based industry for *Camelina sativa* is driving the introduction of this new oilseed crop into different domestic environments, in relation to the widespread adaptability of this species, including both winter and spring genotypes. In this view, the effect of temperatures on the final oil quality needs to be carefully studied to define the most appropriate agronomic management (i.e., sowing date) as well as the most suitable growing area.

In camelina, the significant increase in PUFAs (membrane FAs) in response to low temperatures, associated with an increased expression of the *CsFAD2* and *CsFAD3* genes in developing seeds, might be presumably related to mechanisms for maintaining cell membrane fluidity. As documented in the literature, low temperatures decrease the C18:2n6 content in camelina oil while increasing the amount of C18:3n3. Interestingly, in this study a significant decrease in C18:1n9 was associated with an increase in C20:1n9 in response to low temperatures, and the latter positively correlated with C18:3n3. Although the oleochemical industry has a growing interest in C20:1n9, as demonstrated by the European Project COSMOS,

the effect of temperature on C20:1n9 metabolism still remains a controversial and little known aspect. With this scope, gene expression analyses were carried to elucidate the effects of temperature on *CsFAE1* genes, but the expression level of these genes was negligibly influenced by temperature. Thus, the increase of C18:3n3 and C20:1n9 at low temperatures might be related to a compensatory effect: the increase of PUFAs with a consequent decrease of their precursor (C18:1n9) led to an increase in the activity of *CsFAE1* to produce C20:1n9 in order to balance the total amount of MUFAs. However, before excluding any temperature effects on *CsFAE1* expression, further studies considering a wider "time frame" in camelina developing seeds needs to be carried out.

The study of FA metabolism in camelina developing seeds allowed to precisely determine the "time frame" in which the main variations in FA composition are occurring. The definition of this "time frame" permitted developing empirical relationships, based on the final camelina oil quality in seeds harvested in a wide range of climates in Europe and Canada and mean T_{min} during the critical post-anthesis period, which was specific for each FA. The adoption of this empirical model, based on a multi-location (*n*=11) and multi-year (*n*=2) study, permitted

early evaluations of the final oil composition of camelina many days before harvest with important implications for bio-based industry, thus determining in advance its oleochemical value. The same empirical model applied to camelina plants grown in the same environment (i.e., Bologna) but subjected to different sowing dates, from early autumn to spring, confirmed the strong relationship between final FA composition and mean T_{min} during the defined "critical periods". In this case, the relations between FAs and temperature were even more significant (i.e., higher R^2) compared to the multi-location study, presumably in response to more restrained environmental and climatic variability included in this trial, testing different sowing dates in one location. Furthermore, this latter study showed a higher elevation of the linear regression lines for C20:1n9, thus confirming that the increased C20:1n9 content in camelina seeds may be related to anticipation of sowing in autumn. Autumn sowing of spring camelina would appear to be a feasible agronomic option in the northern Mediterranean climate, such as that in Bologna, to increase both yield and oil quality. The prolonged crop cycle of autumn sown camelina compared to late spring sown ones (+130 days in the field) is associated with an increased plant biomass ("carbon stock") as well as to higher accumulation of

carbon at sink level (TKW, g). The higher amount of carbon available in seeds, together with the lower temperatures during seed filling associated with early autumn sowings, was presumably able to saturate the activity of the enzymes FAD2 and FAD3 with a consequent, temporary, increase in C18:1n9, which being the substrate for ELO1, leads to an increased final content of C20:1n9. In conclusion, sowing dates strongly affected the final FA composition of camelina in relation to the synergic effects of low temperatures, during seed filling, and the carbon stocked in plants.

The acquired knowledge on the response mechanisms of camelina to different growing conditions will undoubtedly have impact not only on the present understanding of the biochemical and physiological processes and their regulation, but also in defining proper agronomic crop management.

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Appendix

1 Statistical analysis

Analyses of variance were performed on the obtained results for oil content (%), principal FA (C16:0, 18:0, C18:1n9, C18:2n6, C18:3n3, C20:1n9) contents (%) and on oil components (FFAs, STs, DGs and TGs, %). Prior to perform the ANOVA analysis, the homogeneity of variance was tested with Bartlett's test. The results were then subjected to one-way ANOVA considering the "extraction method" as an independent factor. Finally, the LSD test was carried out to separate means when the analyses of variance revealed statistical differences between the different methods ($P \leq 0.05$).

2. Definition of the optimal oil extraction method for camelina immature seeds

The amount of oil (%) extracted with the three analytical methods was not statistically different (*tab. 1*). Moreover, no significant differences were seen in principal FA contents (%) among the three methods except for C18:2n6, which showed a significantly lower % in the oil extracted with the Hara and Radin (1978) method (*tab 1*). Oil content (%) extracted with the three methods was lower compared with the values found in the literature (Vollmann et al., 2007; Pecchia et al., 2014). The three different methods did not show any significant differences for the contents of FFAs, STs, DGs and TGs (*fig. 1*).

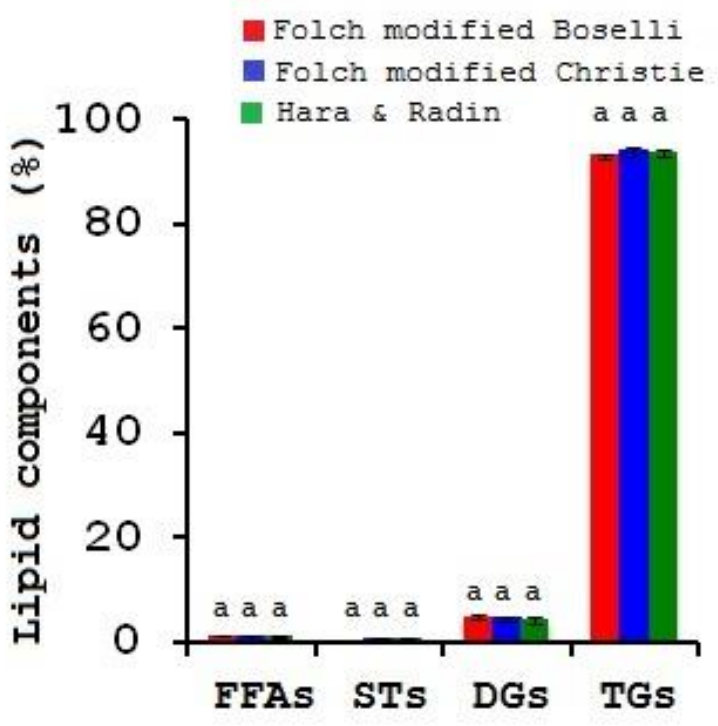

Figure 1 Comparison of FFAs, STs, DGs, TGs (%), contained in camelina seed oil extracted with Folch modified Boselli (Boselli et al., 2001) in blue, Folch modified Christie (Christie, 1989) in red and Hara and Radin (1978) methods in green. Different letters within each column: significant different values (P ≤0.05, LSD test). Vertical bars: standard deviation

Table 1 In the upper part of the table the comparison of camelina seed oil content and principal fatty acid composition (% ± Standard Deviation) of the three tested extraction methods are presented: [1] Boselli et al., 2001 (Folch modified Boselli); [2] Christie, 1989 (Folch modified Christie); [3]Hara and Radin, 1978 (Hara and Radin). Different letters within each column: significant different values (P ≤0.05, Newman-Keuls test). Bottom part of the table reports literature reviewed: [4]Vollmann et al., 2007 (FT-NIRS); [5]Pecchia et al., 2014 (Hexane extraction).

Methods	Oil	C16:0	C18:0	C18:1n9	C18:2n6	C18:3n3	C20:1n9
				%			
[1]	28.11±3.49**a**	6.73±0.10 **a**	2.03±0.02**a**	14.61±0.02**a**	19.48±0.0**a**	30.78±0.16**a**	12.79±0.26**a**
[2]	28.30±1.27**a**	6.65±0.12 **a**	2.04±0.01**a**	14.65±0.17**a**	19.35±0.1**a**	30.73±0.31**a**	12.72±0.02**a**
[3]	23.88±1.15**a**	6.61±0.12 **a**	2.08±0.09**a**	14.43±0.23**a**	19.03±0.0**b**	31.05±0.22**a**	12.64±0.03**a**
References	Oil	C16:0	C18:0	C18:1n9	C18:2n6	C18:3n3	C20:1n9
				%			
[4]	43.90	6.45	2.75	14.48	16.74	32.41	16.91
[5]	32.48	2.95	0.67	9.34	13.63	61.88	8.81

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