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University of Alberta

EFFECT OF GERMINATION ON CANOLA SEED CONSTITUENTS

By

HAIYAN ZHANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of **Master of Science**

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

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ABSTRACT

Content and quality changes of canola seed components throughout 20 days of germination under illuminated and dark conditions were studied. Samples of whole seedlings, seedling tops and bottoms were collected at different time intervals and subjected to lipids, sterols, tocopherols and protein analyses.

The oil and protein reserves were depleted and solubility of protein improved during germination. The content of triacylglycerols was depleted while that of free fatty acids, monoacylglycerols and diacylglycerols was increased. Under illuminated conditions, new tocopherols were synthesized and conversion of γ-tocopherol to α-tocopherol occurred regardless of conditions. Sterols content in seedlings showed negligible changes while a 5- and 4-fold increase in total sterols was evident in oil under illuminated and dark conditions, respectively. Enrichment of free fatty acids, sterols and tocopherols in oil from the germinating seeds as natural means of concentrating nutraceutically important substances can be important to functional food industry.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphophate
ALA	α-Linolenic acid
BSTFA	N, O-bis(trimethylsilyl)trifluoroacetamide
DAG	Diacylglycerols
DHA	Docosahexaenoic acid
EPA	Eicosspentaenoic acid
FFAs	Free fatty acids
GC	Gas chromatography
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
MAG	Monoacylglycerols
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PUFA	Polyunsaturated fatty acid
TAG	Triacylglycerols
TE	Tocopherol equivalent
TMCS	Trimethylchlorosilane
γ-TMT	γ-Tocopherol methyltransferase
VLDL	Very low-density lipoprotein
WAC	Water absorption capacity

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1.0 INTRODUCTION

1.1 History of canola

The rapeseed (*Brassica campestris*) was first introduced to Canada in 1936 and has been used for edible purposes since 1948. In 1968, the first low-erucic acid rapeseed cultivar was bred in Canada. The first "double-low or double-zero" cultivar, which was low in the content of both erucic acid and glucosinolates, was introduced in 1974. The term Canola, adopted in 1979, refers to rapeseed varieties that contain less than 2% erucic acid in the oil and less than 30 μmol/g of aliphatic glucosinolates in the defatted meal (Daun, 1993). Summer variety *B. napus* L. and *B. rapa* (*campestris*) L. are major species grown in Canada (Daun, 1993). In 1985, the Food and Drug Administration (FDA) of the United States granted GRAS (generally recognized as safe) status to canola (Shahidi, 1990).

1.2 Production and utilization of canola

Canola/rapeseed, soybean, flaxseed and sunflower are the major oilseeds grown in Canada (Daun, 1998). Oilseeds are used in the production of vegetable oils and high-protein meals. Vegetable oil may be used for edible purposes, such as salad or cooking oils, shortening and margarines, and also for industrial purposes, primarily as a source of fatty acids and related derivatives, and as polymerization agents in the coatings and linoleum industries (Daun, 1993). The high protein meals are used as animal feed and fertilizer.

The total annual production of canola in Canada is 6 million tonnes (Table 1.1). Provinces of Alberta, Saskatchewan and Manitoba are the major geographical areas of

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canola production in Canada. Canadian production of deodorized canola oil reached nearly 1.0 million tonnes per year (Table 1.2), which accounts for nearly 75% of all edible oils deodorized in Canada. Canola oil represents nearly 90% of domestically produced salad and cooking oils, 50% of shortening and shortening oils, and 45% of margarine and margarine oils (Malcolmson and Vaisey-Genser, 2001).

Canola oil is used mainly as edible oil and a small proportion is for industrial purposes. In the western world, canola oil is consumed mainly as margarine, shortening, and salad/cooking oil. Manufacturing of the rubber substitute factice is probably the principal industrial use of rapeseed/canola oil. Besides, canola oil also has found applications in lubricants, leather tanning, textile chemicals, inks and printing processes, detergents and plasticizers. High protein canola meal is used extensively as animal feed.

1.3 Chemical composition of canola

As shown in Table 1.3, lipids (oil), protein and carbohydrates are the major constituents in canola seeds. Oil accounts for 40-50% of dry canola seed, which consists mainly of triacylglycerols (TAG), smaller amounts of monoacylglycerols (MAG), diacylglycerols (DAG), free fatty acids, polar lipids, and minor components such as tocopherols, sterols and pigments (Table 1.4). The residue after oil extraction is called canola meal and it contains protein, fiber, minerals and smaller amounts of the less desirable components such as glucosinolates, phytic acid and sinapine (Naczk et al., 1998).

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Triacylglycerols (TAG)

This class of lipids is the predominant one and account for 94.4 to 99.1% (w/w) of canola oil (Przybylski, 2001). TAG is the ester of one molecule of glycerol and three molecules of fatty acids. TAG composition is governed by the type and amount of fatty acids present in the oil. Major fatty acids in canola are given in Table1.5. Canola is characterized by its very low saturated fatty acid content (mainly palmitic and stearic acids) that accounts for only 6% (w/w). Oleic acid content in canola oil is about 60% (w/w) of the total fatty acids as compared to 77% (w/w) in olive oil. Canola oil is intermediate among the vegetable oils in its level of polyunsaturated fatty acids (30%, w/w), mainly linoleic and linolenic acids, which is higher than that in peanut and palm, but lower than that in soybean, sunflower and corn (McDonald, 2001). In regular canola oil, four TAGs, namely olein-dilinolein, linolenin-dilinolein, triolein and linolein-diolein are present in almost equal amounts. In high oleic acid canola oil, the main TAG was triolein. Long chain (C20:0–C24:0) and saturated fatty acids occur mostly in the sn1- and sn3- positions, while the octadecanoic (C18) acids, especially linoleic and linolenic, are integrated in the sn2- position.

Minor fatty acids

Most of the minor fatty acids are present at <0.1% (w/w) level (Przybylski, 2001). Minor fatty acids differ from common fatty acids by the location of the double bond. Most of the minor fatty acids in canola oil are the n-7 series, but n-9 isomers are also present in varying amounts (Ackman, 1990). Conjugated C18:2 fatty acids have also been found in canola oil. Some of these components are artifacts of refining, although

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some were observed as natural components in the seeds. Some fatty acids with sulfur as the integral part of the molecule are also found in canola oil (Wijesundera and Ackman, 1988). Przybylski and his coworker (1993) reported minor fatty acids with 26-32 carbon atoms in the sediment from industrial winterization (a secondary processing step in canola oil refining). These compounds may be from the seed coat (Hu and Scarth, 1994).

Polar lipids

Polar lipids in canola oil consist primarily of phospholipids and glycolipids (particularly galactolipids). Phospholipids constitute the main lipid fraction among lipoprotein lipids. They are comprised of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol. The phosphorus content of the oil is an approximate index of the amount of phospholipids.

Glycolipids are a group of compounds that have glucose bound through a glycoside bond to the primary hydroxyl group of sphingosine. These components are removed during degumming, forming so-called hydration sludges. Galactolipids is a subgroup of glycolipids.

Tocopherols

The main nonsaponifiable components in vegetable oils are tocopherols and sterols. The tocopherols content in canola and some common vegetable oils is summarized in Table 1.6. Canola oil contains mainly two isomers of tocopherols, α- and γ-, and the γ-isomer is normally present in higher amount. A huge loss of tocopherols during the industrial oil processing has been reported in literature. The degumming

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process is responsible for about 20% loss of total tocopherols. When degumming was followed by refining and bleaching, about 52% of total tocopherols were lost and α-tocopherol was more sensitive to destruction by bleaching than γ- or δ- tocopherol (Prior, et al., 1991).

Sterols

Sterols are present in canola oil in equal amounts in two forms, free and esterified (Ackman, 1983). The composition of major sterols founded in common vegetable oils are presented in Table 1.7. The total amount of sterols in rapeseed and canola oils ranges from 0.53 to 0.97% (w/w), which is about 50% higher than that in soybean oil, but lower than that in corn oil (Przybylski, 2001). Two of the major sterols, campesterol and sitosterol, are equally distributed in the esterified and free sterol fractions in canola oil. Brassicasterol is a unique sterol present in the *Cruciferae* family, and the amount of free brassicasterol is twice that of its esterified form.

Pigments

Pigments present in canola and other oilseeds are important as they can impart undesirable color to vegetable oils, promote oxidation in the presence of light, and inhibit catalysts used for hydrogenation. Chlorophyll is one of the pigments, and its type and content is dependent upon the stage of maturity of seeds. In fully matured seeds, only 2 ppm of chlorophyll was observed (Przybylski, 2001). In addition to chlorophyll pigments, canola oil also contains carotenoids, which include lutein, neolutein A,

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neolutein B and β-carotene (Appelqvist, 1976). A bleaching step is necessary during oil processing to remove chlorophylls, other pigments and color bodies.

Triterpenoids

The triterpenoids fraction of canola oil is very complex and at least 13 components have been detected. The major constituents are: β-anyrin, cycloartenol and 24-methyl-cycloartenol (Hougen and Stefansson, 1982). Some of the triterpenoids are related to biosynthesis of sterols.

Protein

Nitrogen-containing substances in canola consist of 87% (w/w) proteins and 13% (w/w) peptides and amino acids. The canola proteins can be divided into three functional groups, storage proteins, structural proteins and enzymes. Storage proteins are the largest class of proteins that act mainly as a source of amino acids and nitrogen during seed germination. The structural proteins are the second largest class that consists of materials forming the membranes. The smallest group is the enzyme proteins that participate in enzymatic reactions. Myrosinase and lipase are the typical enzymes found in canola. The proteins in meal have a favorable amino acid composition. The content of such essential amino acids as lysine, methionine, cysteine, threonine and tryptophan in canola compares favorably with that found in other crops (Ohlson, 1978; Sarwar, 1984).

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Carbohydrates

Canola contains carbohydrates in the form of mono-, di- and polysaccharides as well as sulphur, steroid and anthocyanidin glycosides (heterosides). Sucrose is the major disaccharide occurring in canola. The mature seed contains very little starch. Polysaccharides include cellulose, hemicellulose, lignin, arabinogalactan, arabinan, amyloid and pectins. Most of the cellulose and hemicellulose are in the hull of the seed.

Glucosinolates

Plants of the *Cruciferae* family characteristically contain glucosinolates, a large group of sulphur-containing compounds. Glucosinolates are stable within the cytoplasm until brought into contact with myrosinase following tissue disruption. Myrosinases are enzymes, which can hydrolyze glycosinolates. Upon hydrolysis, glucosinolates yield glucose, aglycone and sulphate. The aglycone is not stable, leading to further reactions that yield thiocyanates, nitriles, isothiocyanates etc. These compounds inhibit the addition of iodine in the biosynthesis of certain hormones in the thyroid gland, thus resulting in their deficiency (Mithen et al., 2000). Glucosinolate content has been substantially reduced in the low-glucosinolate cultivars. In canola meal the glucosinolate content is less than 30 μmol/g (Daun, 1993).

Phytic acid

Phytic acid and phytates are common constitutes of cereals, legumes and oilseeds. Phytic acid is the main source of phosphate in canola, representing 78.6 to 88.1% (Naczk et al., 1986). Phytic acid typically exists in rapeseed/canola as salts of Mg, Ca and K. It

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is found inside protein bodies within the cells of the radicle, especially the cotyledons (Yiu et al., 1983). Phytic acid level in whole rapeseeds ranges from 2.0 to 4.0% (w/w) (Reddy et al., 1982) while the range for defatted meal is 2.0 to 5.0% (w/w) (Nwokolo and Bragg, 1977). Protein-phytic acid complexes have been reported to occur naturally in rapeseed (Lott, 1985). These complexes can cause poor bioavailability of minerals in animals. Therefore, it is necessary to supplement the feed with these elements.

Polyphenolics

Phenolic acids and condensed tannins are the predominant phenolic compounds found in rapeseed/canola (Naczk, 1998). Phenolics, such as free phenolic acids, sinapines and condensed tannins, may contribute to the bitter taste and astringency of rapeseed/canola products.

Inorganic constituents

Major inorganic constituents include S, P, I, Fe, Mg, and Ca while the trace inorganic constituents include Zn, Pb, Cd and Cu. Rapeseed/canola meal contains from 11.3 to 14.3 mg phosphorus per gram of meal.

1.4 Processing of canola oil

Seed cleaning

Prior to processing, the canola seed must be cleaned to remove foreign matters such as canola plant, weed seeds, other grains and dust etc. Typically, the cleaning equipment consists of aspiration, indent cleaning and sieving.

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Preconditioning and flaking

Preheating of canola seed to 30-40°C is carried out prior to flaking to prevent shattering. Preheating is generally carried out with indirect heating or direct hot air contact. The preheated canola seeds are flaked between two smooth surfaced cast-iron rollers. The objective of flaking is to produce flakes with a larger overall surface area to allow for easy contact of the seeds with solvent during the solvent extraction process.

Cooking/conditioning

Following the flaking operation, the canola flakes are heated to 75-100°C. There are several reasons for the cooking operation. This step adjusts the moisture content in canola flakes to the desirable level for proper screw pressing. During the heating, the minute lipid particles begin to coalesce to form larger oil droplets. Furthermore, the cooking action causes certain seed proteins to coagulate. This facilitates easy separation of protein and oil. Another important function is to deactivate some of the enzymes in canola, especially myrosinase and lipase. Myrosinase can hydrolyze the glycosinolates into various derivatives. Removal of these derivatives from oil is more difficult than the removal of native counterparts. Additionally, some glucosinolate derivatives will result in poor feeding performance of the finished meal. Lipase can hydrolyze phosphatides and TAG and the breakdown products make refining step more difficult.

Screw pressing

The conditioned seeds are subjected to screw pressing. With this unit operation, 60-70% of the oil is removed from the canola flakes. In addition, the screw pressing

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compresses the small fragile canola flakes into a more dense and durable cake to facilitate good solvent contact and percolation in the extractor. The oil and the solid portions are separated in a settling tank. The press cake is then subjected to further extraction with a solvent.

Solvent extraction

Hexane is the solvent most frequently used in modern extraction plants. The liquid hexane is heated to 50-60°C in order to achieve rapid extraction of the oil. The extracted oil consists mainly of TAG and small amounts of oil-soluble components such as phosphatides, chlorophyll break down products, and free fatty acids. This process of solvent extraction can reduce the oil content of the solid meal to about 1% (Unger, 1990).

Desolventizing

After the solvent extraction process, the hexane must be removed from the meal and oil. Several stages of heating and drying under low pressure and live steam are applied to evaporate hexane and to dry the meal. In this process, complete myrosinase inactivation is achieved. Furthermore, some glucosinolates and their break down products are removed from the canola meal.

Degumming

Crude oil recovered from the extraction process and the prepressing operation contains phosphatides, insoluble matter, free fatty acids and other colored bodies along with some other minor impurities. The purpose of degumming is to remove

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phosphatides, which tend to separate from the oil as sludge during storage. Two degumming methods commonly used are the aqueous degumming and acid-assisted degumming (Unger, 1990). Citric and malic acids are popular organic acids used in the latter process. The phosphatides or gums removed during the degumming process are added back to the meal to enhance the feed value of canola meal.

Refining

The crude, degummed oil must be further refined to remove phosphatides and free fatty acids to very low levels. There are two methods used for refining, namely, alkali refining and physical refining (Mag, 1990). In alkali refining, the oil is contacted with a base, to saponify free fatty acids. In physical refining, the free fatty acids are removed by distillation at low pressures and high temperatures.

Bleaching

Bleaching is a further cleanup process following alkali refining or acid degumming. In bleaching, adsorbents are used to remove compounds such as soap, chlorophylloids, carotenoids, phosphatides, oxidation products, and traces of iron and copper from the oil. Chlorophyll removal is the most important aspect of bleaching, since it catalyzes oil oxidation and gives an undesirable green color to the oil (Mag, 1990).

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Hydrogenation

In the process of hydrogenation, double bonds of the carbon chains of fatty acids in TAG are progressively saturated with hydrogen, which eliminates the possibility of oxidation. In addition to imparting oxidative stability to oil, hydrogenation changes the solidification behavior of the oil so as to facilitate the manufacture of margarine, shortening and other specialized products. An adverse effect of hydrogenation is the formation of *trans* fatty acids, which are often implicated in carcinogenesis (Mag, 1990).

Deodorization

The purpose of this process is to remove primarily odorful compounds from the oil in a steam distillation procedure. Other compounds such as free fatty acids are also removed in this process. Vegetable oil deodorizer distillate is a by-product of the refining process. It is a very complex mixture containing aldehydes, ketones, free fatty acids, glycerides and bioactive components such as sterols and tocopherols. Therefore, vegetable oil deodorizer distillate is an excellent source for commercial production of FFA, sterols and tocols. Properly deodorized oil is essentially bland in taste, light yellow in color, and low in the content of free fatty acids.

Winterization

Occasionally, canola oil contains a small amount of waxes (esters of long chain alcohol and long chain free fatty acids), which are crystalline at room temperature thus give a hazy appearance. With winterization, the wax content is brought down to less than 50 ppm, which gives canola oil a clear liquid appearance at refrigerator temperature.

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Interesterification

Like hydrogenation, interesterification is another method of changing the melting properties of the oil. In interesterification, the positions of fatty acids are redistributed randomly among the TAG mixture. New TAG compositions result with altered crystallization properties.

1.5 Nutritional aspects of canola

1.5.1 Canola oil

Canola oil as a recommendable dietary oil is gaining more and more attention. It is low in saturated fatty acids (6%) as compared to other vegetable oils and rich in oleic acid similar to olive oil. In the past, only polyunsaturated fatty acids were known to reduce the plasma cholesterol levels, whereas monounsaturated fatty acids were thought to have no effect on plasma cholesterol. However, in 1985, Mattson and Grundy first reported that monounsaturated fatty acids, namely oleic acid, were as effective as PUFA in reducing plasma total and LDL cholesterol levels. This finding helped gain canola oil gain more attention.

Canola oil contains higher levels of linoleic acid, an essential fatty acid, compared to olive oil. This fatty acid is a major component of brain tissue and cell membranes. It plays a major role in the growth and development of infants. Linoleic acid deficiency can lead to impaired organ growth and function. The central nervous system, the eye and platelets in the bloodstream are particularly vulnerable to a deficiency of linoleic acid (Watkins and German, 2002).

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In addition, canola oil contains high levels of α-linolenic acid (ALA), which is essential for humans. In the human body, linoleic acid and linolenic acid can be converted to other omega-3 fatty acids, namely eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA is a major constituent of lipids in the brain and the retina of the eye. They have been shown to lower blood cholesterol and triglyceride levels (Watkins and German, 2002). These omega-3 fatty acids protect against heart attacks and strokes, and they also play a role in reducing the inflammation of joints associated with rheumatoid arthritis.

ALA has been shown to decrease cytokines, which act as cellular mediators in the pathology of atherosclerosis in human and in mice. Moreover, n-3 ALA in vegetable oils has been shown to cause less serum lipid oxidation in rats as compared to fish oils. Compared to other dietary vegetable oils, the specific fatty acid composition of canola oil makes it a healthy choice for consumers.

According to the 2002 World Health Report, cardiovascular disease is the number one killer in the world, leading to over 12 million deaths per year. Over 4 million of these deaths can be attributed to the high blood cholesterol levels. High levels of blood cholesterol, particularly LDL cholesterol, are a major risk factor in cardiovascular disease.

Diets enriched with canola oil can reduce total cholesterol and LDL cholesterol in healthy humans (McDonald, 1989; Chan, 1991; Wardlaw, 1991) and are as effective as diets enriched with other vegetable oils. Canola oil was also found to be antithrombic, and has the ability to decrease platelet aggregation (Renaud et al., 1986; Lenz et al., 1990; Kwon et al., 1991)

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According to a recent study by Gulesserian and Widhalm (2002), a low fat/low cholesterol diet enriched with rapeseed oil led to a reduction of total cholesterol and LDL cholesterol of 10% and 7%, respectively, on the subjects with familial hypercholesterolemia. Moreover, TAGs were reduced by 29% and very low-density lipoprotein (VLDL)-cholesterol by 28% over a period of five months. HDL-cholesterol remained mainly unaffected during the whole study.

Mclennan and Dallimore (1995) carried out a study to compare canola oil and soybean oil, which has the same level of ALA as in canola, but a much higher level of linoleic acid. They concluded that only the experimental animals fed canola oil showed antiarrhythmic effect and experimental animals fed other oils had no effect. They suggested the balance between the dietary n-6 and n-3 fatty acids is an important factor. Canola oil contains a lower ratio of n-6 to n-3 (approx. 2:1, soybean oil is nearly 7:1) fatty acids, which may allow for better conversion of linolenic acid to eicosapentaenoic acid (EPA) known to protect against induced arrhythmias.

1.5.2 Vitamin E

Evans (1922) first discovered vitamin E as a nutritional factor that prevented the death and resorption of foetuses in pregnant rats. Vitamin E is a family name, which consists of eight natural compounds, namely α-, β-, γ-, and δ-tocopherols, and α-, β-, γ-, and δ- tocotrienols (Fig. 1.1). The richest sources of natural vitamin E are lipid-rich plant products, including cereals, oilseeds, nuts and vegetables (Bramley et al., 2000). In pure form, these compounds are yellow viscous liquid at room temperature. They are easily decomposed in the presence of light, oxygen, alkaline pH, or traces of transition

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metal ions (Bramley et al., 2000). Tocopherols and tocotrienols are the most important natural antioxidants in fats and oils.

Vitamin E is an important antioxidant in the body that deals with free radicals, the natural products of oxidative reactions in the body. These radicals can attack proteins, nucleic acids and phospholipids, causing impairment of normal cell activity and DNA damage (Bramley et al., 2000). Oxidized fatty acids or mutagens can lead to heart disease or cancer. Tocopherols and tocotrienols can donate hydrogen atoms from their phenolic groups to free radicals to neutralize them. This process makes tocopherol and tocotrienol molecules to form free radicals themselves, but these are stabilized through the electron delocalising within the aromatic ring. Generally, the antioxidant activity of tocopherols is in the order of δ-> γ-> β-> α- and tocotrienols exceed the activities of tocopherols (Shahidi, 2000). In addition to structural features, the antioxidant activities of tocols also depend on the concentration and the temperature employed. Some authors reported that tocopherols might exhibit prooxidant activity at high concentrations (Jung and Min, 1990). Furthermore, vitamin E and the products of membrane lipid hydrolysis, such as lysophospholipids and free fatty acids, can form complexes, which can stabilize membranes (Wang, 2000). The effectiveness of tocopherols as vitamins is different from their antioxidant potential. Alpha-tocopherol is the most important to human health because it has the highest vitamin activity (Kamal-Eldin and Appelqvist, 1996) and only (R, R, R)- α- tocopherol is preferentially retained and distributed throughout the body (Traber and Sies, 1996). Currently, the vitamin E activity is defined in terms of α-tocopherol equivalents (α- TE). RRR-α- tocopherol has an activity of 1 mg α-TE per mg compound and RRR-β-, RRR-γ- and RRR-δ- isomers have activities of 0.5, 0.1, 0.03 mg

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α-TE per mg compound, respectively (Sheppard, 1993). Of the tocotrienols, only α-tocotrienol has significant biological activity that is equivalent to 0.3 mg α-TE per mg compound (Eitenmiller, 1997). The activity of the naturally occurring α-tocopherol is about 30% higher than that of all the synthetic racemic α-tocopherols.

There have been a multitude of research activities surrounding the health benefits of vitamin E in the last two decades. Sodergren and his coworkers (2001) compared the effects of a rapeseed oil-based diet containing an increased proportion of easily oxidisable polyunsaturated fatty acids (PUFA) such as ALA with a diet rich in saturated fatty acids on the degree of lipid peroxidation in the human body. They found that a rapeseed oil-based diet rich in ALA does not seem to increase the degree of lipid peroxidation in plasma and urine compared to a diet rich in saturated fats. They attributed this effect to dietary rapeseed antioxidants that increased plasma concentrations of antioxidants.

In humans, vitamin E-deficient diets do not lead to a characteristic disease state or disorder. However, recent research suggests that vitamin E deficiency is associated with an elevated risk of atherosclerosis and other degenerative diseases. Meydani and his coworkers (1993) observed that vitamin E plays an important role in the maintenance of the immune system. Additionally, administration of vitamin E can significantly relieve symptoms in patients suffering from several types of acute or chronic inflammatory diseases (Blankenhorn, 1993). Moreover, vitamin E may protect against certain other diseases, such as cardiovascular disease, cancer, neurological disorders, cataract and age-related macular degeneration. The recommended dietary allowance for vitamin E is 30 mg / day (Dunford, 2001).

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1.5.3 Plant sterols

Plant sterols, also called phytosterols, are compounds, which are structurally related to cholesterol, differing in the side chain (Fig. 1.2). Sitosterol, stigmasterol, and campesterol are the three main plant sterols. Plant sterols can exist in four forms in plant materials, namely, free and esterified in the form of steryl glycosides and acylated steryl glycosides (Fig. 1.3) (Piironen et al., 2000). Plant stanols are hydrogenated products of the respective plant sterols, e.g. campestanol/campesterol and sitostanol/sitosterol, and are found in the plant kingdom at very low concentrations. At room temperature, plant sterols are solid. For instance, sitosterol, campesterol and stigmasterol have melting points of 140, 157-158 and 170°C, respectively (Akihisa, 1991). Plant sterols are essential components of the membranes of all eukaryotic organisms. Sterols regulate the fluidity and permeability of membranes and participate in the control of membrane-associated metabolic processes. Some plant sterols also have a specific function in signal transduction. In addition to their important role in plant cell membranes, plant sterols are also precursors of a group of plant growth factors and they also act as substrates for secondary metabolites such as the glycoalkaloids, cardenolides and saponins (Piironen et al., 2000). Sterols also play a role in cellular differentiation and proliferation. They may provide a reservoir for the growth of new cells and shoots during seed germination.

Vegetable oils are the richest natural sources of sterols, followed by cereal grains, and nuts. The commonly consumed plant sterols are sitosterol, stigmasterol and campesterol. Brassicasterol is a typical sterol in rapeseed/canola and other *cruciferaceae*. Sterols in vegetable oils occur mainly in free and ester forms. In soybean, olive and sunflower oils, free sterols are predominant (57-82%) while in canola and corn oils, free

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sterols account for only 33-38% (Phillips et al., 1999). During the industrial refining process; a substantial portion of sterols are lost due to direct removal and converted to other forms via oxidation, isomerisation and other transformation. Compared to crude corn, soybean and rapeseed oils, the total sterol content in refined counterparts is 36%, 18% and 24%, respectively, lower (Ferrari et al., 1996).

Several studies have demonstrated that plant sterols can reduce serum or plasma cholesterol and low-density lipid cholesterol levels in normal and mildly hypercholesterolaemic subjects (Peterson, 1951; Pollak, 1953; Weststrate and Meijer, 1998; Hendrinks et al., 1999). The consumption of average amounts (approximately 20 g) of spread per day supplemented with 8-10% (w/w) plant sterols can lower serum total cholesterol and LDL cholesterol by 8-13% (Piironen et al., 2000). This is equivalent to the consumption of 1.6-2.0 g of plant sterols per day. Although people consume plant sterols every day in their normal diet, the amount is not sufficient enough to have a significant blood cholesterol lowering effect. In order to achieve a cholesterol lowering effect, approximately 2 g of plant sterols have to be consumed daily (Hendriks et al., 1999). A recent study by Nestel (2001) found that sterol/stanol esters are more effective than sterol/stanol. After esterification, a 10-fold increase in solubility is achieved and that in turn made sterols/stanols more miscible with food constituents in a variety of products.

Phytosterols are also effective in the treatment of benign prostatic hyperplasia, rheumatoid arthritis, allergies, and stress related illness and inhibit the development of colon cancer (IFST, 2000).

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The exact mechanism by which plant sterols and stanols cause a decrease in serum cholesterol concentration is poorly understood. One explanation is that their interference with cholesterol incorporation in the micelles and, subsequently, inhibiting the absorption of cholesterol from the digestive tract (Ikeda, 1983). The formation of water-soluble micelles is a pre-requisite for the absorption of cholesterol. Because of the structural similarity between sterol molecules, plant sterols and stanols are able to compete with cholesterol for incorporation into micelles. The cholesterol that has not been incorporated in the micelles cannot be absorbed and is therefore excreted from the body.

There exist two common sources of sterols for commercial production. One is from soy oil refining by-product and another is tall oil, which is a byproduct from the process of paper production from wood. When edible oils undergo normal refining, plant sterols are partially extracted together with some tocopherols (in the process of natural vitamin E production). It is estimated that 2500 tonnes of vegetable oil needs to be refined or approximately 2500 tonnes of pinewood is required to produce 1 tonne of plant sterols. Plant stanols are obtained by hydrogenation of the plant sterols.

There are several sterol/stanol-based commercial products available in the market. "*Benecol*", manufactured by *McNeil* Consumer Healthcare, has been available in Finland since 1995. "*Take Control*" is a similar product manufactured by *Lipton*. The lipid-lowering properties of these products are attributed to the plant sterols or stanols ester, incorporated into the margarine base. "*Benecol*" contains sitostanol ester derived from wood pulp, whereas "*Take Control*" spread uses unsaturated plant sterols, mainly sitosterol ester, derived from soybean.

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1.5.4 Canola meal

The utilization of rapeseed/canola as a source of food-grade proteins is still limited by the presence of glucosinolates, phytates, hulls and phenolics in the reside meal (Naczk et al., 1998). Compared to soybean meal, canola meal contains lower levels of crude protein and some essential amino acids and has lower digestible or metabolizable energy (Bell, 1993). It contains more crude fiber and is richer in several minerals and several vitamins such as tocopherols, niacin, choline, riboflavin, biotin, folic acid and pyridoxine (Bell, 1993).

Canola meal contains glucosinolates but at much lower levels than were present in its predecessor, rapeseed meal. Glucosinolates and their hydrolysis products are responsible for the enlargement of thyroid glands (Heaney, 1995) inducing goitrogenic effects in humans (Langer, 1971). Apart from their goitrogenic properties, glucosinolates breakdown products induce other less specific toxic effects in laboratory rodents, including embryotoxicity and impairment of growth (Nishie, 1980), and enlargement of the liver without detectable evidence of histological changes (Nishie, 1982). On the contrary, according to some recently published reports (World Cancer Research Fund, 1997; Verhoeven, 1997; Hecht, 1999), glucosinolate breakdown products may exert anticarcinogenic activity in experimental models. The dark color and poor palatability of the meal are also associated with these products. The nutritional and biochemical properties of glucosinolates have been well reviewed by Mithen et al. (2000).

Canola meal may contain up to 12% (w/w) crude fiber as compared to 7% (w/w) in soybean meal. Much of the fiber is in the hull, which comprises about 16% by weight of canola seed, or about 30% in the extracted meal (Bell, 1993). Higher fiber

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content reflects lower value of the digestible or metabolizable energy of canola meal because fiber is largely indigestible by nonruminant animals (Eapen et al., 1969). Besides the crude fiber in the hull, the complex carbohydrates in the embryo are not very susceptible to enzymatic digestion in the gastrointestinal tract of monogastric animals such as pigs and poultry.

Concern about fiber in canola meal led to various studies on hull removal. Some researchers tried to soak the grains in water before milling (Sosulski, 1974) or grind whole canola seed in a stream of water (Eapen, 1969) while others investigated hydrothermal pretreatment for loosening the hull (Thakor et al., 1994). But to date, none of these treatments has appealed to the crushing industry. Various obstacles have arisen, including (1) loss of oil in the hulls and excessive fineness of the final meal if the hulls are removed before extracting, (2) small and uneven seed size make dehulling more difficult, and (3) poor separation of the meal and hulls.

Phenolic acids and condensed tannins are the predominant phenolic compounds found in rapeseed. The content of phenolic compounds in rapeseed/canola products is much higher than that found in corresponding products from other oleaginous seeds and up to five times higher than that in soybean meal (Kozlowska et al., 1991). Phenolics such as free phenolic acids, sinapines and condensed tannins may contribute to the dark color, bitter taste and astringency of rapeseed products (Ismail et al., 1981; Shahidi and Naczk, 1992). In addition, both phenolic acids and condensed tannins may form complexes with amino acids and proteins, thus lowering the nutritional value of rapeseed products. Phenolic compounds have been indentified as possible inhibitors of iron adsorption (Gillooly, 1983). This inhibition may be due to the formation of insoluble

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iron-phenol complexes in the gastrointestinal tract, thus making the iron unavailable for absorption (Brune et al., 1989).

Phytic acid is found mainly in the embryo/cotyledons, which is the main source of phosphorous and also a carrier of essential minerals, such as Mg, Ca, Mn and Zn (Morris, 1986). Phytates occur widely in cereal grains and oilseeds and are known to reduce the bioavailability of minerals through metal-chelating mechanisms that lead to Zn deficiency. Therefore, it is necessary to supplement the feed with these elements. In foods and feeds, the binding of phytic acid with proteins may inhibit the action of proteolytic enzymes, thus affecting the digestibility of proteins and thereby the availability of amino acids (Knuckles, 1985). Moreover, phytic acid has also been shown to influence the rate of starch digestion and absorption in vivo (Yoon et al., 1983).

1.6 Germination

Due to the very low level of moisture, the dry seeds have remarkably low rate of metabolism, a very low rate of respiration and a few insignificant changes in their chemical composition (Forbes and Watson, 1999). As soon as the seeds are hydrated, marked changes of composition in their various parts occur. These changes consist of three main processes, namely, the breakdown of certain reserve materials, the transportation of the breakdown products from one part of the seed to another (mainly from the endosperm to the embryo or from the cotyledons to the growing parts), and the synthesis of new materials from the breakdown products. In the initial stage, seeds normally take up water and oxygen. In this stage, dry matter loss occurs due to oxidation of substances and leakage of certain substances (e.g. germination inhibitors) out of the

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seed. Only when the seedling has formed, i.e. when a root has emerged and takes up minerals, and the cotyledons or first leave are exposed to light and are capable of photosynthesis, does an increase in dry weight begin.

The first step of germination of canola seed is the growth of radicle within the seed coat. The radicle extends mainly by elongation rather than the multiplication of cells. As soon as it bursts through the seed coat, the radicle quickly begins to function as a root, absorbing water and minerals and soon providing firm anchorage for the seedling. Following the emergence of the radicle, there is little growth of the plumule. Plumule is a portion of the embryo axis just below the cotyledons. The stem-like structure is known as the hypocotyls, which pushes its way up through the soil, carrying the entire plumule and the cotyledons. It is bent in a hook shape as it grows up. Once above the ground, it straightens out, which is triggered by light, and cotyledons expand greatly to become the first functional leaves of the new plant. Only at this stage does the plumule itself begin to grow, true leaves start to appear and eventually taking over from the cotyledons as the photosynthetic system. During germination, cotyledons show a loss of many nutrients, while all the other organs show an increase in the various constituents. Particularly striking is the increase in the size and dry weight of the rapidly growing hypocotyls.

During germination, two processes occur simultaneously, namely photosynthesis and respiration. Photosynthesis is a process that synthesizes the organic compounds by fixation of carbon using the energy of light. Light provides the energy to transfer electrons from water to nicotinamide adenine dinucleotide phosphate ($NADP^+$) forming NADPH that generates adenosine triphophate (ATP). ATP and NADPH provide the energy and electrons to reduce carbon dioxide (CO_2) to organic molecules. These

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organic molecules serve as the starting material for the synthesis of glucose and fructose by the Calvin cycle (Forbes and Watson, 1999). Glucose and fructose make the disaccharide sucrose, which travels in solution to other parts of the plant (e.g. fruit, roots). Glucose is also the monomer used in the synthesis of the polysaccharides starch and cellulose.

In terms of the inputs and outputs, respiration may be considered as the reverse of photosynthesis. In the process of respiration, the oxygen is taken in; carbohydrate is broken down and carbon dioxide is released. Similar to photosynthesis, respiration involves long metabolic pathways, requiring many enzymes and forming many intermediate products.

In the light, photosynthesis proceeds faster than respiration and therefore, carbohydrates are synthesized faster than it is broken down. This is the basis of dry matter production in crops (Mayer and Poljakoff-Mayver, 1989). In absolute darkness, the rate of photosynthesis is zero, however respiration continues, leading to dry matter loss.

The metabolic changes occurring in the early stages of germination are the result of the activity of various enzymes, which are either present in the dry seed or very rapidly become active as the seed imbibes water (Mayer and Poljakoff-Mayver, 1989). Generally, enzymes break down starch, proteins, hemicellulose, polyphosphates, lipids and other storage materials, and their activity rises fairly rapidly as germination proceeds.

The main storage carbohydrates are starch, oligo- and polysaccharides of the cell wall and soluble sugars. In a cereal seed, virtually all the energy is stored in the form of starch granules, which is insoluble and therefore cannot be moved from cell to cell. The

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seed needs α- and β-amylase to convert starch to sugars, the form that is transported via the scutellum to the embryo axis. Much of the sugar resulting from the mobilization of the reserves is not respired away but provides carbon skeletons for a wide rage of compounds, such as protein, lipids, nucleic acids etc. required by the growing plant. A large proportion of the sugar is assembled into cellulose for the building of cell walls.

Lipids are generally present in special organelles referred to as lipid bodies or sometimes as spherosomes. With the germination, the lipid is hydrolized by lipase to fatty acids and glycerol. The fatty acids are broken down further by β-oxidation, while the glycerol becomes part of the general carbohydrate pool present in the seed and as such becomes available for various processes including respiration. The process of β-oxidation cleaves fatty acids into two carbon units in the form of acetyl-CoA, which enters the tricarboxylic acid cycle or glyoxylate cycle where carbohydrates are synthesized (Trelease and Doman, 1984). In many seeds disappearance of lipids is accompanied by the appearance of carbohydrates. The course of fatty acid breakdown differed in the light and dark. In the light, all fatty acids are broken down at the same rate while in the dark linolenic acid disappeared at a greater rate than in the light (Mayer and Poljakoff-Mayver, 1989). Certain fatty acids may be released selectively by lipase attack. The lipase from *Brassica campestris* attacked triolein more slowly than either triacetin or tributyrin (Wetter, 1957). In seeds of cotton, linoleic and palmitic acids are broken down preferentially compared to stearic and oleic acids (Joshi and Doctor, 1975).

The storage proteins of most seeds are found primarily in subcellular organelles called protein bodies (Ashton, 1976). During germination the storage proteins are broken down and the protein bodies emptied. The sequence of breakdown of protein bodies in

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some of the cereals (monocotyledons) differs from that in seeds of dicotyledonous plants. The detailed mechanism of protein metabolism is still not very clear. It is generally agreed that the storage protein is hydrolyzed to amino acids by proteolytic enzymes, which can attack different size protein molecule at different site. The development of proteolytic enzyme activity appears to be under hormonal control. In the seeds of dicotyledonous plants, the hormones usually originate in the embryonic axis while the enzyme is formed in the cotyledons. The broken down amino acids may remain in the storage tissue but most are translocated to the developing axis tissues. They are used for the synthesis of various enzymes and structural proteins. Some of the amino acids may also undergo deamination. These products may then be used for the synthesis of non-nitrogen containing compounds or further metabolized to yield energy (Ashton, 1976). Breakdown of storage protein in the cotyledons is accompanied by the appearance of new protein in other parts of the seedling. But usually there is little change in the total nitrogen content of the seed or seedling during germination, although slight losses may occur, especially due to leaching out of nitrogenous substances.

1.7 Literature review and thesis objectives

It has been reported that germination has some beneficial effects on rapeseed. Mahajan (1997) reported that some antinutritional factors, such as glucosinolates, phenols and phytic acid decrease with the germination of rapeseed. Also reported was an increase of total protein content from 25.1% to 32.6% (w/w) after 8 days of germination in the dark at 22±2°C.

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Thompson and Serraino (1985) reported that 2 days of germination of rapeseeds in the dark at 20-22°C reduced phytic acid by 13% and slightly increased the fat and protein by 6 and 4%, respectively. After 7 days, a 65% decrease in phytic acid, 46% decrease in fat and 10% increase in protein was reported. The FFA concentration increased quite significantly.

Some researchers reported that the content of lysine, threonine, valine and leucine increased while that of methionine and phenylalanine decreased with rapeseed germination (Cho-BM, 1985). Kim and his coworkers (1988) found that the content of sugars, such as fructose, glucose and sucrose decreased at the beginning and then recovered with an overall decrease. They (1997) also reported that the levels of vitamin C greatly increased while the level of vitamin B group decreased.

Riout (1973) observed that the fat content decreased from 47.2% to 10.10% in the 7- day-old rapeseed seedlings while acidity index increased from 1.7 to 8.9. There was little change in the gadoleic, erucic, oleic and linoleic acid contents, but the level of gadoleic and erucic acids decreased while oleic and linoleic acid concentrations increased during day 4-7. Chung et al. (1989) reported that the fatty acid and amino acid composition in canola remained unchanged while the lipid fractions underwent a considerable change. There was a significant decrease in TAG content while FFA, DAG and phospholipids contents increased.

Mahajan et al. (1999) reported that meals obtained from germinated rapeseeds had improved water absorption capacity (WAC), protein solubility, foam capacity and viscosity.

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There is little information on the variation of minor valuable compounds such as sterols and tocols content during the germination of canola seeds. Understanding of these compositional changes during germination may lead to novel value-added opportunities for Canadian canola industry. Therefore, the **objectives** of this study were:

1. To determine the effect of germination under light and dark conditions on the compositional changes in canola grain/seedling, which include:
    - Oil profile (TAG, DAG, MAG and FFA)
    - Protein content and solubility
    - Sterols
    - Tocols
2. To determine the composition and distribution of these components in the seedling tops and bottoms
3. To suggest new value-added opportunities by evaluating the compositional changes during germination

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Table 1.1 Canola production in Canada by province (×10^3 Tonnes)[1]

Year	Ontario	Manitoba	Saskatchewan	Alberta	British Columbia	Canada
1990	43.1	460.4	1,451.5	1,281.4	29.5	3,265.9
1991	45.4	796.1	1,723.7	1,621.6	37.4	4,224.2
1992	29.5	986.6	1,474.2	1,349.4	32.7	3,872.4
1993	38.6	907.2	2,381.4	2,109.2	43.1	5,479.5
1994	45.4	1,485.5	3,175.1	2,472.1	54.4	7,232.5
1995	68	1,227.0	2,653.5	2,426.7	61.2	6,436.4
1996	45.5	1,068.2	2,245.3	1,678.3	19.1	5,056.4
1997	54.4	1,496.9	2,651.2	2,041.2	22.7	6,266.4
1998	56.7	1,803.0	3,220.5	2,449.4	61.2	7,587.8
1999	54.4	1,707.80	3,975.7	2,971.0	62.4	8,798.3
2000	32.9	1,486.6	3,354.3	2,154.6	45.4	7,085.8
2001	31.3	1145.3	2,109.2	1,723.7	42.5	5,062.0
2002	44.2	1,406.1	1,304.1	793.8	15.9	3,577.1
2003	45.4	1,474.2	2,653.5	1,995.80	36.3	6,229.7

[1]Source: Field Crop Reporting Series - Statistics Canada (2003)

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Table 1.2 Canadian canola crushing totals for the year 2003 ($\times 10^3$ Tonnes)[1]

	Total Crush	Oil Production	Meal Production
Aug-02	191	81	118
Sep-02	217	92	136
Oct-02	193	80	121
Nov-02	168	68	105
Dec-02	145	61	92
Jan-03	146	61	92
Feb-03	186	77	116
Mar-03	192	80	120
Apr-03	188	78	115
May-03	181	76	113
Jun-03	198	81	122
TOTAL	2,004	834	1,252

[1]Source: Cereals & Oilseeds Review - Statistics Canada (2003)

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Table 1.3 Chemical composition of canola and soybean grains (Slominski et al., 1999)

Component (%)	Canola	Soybean
Protein	25.7	41.4
Oil	41.5	20.2
Sucrose	4.1	5.2
Oligosaccharides[1]	2	5.1
Starch	0.4	nd[3]
Total fiber:	20.4	18.8
- Non-starch polysaccharides	11.8	14.7
- Lignin and others[2]	8.6	4.1
Ash	5	5.4
Calcium	0.46	0.39
Phytate phosphorus	0.55	0.51
Non-phytate phosphorus	0.34	0.12

[1] Includes raffinose and stachyose; [2] Includes lignin with associated polyphenols, cell wall protein and minerals associated with the fiber fraction; [3] Not detected

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Table 1.4 Constituents of some vegetable oils (Mag, 1990)

	Canola	Rapeseed	Soybean	Sunflower
Triglycerides (%)	94.4-99.1	91.8-99.0	93.0-99.2	-[1]
Free fatty acids (%)	0.4-1.2	0.5-1.8	0.3-1.0	0.5-1.0
Phospholipids (%)				
Water degummed	Up to 0.6	Up to 0.8	Up to 0.4	-
Acid – water degummed	Up to 0.1	-	-	-
Unsaponifiables (%)	0.5 – 1.2	0.5 – 1.2	0.5 – 1.6	0.3-1.5
Chlorophylls (ppm)	5-35	5-35	trace	trace
Sulphur compunds (as S, ppm)	3-15	5-25	nil	nil
Tocopherols (ppm)	700-1,000	700-1,000	1,700-2,200	~700
Total sterols (g/kg)	2.5-7.3	2.5-7.3	2.21-3.28	3.74-7.25

[1] data is not available

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Table1.5 Comparison of major fatty acids in some vegetable oils (w/w %) (Ackman, 1990)

Fatty acid	Canola	HEAR	Soybean	Sunflower	LLFlax	Corn
C10:0	-	-	-	-	-	-
C12:0	-	-	-	-	-	-
C14:0	0.1	-	0.1	-	0.1	-
C16:0	3.5	4.0	10.8	6.2	6.3	11.4
C18:0	1.5	1.0	4.0	4.7	4.1	1.9
C20:0	0.6	1.0	-	-	0.1	-
C22:0	0.3	0.8	-	-	0.1	-
Total saturated	6.0	6.9	14.9	10.9	10.4	13.3
C16:1	0.2	0.3	0.2	0.2	0.1	0.1
C18:1	60.1	15.0	23.8	20.4	16.5	25.3
C20:1	1.4	10.0	0.2	-	0.1	-
C22:1	0.2	45.1	-	-	-	-
Total MUFA	61.9	70.1	24.2	20.6	16.7	25.4
C18:2n-6	20.1	14.1	53.3	68.8	69.5	60.7
C18:3n-3	9.6	9.1	7.1	-	1.8	-
Total PUFA	29.7	23.2	60.4	68.8	71.4	60.7

Abbreviations: HEAR- High erucic acid rapeseed; MUFA-Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids; LLFlax – Flaxseed oil with reduced content of linolenic acid.

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Table 1.6 Tocopherol contents of selected vegetable oils (ppm)[1]

Oil	α	β	γ	δ	P-8
HEAR	268.0	-	426.0	-	96.8
Canola	272.1	0.1	423.2	-	74.8
LLCanola	149.8	-	313.6	7.1	46.5
HOCanola	226.3	-	201.6	2.7	42.2
HOLLCanola	285.8	-	607.2	8.2	82.5
Soybean	116.0	34.0	737.0	275.0	-
Sunflower	613.0	17.0	18.9	-	-
Corn	134.0	18.0	412.0	39.0	-
LLFlax	25.8	-	212.6	9.2	129.3

[1]Adpted from Canola Council of Canada. Abbreviations: P-8-Plastochromanol-8; HEAR- high erucic acid rapeseed; LLCanola – low linolenic acid canola; HOCanola- high oleic acid canola; HOLLCanola- high oleic acid and low linolenic acid canola; LLFlax- low linolenic acid flax; P-8- Plastochromanol-8.

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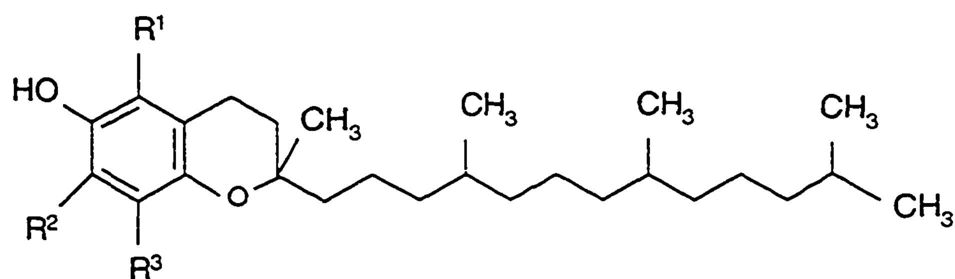
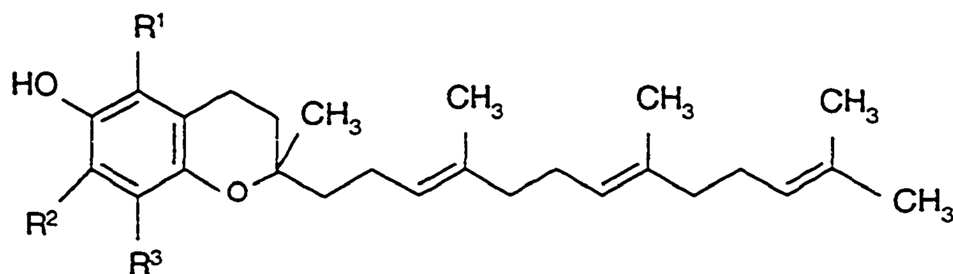
Table 1.7 Composition of major sterols in selected vegetable oils (w/w %)[1]

Sterol	HEAR	Canola	LLCanola	HOCanola	HOLLCanola	Soybean	Corn
Cholesterol	0.4	0.1	0.1	0.1	0.1	0.3	0.1
Brassicasterol	13.2	13.8	12.2	10.8	16.2	-	-
Campesterol	34.4	27.6	31.2	33.9	28.8	18.1	17.2
Stigmasterol	0.3	0.5	0.2	0.8	0.1	15.2	6.3
β-sitosterol	47.9	52.3	51.3	48.7	50.9	54.1	60.3
Δ5-avenasterol	2.1	1.9	1.9	1.8	2.1	2.5	10.5
Δ7-avenasterol	1.6	1.1	1.1	1.9	0.8	2.0	1.1
Δ7-stigmasterol	2.1	2.3	2.1	2.1	2.3	1.4	1.8
Total (mg/kg)	8810.0	6900	6326.0	7102.0	6892.3	4600	9700
Esterified (mg)	4356.8	4231.5	3987.6	4356.8	4156.2	576.4	5654.8

[1]Adpted from Canola Council of Canada. Abbreviations: HEAR- high erucic acid rapeseed; LLCanola – low linolenic acid canola; HOCanola- high oleic acid canola; HOLLCanola- high oleic acid and low linolenic acid canola.



Compound	R^1	R^2	R^3
α -Tocopherol	CH_3	CH_3	CH_3
β-Tocopherol	CH_3	H	CH_3
γ-Tocopherol	H	CH_3	CH_3
δ-Tocopherol	H	H	CH_3



Compound	R^1	R^2	R^3
α -Tocotrienol	CH_3	CH_3	CH_3
β- Tocotrienol	CH_3	H	CH_3
γ- Tocotrienol	H	CH_3	CH_3
δ- Tocotrienol	H	H	CH_3

Figure 1.1 Structure of tocols

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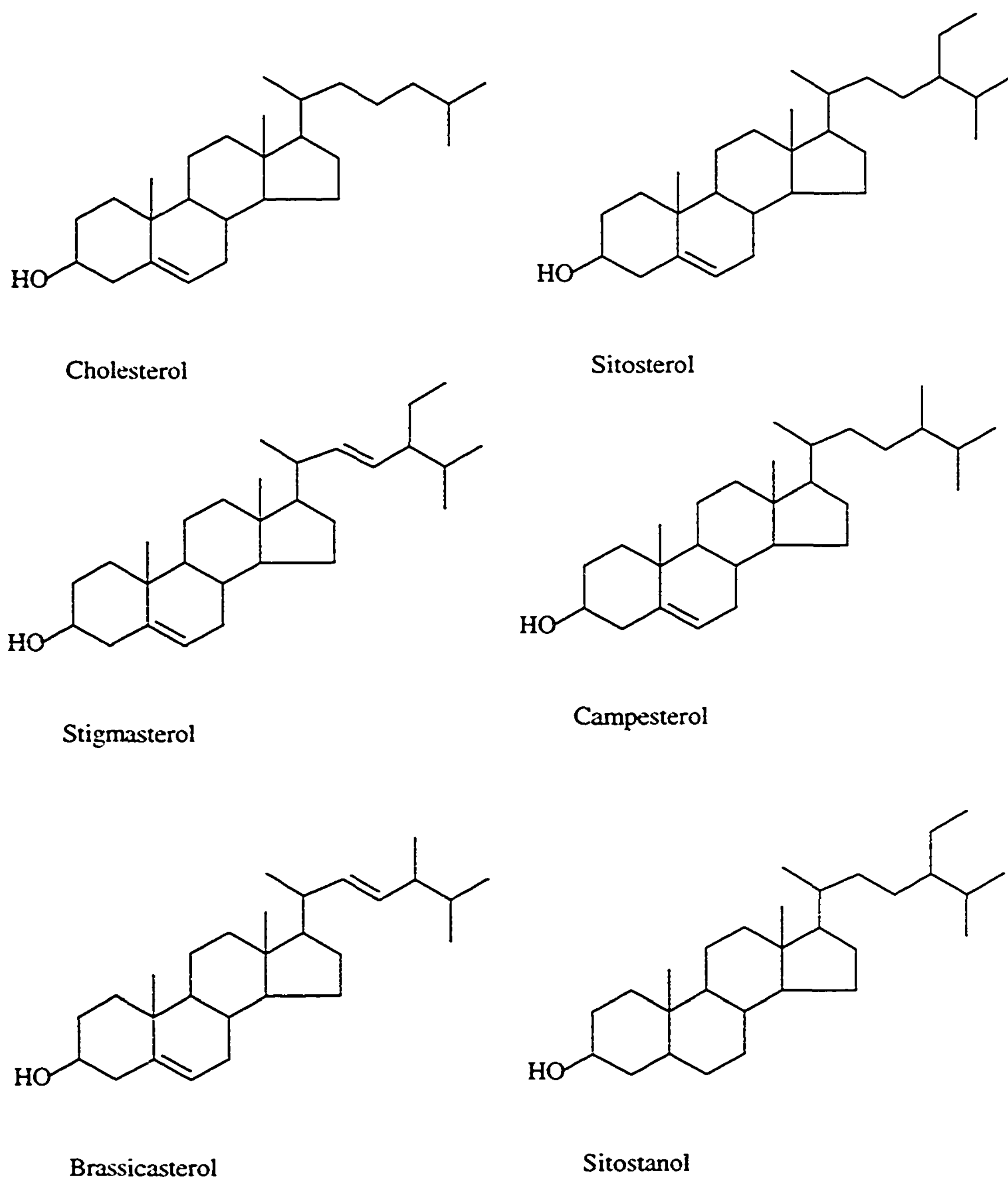

Figure 1.2 Structures of major plant sterols/stanols

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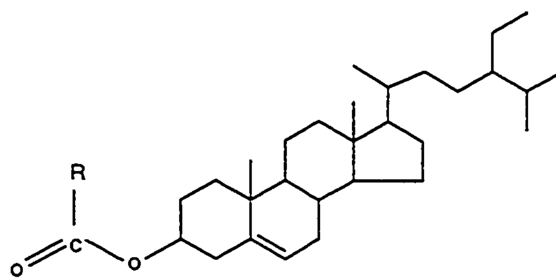
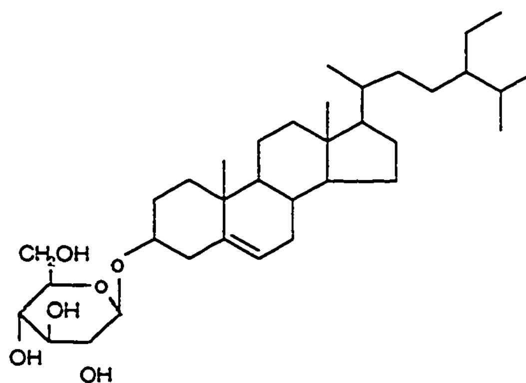
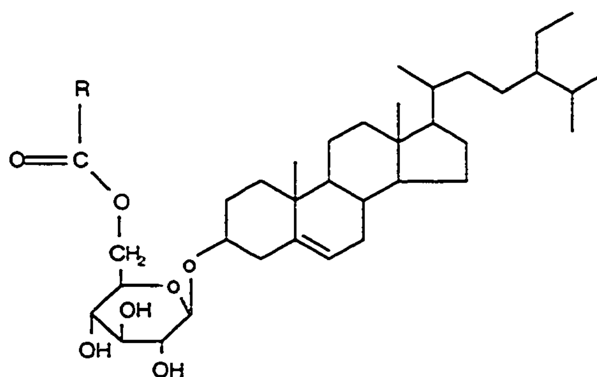


Sitosterol ester



Sitosteryl β-D-glucoside



Acylated sitosteryl β-D-glucoside

Figure 1.3 Examples of steryl conjugate structures

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2.0 DRY MATTER, LIPIDS AND PROTEINS OF CANOLA SEEDS AS AFFECTED BY GERMINATION AND SUBSEQUENT SEEDLING GROWTH IN ILLUMINATED AND DARK ENVIRONMENTS

2.1 Introduction

Canola is a member of the *Cruciferae* family and is an important cash crop in Canada (Daun, 1993). Canola seed contains both nutrients, such as lipids, proteins, and vitamins, and antinutrients, such as phytic acid and glucosinolates. Therefore, an understanding of the mobilization of seed reserves is important to explore the possibility of employing germination as a means for reducing antinutrients while increasing the content of nutritionally important constituents. The amount and proportions of seed reserves determine the nutritional value of seeds used for human and animal consumption. Effects of germination on major components in seeds from several crops have been investigated (Riout, 1973; Thomposn and Serraino, 1985; Mostafa and Rahma 1987; Kim, 1988). For instance, the changes in the cell wall polysaccharides of white mustard have been reported (Gould et al., 1965). Mobilization of lipids in mustard and rapeseed has also been investigated (Goulds and Rees, 1965; Lin and Huang, 1983; Hills and Murphy, 1988). Changes in protein reserves of mustard seeds have been reported (Bhandari and Chitralekha, 1984).

Majority of these investigations are limited to gross changes in cotyledons only. In this study, the main objective was to investigate the effect of germination stage and light on changes in major seed reserves, namely lipids and proteins. In addition, attempts have been made to understand the changes in reserves in leafy parts (seedling tops) and

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stem/root parts (seedling bottoms) of the seedlings at various stages of germination growth under illuminated and dark conditions.

2.2 Materials and Methods

2.2.1 Materials

Canola seeds (pls) Q2 summer rape (*Brassica napus* L.) were donated by Agricore United (Calgary, AB, Canada). Authentic C18: 1 glycerides standard mixture, dihydrocholesterol and bis(trimethylsily)-trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) (99:1) were procured from Sigma Chemical Co. (St. Louis, MO) whilst hexane was from Fisher Scientific Co. (Nepean, ON).

2.2.2. Methods

2.2.2.1 Sample preparation and dry matter analysis

Five grams of canola seeds were soaked in 200 ml distilled water for 4 h. After draining excess water, three seed samples were used as the control and the others were allowed to germinate in Petri dishes lined with moistened filter paper and paper towels. The germination was carried out in the presence and absence of light in a greenhouse at 20°C (Nykiforuk and Johnson-Flanagan, 1994). For the former, light was provided for 16 h/day while for the latter, the dish was covered with aluminum foil to block the light. The light was supplied by 400W high pressure sodium bulbs. The light intensity is 200-250 millivolt. The germinating seeds and seedlings were hydrated twice daily using tap water until the seedlings were harvested at intervals of 2, 5, 10, 15 and 20 days of germination, and subjected to analysis. Portions of 10-, 15- and 20-day samples were dissected into

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two sections as shown in Fig. 2.1. The leafy top portion was labeled as "seedling tops" while the remaining portion (stem, roots and seed coat) was labeled as "seedling bottoms". Whole seedlings were also subjected to analysis. The harvested samples were frozen in liquid nitrogen, lyophilized and then weighed. The dry matter loss during germination was determined using weight difference.

2.2.2.2 Determination of crude oil content

Crude oil content was determined gravimetrically. Approximately 2 g sample were ground to powder in a coffee grinder. The oil was extracted by refluxing the sample with hexane for 6 h using a Goldfisch apparatus according to AACC Method 30-20 (AACC, 1982). After the extraction was completed, the mixture was transferred to a pre-weighed glass tube. The extracted oil was then recovered by evaporation of the solvent under a stream of nitrogen at 40°C water bath in fume hood. The oil and glass tube were weighed to determine the oil content of the sample. The oil was then stored at -18°C under nitrogen in an amber glass vial until used. The defatted meal was also kept at -18°C an amber glass container until used.

2.2.2.3 Determination of lipid profiles

2.2.2.3.1 Sample preparation

The content of MAG, DAG, TAG and FFA were determined. Approximately 1 mg oil and 0.1 mg dihydrocholesterol internal standard were derivatized in 0.25 ml pyridine and 0.25 ml BSTFA containing 1% TMCS at 70°C for 30 min. The mixture was then dissolved in hexane and analyzed by gas chromatography.

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2.2.2.3.2 GC analysis

DB-5HT fused silica capillary column (J&W Scientific) (30m × 0.25mm i.d., 0.1µm film thickness, Agilient Technologies) in a Varian 3400 gas chromatograph (Walnut Greek, CA) equipped with a flame ionization detector and a Varian 8100 autosampler were used. Helium was the carrier gas used at a head pressure of 25 psi. Chromatograms were recorded and the peak integration was carried out using Shimadzu Class-VP software (version 4.2, Shimadzu Scientific Institutes, Inc., Columbia, MD). An initial column temperature of 60°C was maintained for 2 min, ramped first at 30°C min^{-1} to 140°C and then at 5°C min^{-1} to 235°C where this temperature was maintained for 7 min. The column temperature was then ramped to 350°C at 5°C min^{-1}, maintained for 3 min before ramping at a rate of 10°C min^{-1} to a temperature of 380°C where this temperature was maintained for 14 min. An initial injector temperature of 70°C was maintained for 0.1 min and then ramped to 370°C at 150°C min^{-1} where this temperature was maintained for 69 min. The detector temperature was set at 370°C. Authentic standards of FFA, and MAG, DAG and TAG of C16:0, C18:1 and C20:0 were run along with the same batch as samples for qualitative and quantitative analyses.

2.2.2.3.3 Qualitative analysis

Canola oil is a very complex mixture, which contains more than 100 components of various lipid classes. During germination, the breakdown of some of the components further increases the complexity, resulting in poor resolution of peaks. The carbon chain-length of almost 99.5% fatty acids in canola oil is between 16 to 20 carbons (Table 1.5) and therefore, this study used authentic C16 and C20 fatty acids and acylglycerols to

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identify different lipid classes. The unidentified components with retention times less than that of C16 fatty acids were also grouped as "volatiles". Almost 95% - 99% of components in the samples were identified using this approach. A representative gas chromatogram is shown in Fig. 2.2.

2.2.2.3.4 Quantitative analysis

Quantification of each group of lipids was carried out using relative response factor (RRF) for internal standards of fatty acids from the C18 family and C18:1 glycerides standard mixture (containing equal amount of FFA, and MAG, DAG and TAG). All calculations were based on the following formulae:

RRF= (amount of std. × area of I.S.) / (amount of I.S. × area of std.)

Amount of X = RRF × amount of I.S. × area of X / area of I.S

Note: X is the target compound.

2.2.2.4 Determination of protein content and their solubility

Total (crude) protein content of the defatted meal was calculated by multiplying the nitrogen content with a conversion factor of 6.25. The nitrogen content was determined using a nitrogen analyzer (Model FP-428, Leco Instruments Ltd., Mississauga, ON). The solubility of proteins was assessed after dissolving 0.7 g of defatted meal in 30 ml of distilled water. The mixture was shaken for 2 h at room temperature (20°C) and then centrifuged at 3000 rpm for 15 min. The supernatant and the residue were collected in pre-weighed plastic tubes, lyophilized in a freeze-dryer and weighed. The protein content in each fraction was determined using the same method

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explained for total protein content. The protein solubility was calculated based on the dry weight of soluble and insoluble fractions.

2.2.2.5 Statistical analysis

All germination experiments were carried out in replicates and analyses were carried out in duplicates. Analysis of variance of the results was performed using General Linear Model procedure of SAS Statistical Software, Version 8 (SAS Institute Inc., 1999). The effect of germination, light and seedling part on the content and composition of all components were evaluated at a significance level of 0.05.

2.3 Results and discussion

2.3.1 Changes in dry matter content

Figure 2.3 shows the significant ($p<0.05$) changes in dry matter content of germinating seed/seedling in the presence and absence of light. Ratio of dry matter (seedling wt./seed wt.) in the presence of light slightly decreased up to day-2 and then increased to 1.25 on day-20 of seedling growth whilst that in the absence of light decreased from 1.00 on day-0 to 0.66 on day-20. These results indicate the accumulation of biomass in the presence of light and may be attributed to the synthesis of structural carbohydrates through photosynthesis after the emergence of first leaves. The decrease in the ratio of dry matter observed for seedlings grown in the dark is consistent with other studies (Huang and Grunwald, 1988; Chung et al., 1989), which may be attributed to the absence of photosynthesis and consumption of carbohydrates in the course of respiration and utilization of food reserves by the growing seedling. Under illuminated conditions, a

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slight decrease in dry matter on day-2 maybe due to respiration of the seed and leaking of certain substances during 4 h soaking before germination. By day-5, the roots have already emerged and started taking up minerals from water. Furthermore, the cotyledons were capable of photosynthesizing causing the accumulation of biomass. On day-5, the accumulation was not enough to compensate the prior loss; therefore, the ratio of dry matter was still slightly lower than 1.

2.3.2 Changes in oil content

Figure 2.4 depicts the oil content of canola seeds and seedlings during the process of germination in the presence and absence of light. There was a drastic decrease ($p<0.05$) in the lipid reserves of seedling during the first 10 days of germination under both light and dark conditions (Fig. 2.4A). Oil content remained stable up to day-2, where it started to deplete rapidly from about 50% (w/w) to about 10-20% (w/w) on day-20. The decrease in total lipid content has been reported by many researchers (Thompson and Serraino, 1985; Huang and Grunwald, 1988; Chang et al., 1989; Qouta et al., 1991). The difference between the oil content (seedling dry wt. basis) in seedlings grown under illuminated and dark conditions may be attributed to differences in dry matter content. For this reason, changes in oil content during germination are best expressed on the basis of initial seed mass (Fig. 2.4B). Based on these results, light is not a crucial factor ($p>0.05$) in terms of the breakdown of lipids. In addition, there was no change in oil content up to 2 days of germination. This implies that the breakdown of lipid does not occur at early stages of germination.

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Seedlings with developed stem and leaves were obtained at 10th day onwards. Under both illuminated and dark conditions, oil content in seedling tops decreased through day-10 and -15 and then increased beyond day-15 (Fig. 2.5A). In contrast, seedling bottoms showed an opposite trend (Fig. 2.5B). This may be due to the translocation of lipids from cotyledons to stems/roots before consumption by the growing seedlings. During day-10 and 20 period under illuminated conditions, the oil content in seedling tops decreased from ~25% (w/w) to ~14% (w/w) while the decrease in oil content under dark conditions was from ~25% (w/w) to ~19% (w/w) (Fig. 2.5A). In contrast, the oil content in seedling bottoms under both illuminated and dark conditions reached to ~9% (w/w) on the day-20 of the trial (Fig. 2.5B). This shows that the stems and roots were highly dependant on the original lipid reserves as compared to the relatively low dependency of leaves and buds. Utilization of more lipids under dark conditions was also evident.

2.3.3 Changes in different lipid classes

Figure 2.6A depicts different lipid classes as a percentage of total lipids extracted from seedlings grown under illuminated conditions. In seeds, the predominant lipid class was TAG (98%) while only traces of free fatty acids and MAG were present. Contribution of DAG to total lipids was approximately 1.4%. Results suggest the heavy dependence of seedlings on TAG ($p<0.05$) regardless of the growing conditions. FFA content dramatically increased ($p<0.05$) from trace to about 47% under light (Fig. 2.6A) and to about 63% in the dark (Fig. 2.6B). For both illuminated and dark conditions, the changes in MAG and DAG content were also significant ($P<0.05$) but not as dramatic as

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in the content of FFA and TAG. Chang et al (1989) and Huang and Grunwald (1990) reported similar observations. This may be due to coordinated mobilization and hydrolysis of lipid reserves by the growing seedlings. The effect of the main factor of light is not significant (P>0.05). The predominant fatty acid found in the FFA fraction was 18:1.

Changes in lipid classes in 10-, 15- and 20-day old seedling tops and bottoms were also monitored for both illuminated and dark growing conditions (Fig. 2.7A-D). Changes were very similar to those observed for whole seedlings. Fifteen days after germination under illuminated conditions, a rapid increase of FFA and concurrent decrease of TAG in seedling tops and bottoms were observed (Fig. 2.7A and B). Under dark conditions, however, a relatively high amount of FFA was evident by day-10 of the trial (Fig. 2.7C and D).

Lipid is the major food reserve in canola seeds and is stored in the form of lipid bodies, which consists of TAG surrounded by a membrane (Yatsu et al., 1971; Yutsu and Jacks, 1972). The biochemistry of lipid reserve mobilization in many oil-rich seeds has been studied in detail. Lipases, located in the surrounding membranes, hydrolyze TAG and liberate fatty acids, which are metabolized through beta-oxidation. The acetate generated is then enters the glyoxylate cycle (Beevers, 1969; Hutton and Stumpf, 1969).

2.3.4 Changes in protein content and solubility

As shown in Fig. 2.8A, crude protein content of seedlings grown under illuminated conditions showed a slight decrease during the first 5 days and then remained stable up to day-15 and then depleted rapidly until day-20. These observations are

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somewhat different from those of other studies (Thompson and Serraino, 1985; Chung et al., 1989; Mahajan, 1997), in which increased protein content were reported. A similar ($p>0.05$) decreasing trend was observed for the crude protein content in seedlings grown under dark conditions (Fig. 2.8A). The only difference was that the protein was depleted within the first 10 days, then kept constant for 5 days and then decreased again.

As shown in Fig. 2.8B, solubility of proteins kept gradually increasing in seedlings grown under illuminated conditions. This increase also happened at a slower rate in the seedlings grown under dark conditions, it stopped at day-10 and then started to decrease. The fact that germination improved the protein solubility of rapeseed meal has been reported by Mahajan et al. (1999). For seedlings grown under both illuminated and dark conditions, insoluble proteins were the major part of the total protein content and contributed the most depletion to the total protein depletion (Fig. 2.9), while soluble protein remained stable during the whole study period under both conditions (Fig. 2.9).

For the tops and bottoms of seedlings grown under illuminated conditions, the crude and insoluble protein content decreased while the soluble proteins remained stable (Fig. 2.10A and B). Under dark conditions, however, an increase in crude proteins and soluble proteins in seedling tops was observed while the proportion of insoluble to soluble reached almost equal amount at day 20 (Fig. 2.10C). A similar trend was observed for the seedling bottoms grown under dark conditions (Fig. 2.10D). Figure 2.11 summarized the protein solubility for the different seedling parts under the two growing conditions. In general, the solubility of proteins for parts obtained from seedlings grown under dark conditions was higher than that under light condition, which was consistent with the observation for whole seedlings. The protein solubility of seedling top part was

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higher than the bottom part under both conditions. The solubility of seedling top part increased with the duration of the germination. However, the solubility of seedling bottom was stable under dark condition, with some fluctuation for the sample grown under light condition.

During the early stages of seedling growth, embryonic tissues obtain energy for metabolism from mobilized seed reserves (Bewley and Black, 1981). The function of seed storage proteins is to provide free amino acids to the growing seedlings. Amino acids are translocated from the membrane-bound aleurone bodies to the growing axis where they are used for the synthesis of enzymes and structural proteins (Ashton, 1976). There is evidence showing that some amino acids are de-aminated and used for the synthesis of non-nitrogen-containing compounds or further metabolized for energy.

2.4 Conclusions

During the 20-day period of germination, oil and protein reserves were depleted to supply energy and building materials for seed growth ($p<0.05$). The factor of light had no significant effect on this depletion ($p>0.05$). Light did, however, have an effect on biomass accumulation, which resulted in a decrease of dry matter of sample grown under dark conditions, and an increase under illuminated conditions. The light also caused some effect on the protein solubility. With light, the protein solubility improved faster than that without light. The breakdown of TAG leaded to a dramatic rise in free fatty acids, and significant changes ($p<0.05$) in MAG and DAG were also observed. A faster rate of lipid breakdown under dark conditions than under illuminated conditions was

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evident. The changes in fat profile in seedling tops and bottoms were similar to that of the whole seedlings.

Hydrolysis of TAG caused FFA levels in oil extracted from the seedlings to rise from almost zero at the beginning to 50-60% at the end of the 20-day period. One negative aspect of this increase in the free fatty acid levels is the potential generation of rancid odor in the oil. However, germination may provide an alternative, less expensive, environmentally friendly natural means of manufacturing FFA for food and non-food uses because the process of germination does not require externally added enzymes or chemicals for TAG hydrolysis.

Increase of protein solubility may find potential applications in food products, in which gelation, emulsification and foaming properties are required (Mahajan et al., 1999). Also, high protein solubility will be beneficial in the animal feeds.

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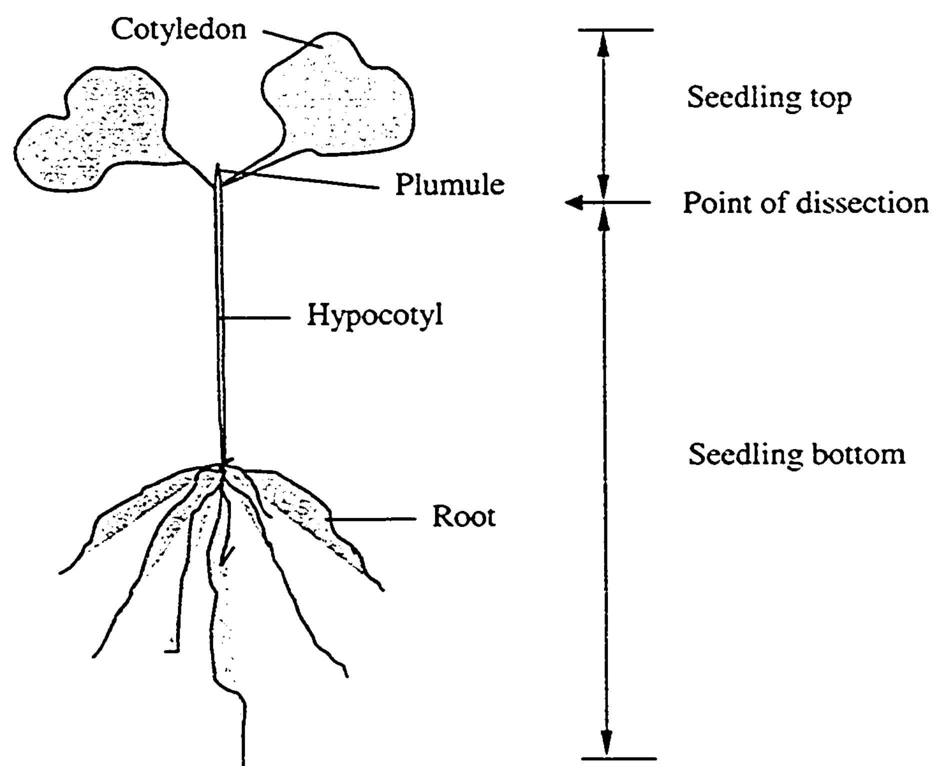

Figure 2.1 Schematic diagram of canola seedling.

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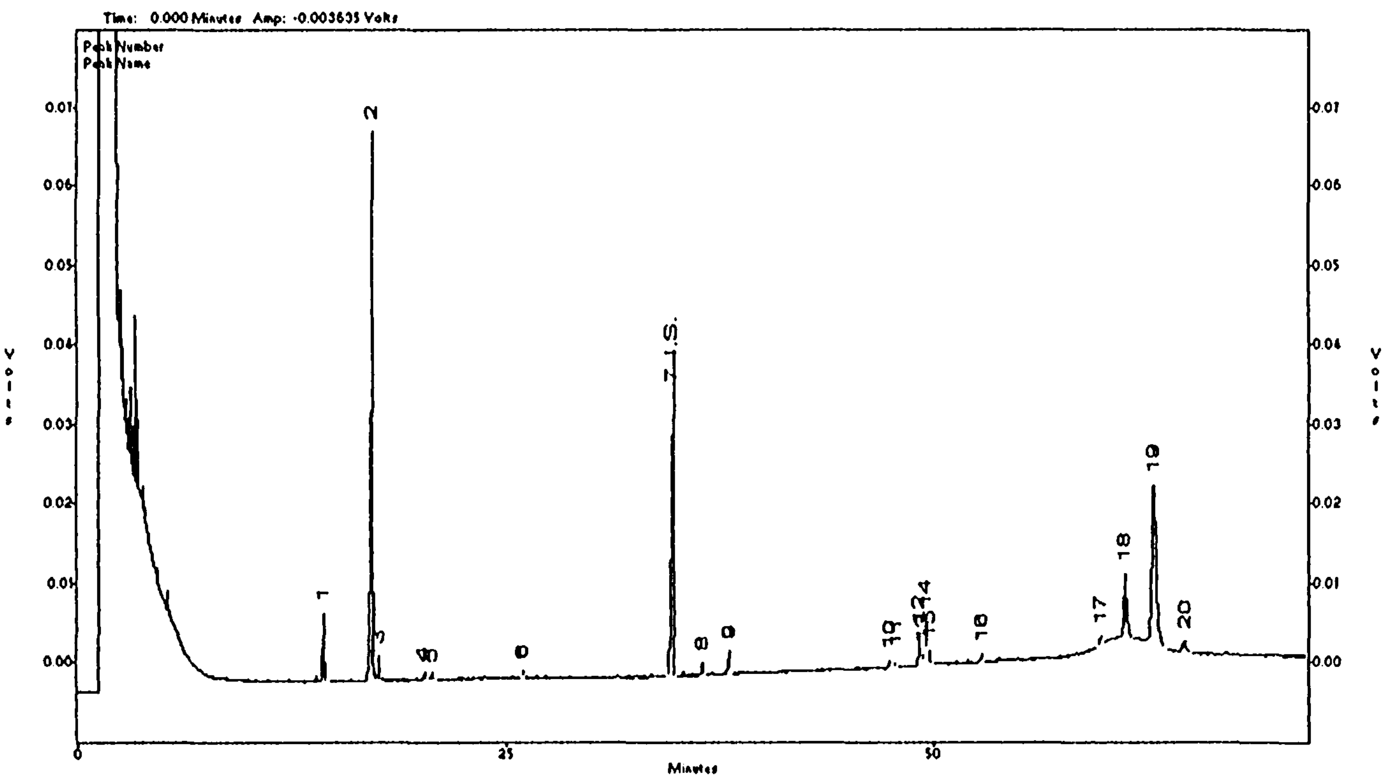

Figure 2.2 Gas chromatogram of canola seedling oil profile. Peak assignment: (2) free fatty acid of C18:1, (7) internal standard (I.S.): dihydrocholesteol, (13) (14) diolein, (19) triolein.

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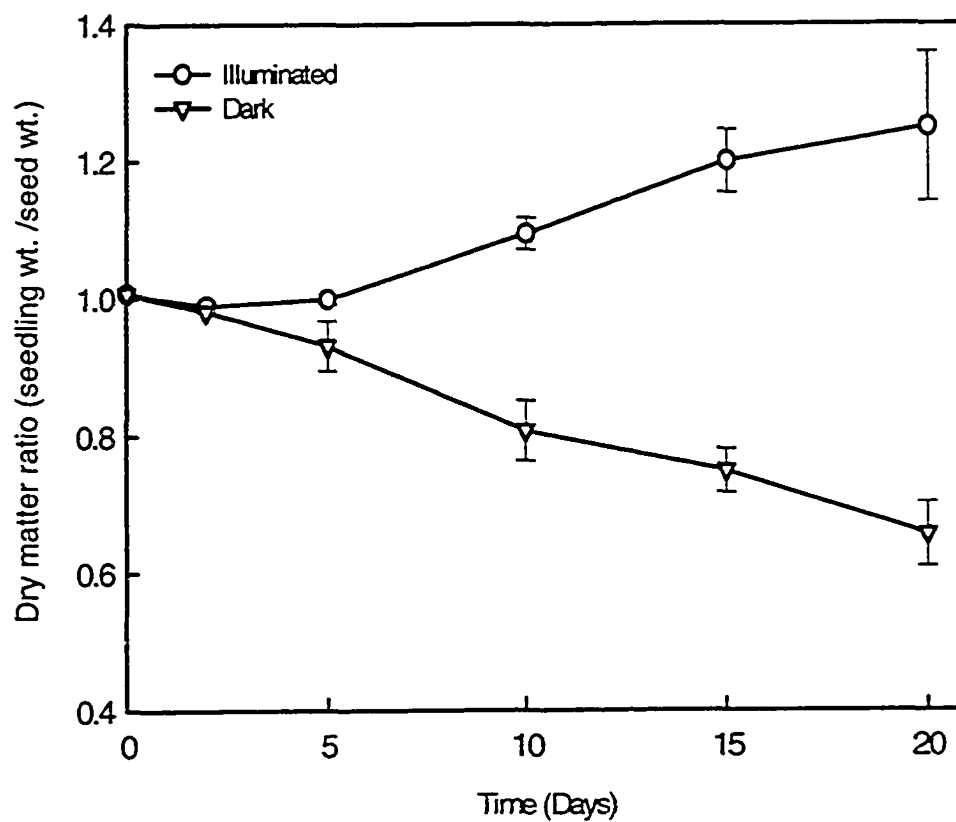

Figure 2.3 Changes in dry matter ratio in seedling grown under illuminated and dark conditions.

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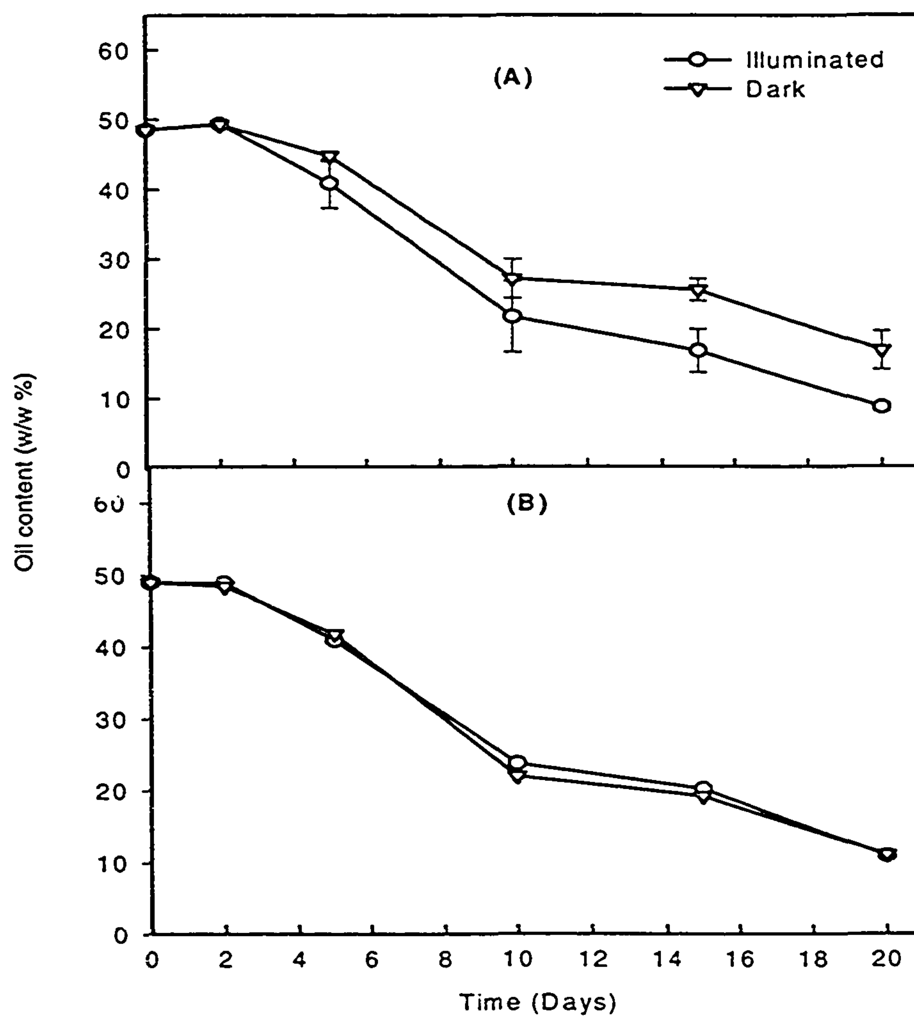

Figure 2.4 Changes in oil content on seedling (A) and seed (B) dry wt. basis.

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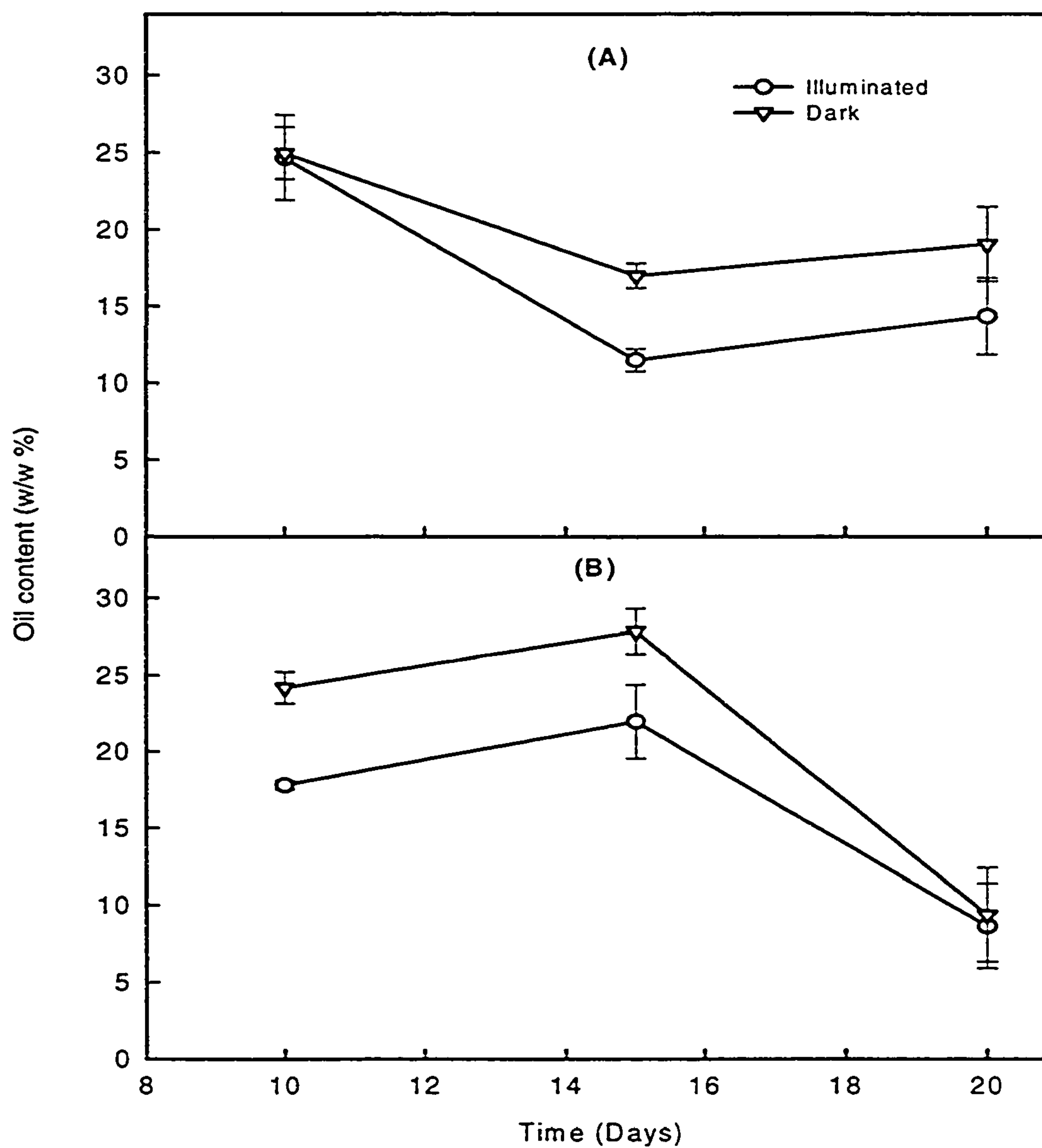

Figure 2.5 Changes in oil content on seedling tops (A) and bottoms (B) dry wt. basis.

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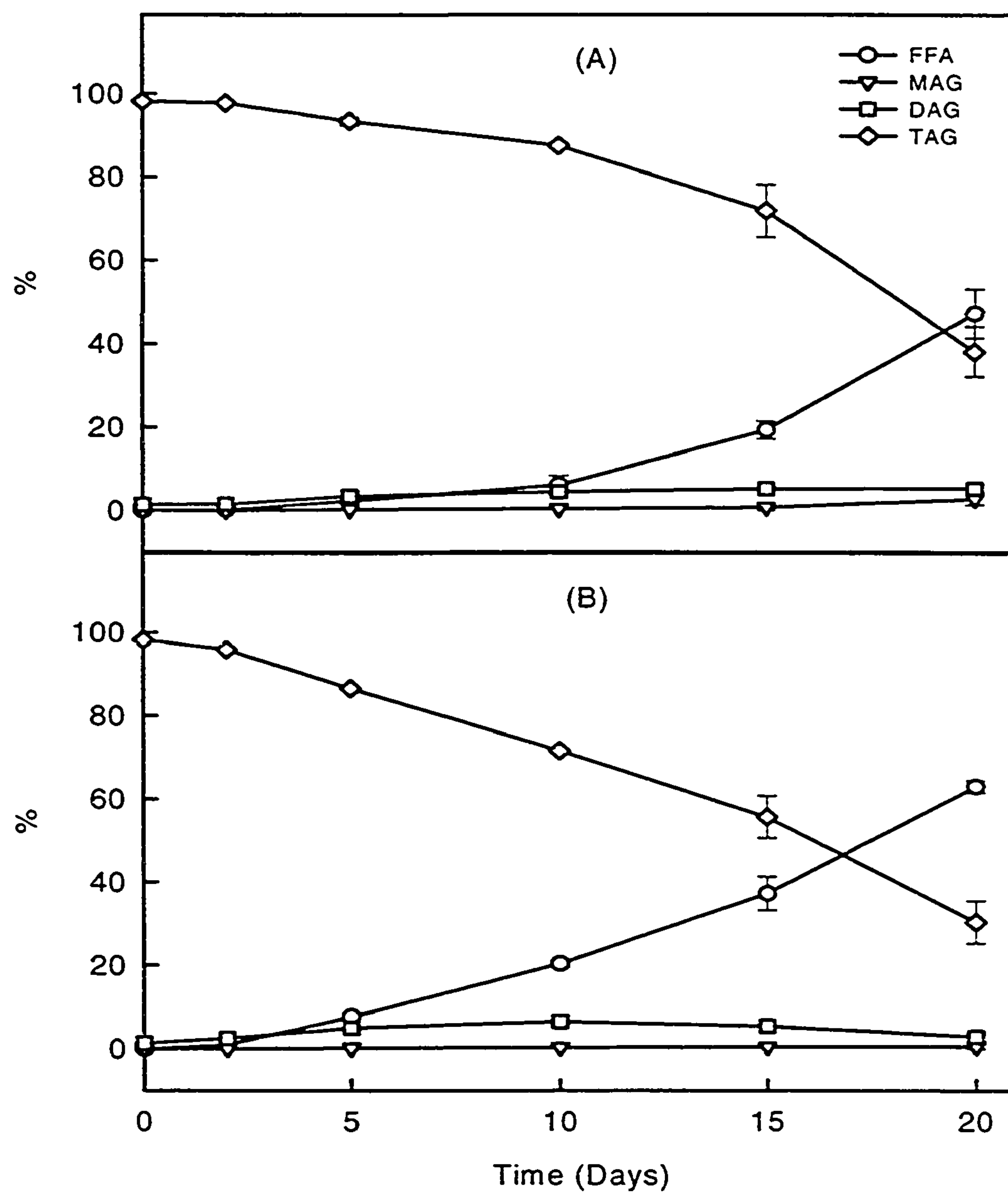

Figure 2.6 Effect of germination on various lipid classes of canola seedling oil. Illuminated (A) and dark (B) condition.

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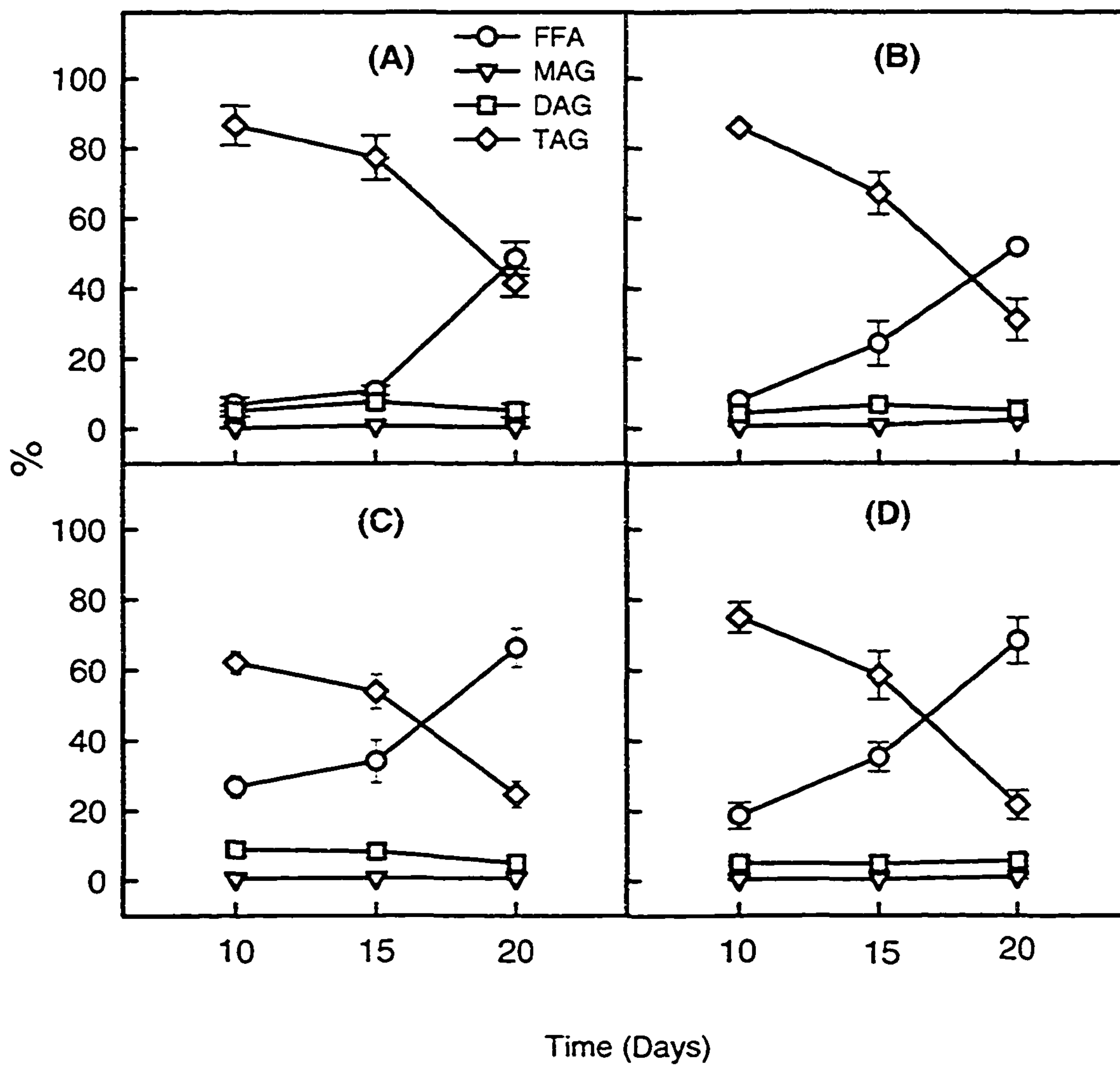

Figure 2.7 Effect of germination on lipid profile of canola seedling tops and bottoms. (A) and (C) depict the seedling tops grown under illuminated and dark conditions, respectively. (B) and (D) depict the seedling bottoms grown under illuminated and dark conditions, respectively.

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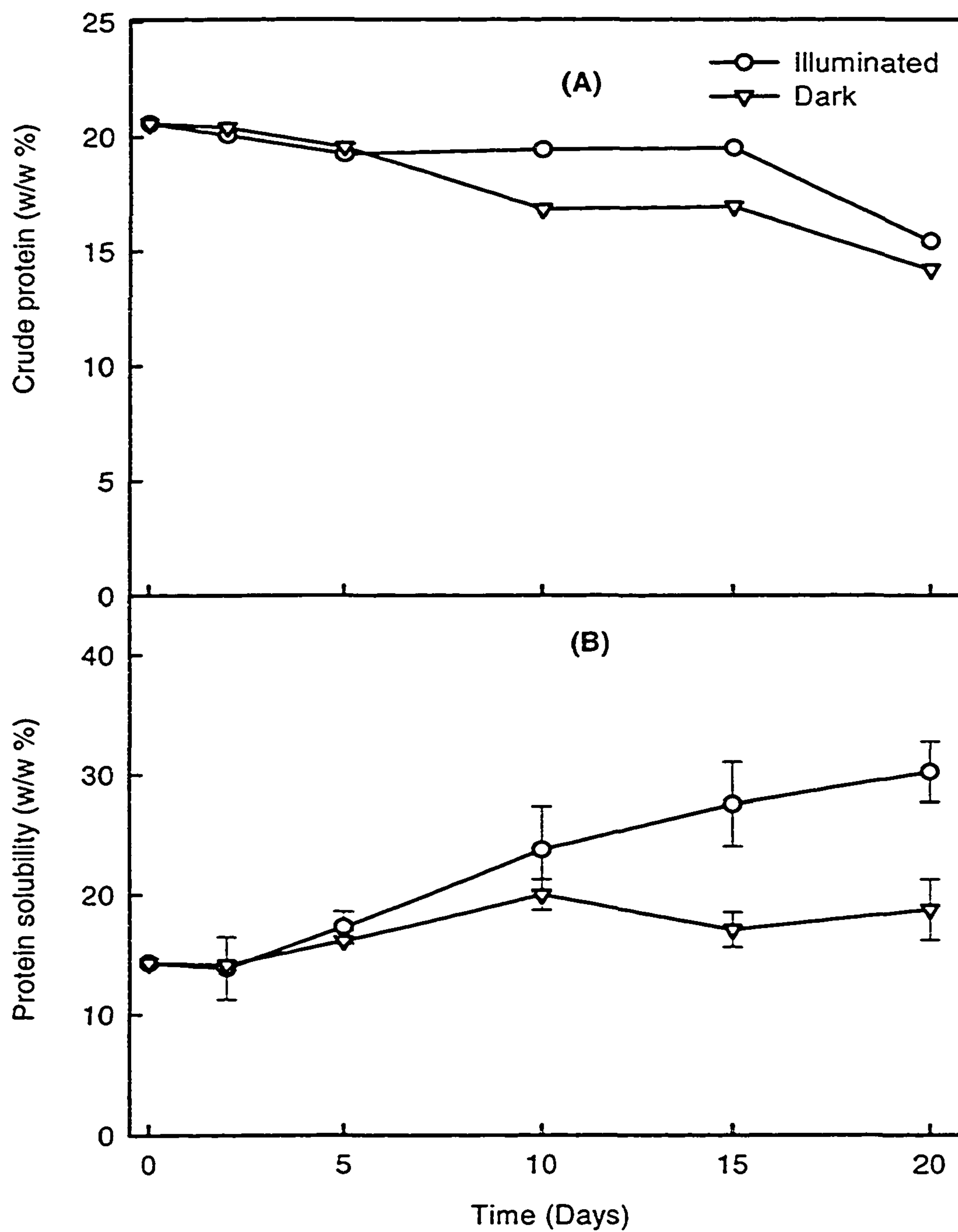

Figure 2.8 Changes of crude protein (A) and protein solubility (B) on seed dry wt. basis.

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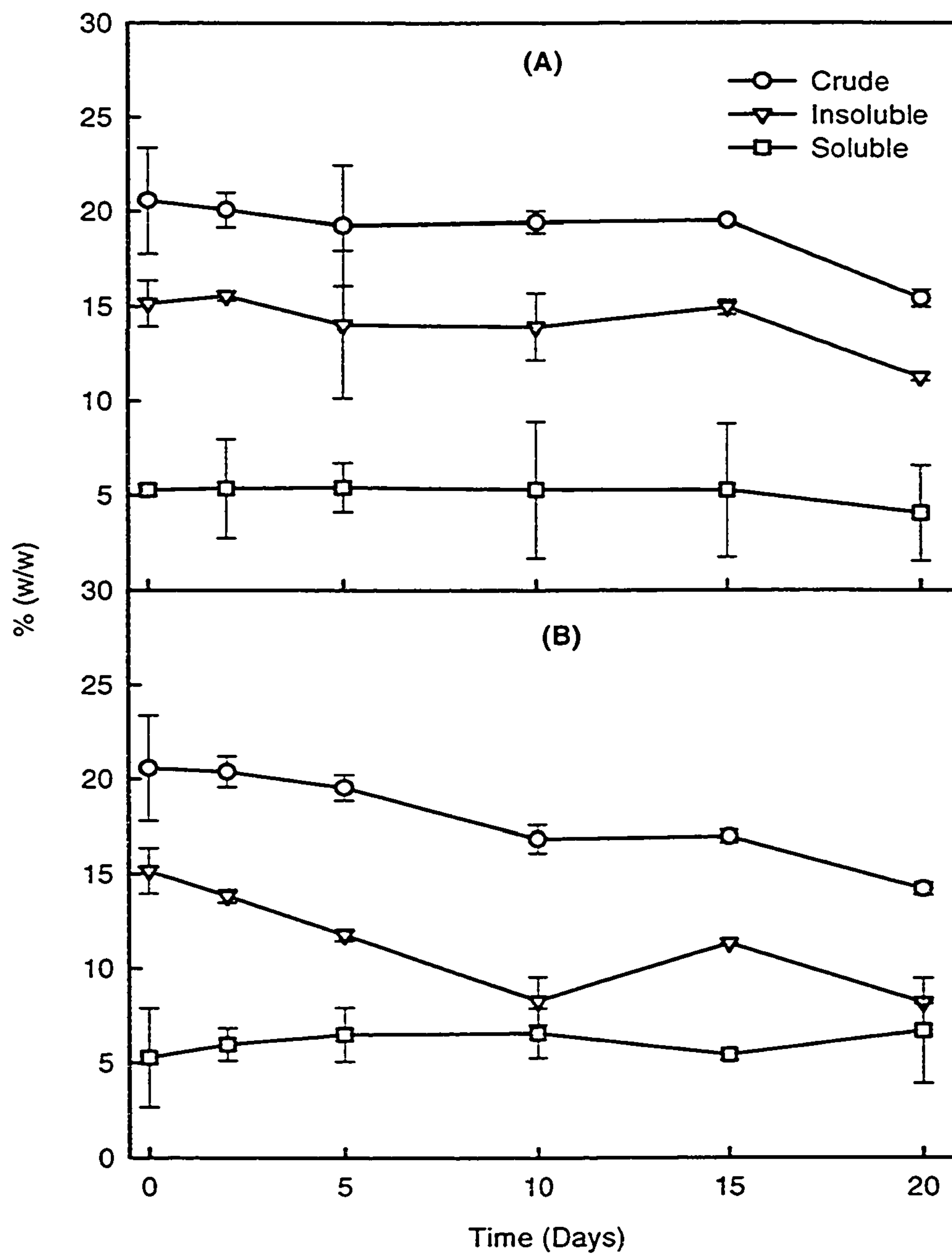

Figure 2.9 Changes in different protein classes in seed dry wt. basis under illuminated (A) and dark (B) conditions.

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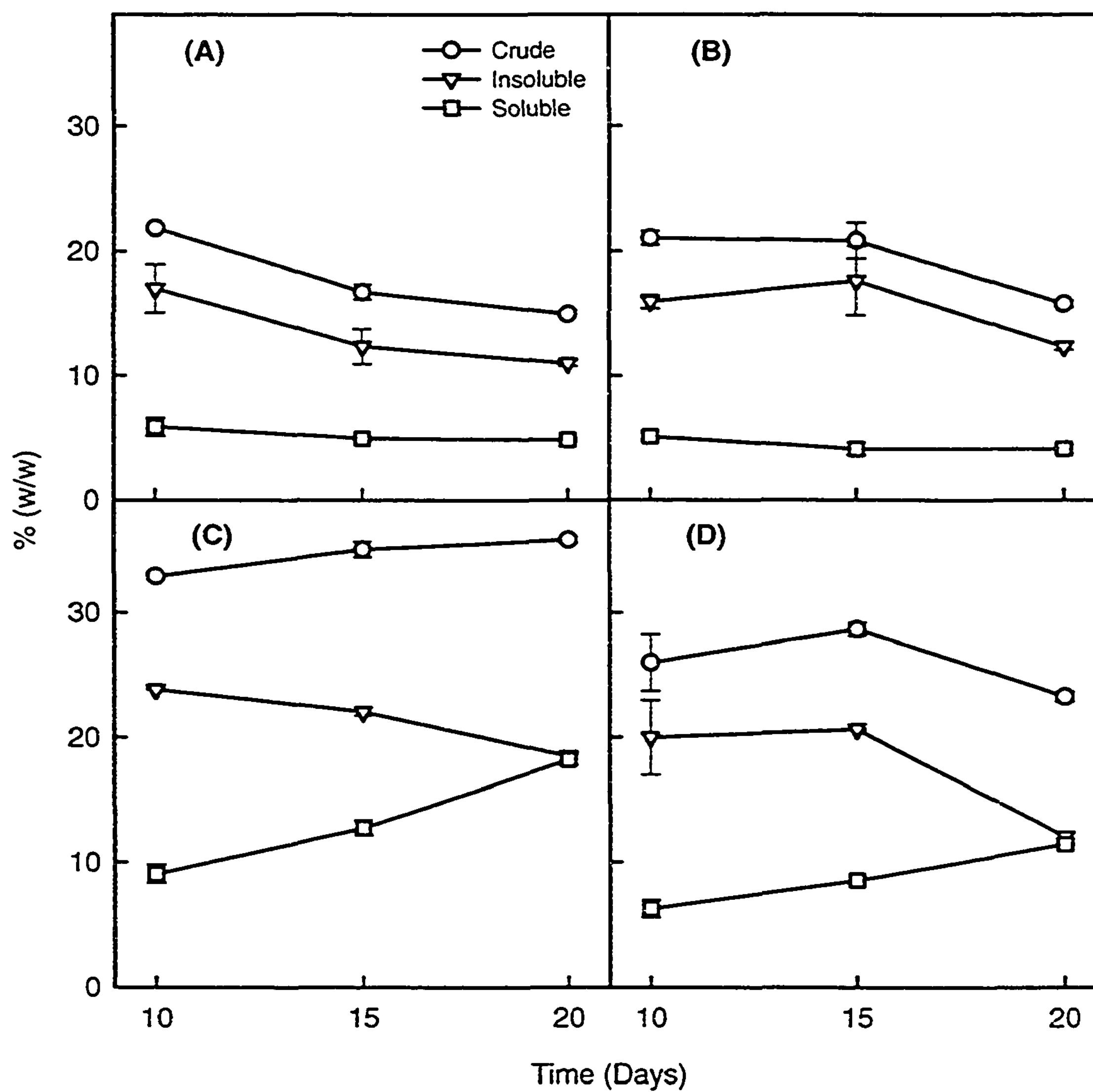

Figure 2.10 Changes in various protein classes of canola seedling tops and bottoms (dry wt. basis). (A) and (C) depict seedling tops under illuminated and dark conditions, respectively. (B) and (D) depict seedling bottoms under illuminated and dark conditions, respectively.

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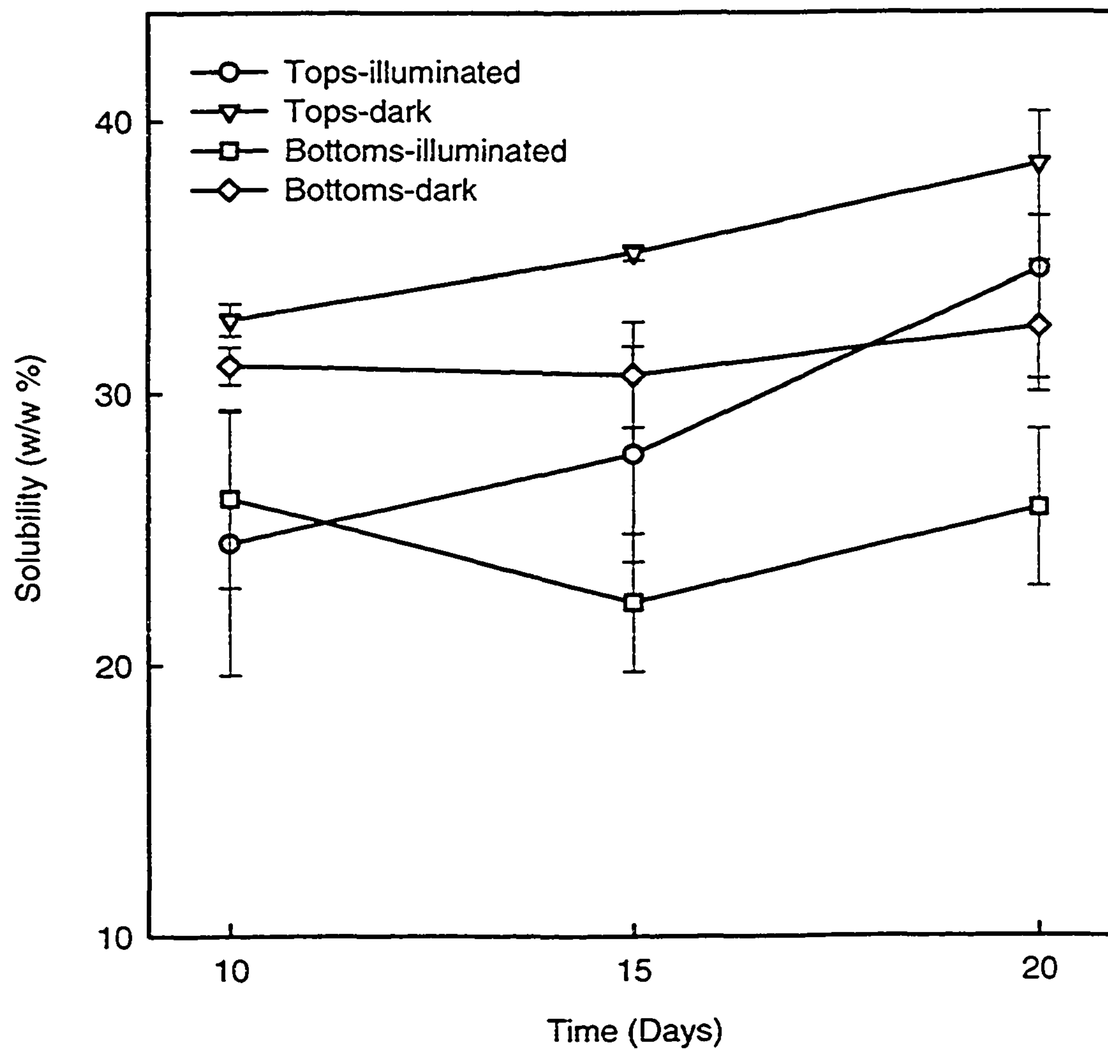

Figure 2.11 Changes in solubility of protein of canola seedling tops and bottoms (dry wt. basis).

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3.0 TOCOPHEROLS AND PHYTOSTEROLS IN CANOLA SEEDS AS AFFECTED BY GERMINATION AND SEEDLING GROWTH UNDER ILLUMINATED AND DARK CONDITIONS

3.1 Introduction

Tocopherols and sterols are important minor constituents of canola seeds. The nutritional value of α-tocopherol or vitamin E is well documented (Packer and Fuchs, 1993; Traber and Sies, 1996; Bramley et al., 2000). The content of tocopherols in canola is approximately 700 ppm and the predominant isomeric form in dry seeds is the γ isomer (Przybylski, 2001). Oilseeds also contain other tocopherol isomers, such as β- and δ-tocopherols. These isomers are not as potent as the α-isomer in terms of vitamin E activity, but confer they have better antioxidant potency than α-isomer (Bramley, 2000). Changes in isomeric forms of tocopherols during germination of rapeseed (Kim et al., 1997), soybean (Mostafa and Rahma 1987) and several cereal grains (Franzen and Haass, 1991; Yang et al., 2001; Liukkonen et al., 2003) have been reported.

Unlike tocopherols, phytosterols are considered non-nutritive components of seeds. Until recently, these components were thought to be biologically inert in humans. This perception started to change with the recognition of non-nutrients as biologically important nutraceuticals for both humans and animals. Phytosterols are ubiquitous in the plant kingdom and play a role in membrane microstructure and cell integrity (Piironen et al., 2000). The sterols of vascular plants are a complex mixture with dominant species being the sitosterol, stigmasterol, and campesterol (Piironen et al., 2000). During seed germination and subsequent plant growth, sterols undergo changes to meet the demands

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of the developing seedling (Kalinowska and Wojciechowski, 1984). Since membrane formation and transformation is an important early event of seed germination, a number of researchers have studied the biosynthesis and inter-conversion of sterols during this phase of development (Holmer et al., 1973; Mostafa and Rahma, 1987; Huang and Grunwald, 1988; Horbowicz and Obendorf, 1992). Malgorzata (1984) reported that the total sterol content of white mustard remains relatively constant on a dry weight basis but there was a distinct increase (about 2.8 fold) per plant basis. An increase in sterol content of germinating alfalfa seeds (Huang and Grunwald, 1988) and soybean (Mostafa and Rahma, 1987) has been reported in literature.

Based on the evidence of substantial increase in sterol content in various plant species during early stages of growth, it was hypothesized that there will be a similar increase in tocopherol and sterol contents in canola during seed germination and subsequent seedling growth. Therefore, the main objective of this study was to investigate the effect of germination under illuminated and dark conditions and stage of growth on various species of tocopherols and sterols.

3.2 Materials and methods

3.2.1 Materials

Canola seeds Q2 summer rape (*Brassica napus* L.) were donated by Agricore United (Calgary, AB). Butylated hydroxy toluene (BHT), potassium hydroxide (KOH), bis(trimethylsily)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) (99:1), and authentic tocopherol and sterol standards were procured from Sigma Scientific Co. (St. Louis, MO). All solvents were from Fisher Scientific Co. (Nepean, ON).

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3.2.2 Methods

3.2.2.1 Sample preparation

The methods of germination, harvesting and sample preparation were same as that described in section **2.2.2.1.**

3.2.2.2 Determination of tocopherols

Determination of tocopherols was carried out according to the modified method of Goffman and Becker (2001). Approximately 0.5 g seed or seedling sample was mixed with 25 ml isooctane (containing 0.01% BHT) in a 50 ml screw-top plastic tube. The sample was ground using Polytron at speed 6 for 1 min and the tube was capped tightly before placing in a mechanical shaker for 4 h in the dark. The mixture was then centrifuged (Model MP4, International Equipment Company, Needham Heights, MA) at 5000 rpm for 10 min. A 5 mL aliquot of the supernatant was transferred into a glass tube and the solvent was removed under a stream of nitrogen at room temperature in a fume hood. The residue was re-dissolved in 1.0 ml of hexane (HPLC grade, Fisher Scientific, Nepean, ON) and the contents were vortexed (Model G-560, Scientific Industries, Inc., Bohemia, NY) for 1 min, transferred into an HPLC vial and subjected to high performance liquid chromatography (HPLC).

A Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD), loaded with a Shimadzu Class-VP chromatography laboratory automated software system for data handling, was used. The system used a Shimadzu RF535 fluorescence detector set at 295 nm for excitation and 330 nm for emission detections. Resolution of tocopherols was achieved on a Supelco LC-Diol column (25 cm × 4.6 cm, 5 μm,

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Supelco, Inc. Bellefonte, PA). Samples (20 µl) placed in a Hewlett Packard 1050 autosampler were injected and the elution under isocratic conditions was carried out using hexane with 0.6% isopropanol as mobile phase at a flow rate of 1 ml/min by Varian 9010 solvent delivery system (Varian Associates, Sugar land, TX). A calibration curve prepared for external standards of α-, β-, γ- and δ-tocopherols (5-150 µg/ml) was used for identification and quantification purposes. The linear regression coefficient for each tocopherol standard curve was greater than 0.99. In addition, three sets of standard solutions were placed at the beginning, in the middle and at the end of each batch. A typical chromatogram for tocopherol analysis is shown in Fig. 3.1. The formula used for quantitative purposes is the same as the formula given in Section 2.2.2.3.4.

3.2.2.3 Determination of total phytosterols

Phytosterols in crude oil were determined using a modified method of Mounts et al. (1996). To approximately 0.05 g oil placed in a glass test tube, 0.2 mg dihydrocholesterol internal standard and 5 mL of 2N alcoholic KOH were added. The contents were vortexed, incubated first at 70°C for 1 h and then allowed to stand overnight at room temperature. After adding 5 mL of deionized water and 15 mL of cyclohexane, the mixture was vortexed vigorously and centrifuged at 1000 rpm for 10 min. The cyclohexane fraction was transferred into a glass tube and washed with water until the pH reached neutral. It was then evaporated under a stream of N_2 at room temperature and the residue re-dissolved in 0.25 ml of pyridine and 0.25 ml of BSTFA containing 1% TMCS. This mixture was heated for 30 min and allowed to stand

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overnight at room temperature. The derivatized sample was dried under N_2, the residue dissolved in hexane and analyzed immediately by GC.

TMS solutions (0.5μl) were injected into a J&W Scientific DB-5HT fused silica capillary column (30m × 0.25mm i.d., 0.1μm film thickness, Agilient Technologies) in a Varian 3400 gas chromatograph (Walnut Greek, CA) equipped with a flame ionization detector and a Varian 8100 autosampler. Helium was the carrier gas used at a head pressure of 25 psi. An initial column temperature of 70°C was maintained for 0.2 min, ramped to 250°C at 20°C min^{-1} and then to a final temperature of 280°C at 15°C min^{-1} where this temperature was maintained for 17 min. The initial injector temperature of 80°C was maintained for 0.2 min and then ramped to 280°C at 150°C min^{-1}. The flame-ionization detector temperature was set at 280°C. Chromatograms were recorded and the peak integration was carried out using Shimadzu Class-VP software (version 4.2, Shimadzu Scientific Institutes, Inc., Columbia, MD). All species of sterols were quantified using an internal standard with the same response factor of 1.0.

Peak identification was achieved by comparing the retention time of authentic sterols in a mixture containing campesterol, stigmasterol, sitosterol and brassicasterol. A representative chromatogram for sterols analysis is shown in Fig. 3.2. For quantitative analysis, stigmasterol standard was used to calculate the relative response factor (RRF) for all the sterols. The formula given in Section 2.2.2.3.4. was used for quantification purposes.

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3.2.2.4 Statistical analysis

All germination experiments were carried out in replicates and analyses were carried out in duplicates. Analysis of variance of the results was performed using General Linear Model procedure of SAS Statistical Software, Version 8 (SAS Institute Inc., 1999). The effect of germination, light and seedling part on the content and composition of all components were evaluated at a significance level of 0.05.

3.3 Results and discussion

3.3.1 Changes in tocopherols in seedlings

During the germination and seedling growth under illuminated conditions, the total tocopherols increased from about 370 ppm on day-0 to about 540 ppm on day-20 (Fig. 3.3A). Gamma-tocopherol, the predominant species in seeds, depleted rapidly until day-10 of the trial and reached a plateau, which also happened in germinated soybean seed (Mostafa and Rahma, 1987). In contrast, alpha-tocopherols followed a reversed trend as compared to gamma-tocopherol. No beta-tocopherol was detected in seeds on day-0, but detectable amounts (17-37 ppm) were present in germinating seeds and seedlings (Fig. 3.3A), which was similar to *Picea abies* seed (Franzen and Haab, 1991). Changes in tocopherol profile may be attributed to the isomeric conversion of individual species from one to another.

As depicted in Figure 3.3B, total tocopherols in seedlings grown under dark conditions remained stable during the entire time course. Individual species of tocopherol followed similar trends as seen in seedlings grown under illuminated conditions. Only notable difference was that the depletion of γ- and increase in α-tocopherols under dark

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conditions occurred earlier at faster rates than those observed under illuminated growing conditions.

Figure 3.4 shows the changes in total tocopherols in seedling tops and bottoms. For both growing conditions, the leafy top parts of seedlings contained more tocopherols and a higher percentage of α-tocopherol than in seedling bottoms ($p<0.05$), which may be due to tocopherol being mainly accumulated in organs and tissues with a high sensitivity to oxidation or photosyhthetically active tissues (Franzen and Haass, 1991). The total tocopherol content in leafy parts obtained from seedlings grown under dark conditions doubled during the last 10 days of growth (Fig. 3.4B).

The decreased level of γ-isomer accompanied by an increased level of α-isomer may be due to the increased activity of γ-tocopherol methyltransferase (γ-TMT). According to Shintani (1998), γ-tocopherol is the biosynthetic precursor to α-tocopherol. In the final step of α- tocopherol biosynthetic pathway, which is catalyzed by γ-tocopherol methyltransferase (γ-TMT), γ-isomer is converted to α-isomer. The emergence of the β-isomer on day-2 is difficult to explain. However, the decrease in absolute amounts of γ-tocopherol was very close to the sum of increase in α- and β-isomers and therefore, it is logical to assume that the γ-isomer is transforming into both α- and β-tocopherols. This observation was made in seedling grown under both conditions, illuminated and dark.

The total tocopherol content in the presence of light did not increase during the first 5 days but it increased dramatically in later stages. At the early stages, there is no evidence to support that new tocols were synthesized, but the results suggest the transformation from one isomer to another. After 5 days, however, an increase in α-

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tocopherol implies that this isomer was newly synthesized. Dark conditions also favored the conversion of γ-isomer to α-isomer. This occurred while there was no increase in the total tocol content. This implies that the synthesis of new tocopherol requires the presence of light. After 10 days of germination and growth under illuminated conditions, the rate of decrease in γ-tocopherol slowed down. This may be due to the cessation of isomeric conversion or near identical rates of degradation and synthesis. The tocol content in the seedling tops is significantly ($p<0.05$) higher than that in the seedling bottoms. This is expected because α-tocopherol is located mainly in the chloroplasts of leafy seedling tops (Bramley, 2000).

3.3.2 Changes in tocopherols in extracted oil

Figure 3.5 shows the tocopherol content of oil extracted from seedlings at different stages of germination and growth. The content of total tocopherol in oil extracted from seedlings grown under illuminated conditions increased from 757 ppm on day-0 to 4944 ppm on day-20 (6.5-fold increase, Fig. 3.5A). Under dark conditions, a 4.3-fold increase in total tocopherols was observed (Fig. 3.5B). For both illuminated and dark conditions, total tocopherol content in the extracted oil remained stable until day-5, started to increase slowly until Day-15 and then increased rapidly until day-20. After 20 days of growth under illuminated and dark conditions, a 50% decrease in gamma-tocopherol in the extracted oil was evident. Beta-tocopherol content in oil extracted from seedlings grown under illuminated conditions increased from 0 to 338 ppm while the increase was from 0 to 182 ppm under dark conditions. These oils can be a good starting

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material for natural vitamin E production. In this aspect, seedlings grown under illuminated conditions are better suited than those grown in the dark.

3.3.3 Changes in sterols in seedlings

There was no significantl change ($p>0.05$) in the total sterol content of seedlings grown under both conditions during 20 days germination (Fig. 3.6B). However, the factor of light showed a significant effect ($p<0.05$). Under dark conditions, a slight loss of sterols was seen up to day-15 and then increased back to the original level by day-20. Under illuminated condition, the total sterols gradually increased, though the trend was not significant ($p>0.05$). As depicted in Fig. 3.7, sitosterol is the predominant sterol species in canola seedlings. Campesterol was the second largest species followed by brassicasterol and avenasterol. Stigmasterol and some unknown sterols present in trace quantities. Statistical analysis showed that the changes in sterol composition with the length of germination were significant ($p<0.05$), whilst, light had no significant effect on it ($p>0.05$). Sterols were reported to remain stable in various germinating seeds, like alfalfa seeds (Huang and Grunwald, 1988) and mustard seed (Kalinowska and Wojciechowski, 1984). However, during soybean (Mostafa and Rahma, 1987) and tobacco seeds (Bush and Grunwald, 1972) germination, the composition of sterols showed some variation. They suggested that the free sterol level in germinating seedlings is regulated through the formation of steryl esters. Under this scenario, the germinating seeds would accumulate sterols in the form of steryl esters, which essentially have no effect on stabilizing membranes. Steryl esters are converted to free sterols when the demand arises.

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3.3.4 Changes in sterol content in extracted oil

For both illuminated and dark conditions, the total sterol content in the extracted oil remained stable up to day-5 and then started to increase in a sigmoidal fashion (Fig. 3.6A). Un-germinated seeds contained only about 10 mg/g sterols. On the day-20 of germination and growth, the sterol content in oil increased to about 52 and 42 mg/g under illuminated and dark conditions, respectively. This effect is clearly associated with the depletion of oil reserves while the absolute amount of sterols remained virtually unchanged. Chung et al. (1989) also reported a similar observation.

Figure 3.8B depicts the sterol content in oil extracted from seedling tops and bottoms on day-10 and onwards. Under illuminated conditions, sterol content in seedling tops first increased from 24 to 34 mg/g and then leveled off by day-20 while that in seedling bottoms first decreased from 26 to 20 mg/g and then increased to 47 mg/g at the end of the trial. Under dark conditions, there was a steady increase in sterol content in the oil extracted from seedling tops whereas the sterols in oil extracted from seedling bottoms remained unchanged throughout the day-10 and day-15 period and then increased rapidly from there on. Unlike in oil, the sterols content in seedling bottom dry matter did not show significant variation although there were some fluctuation for seedling top under both condition (Fig. 3.8A)

3.4 Conclusions

Dry seeds contain only γ and α isomers of tocopherols and β-tocopherol was synthesized only after germination. Under illuminated conditions, new tocopherols were

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synthesized at later stages as compared to the lack of synthesis under dark conditions. Therefore, light is a crucial factor for tocopherol synthesis, but not for isomer conversion, since the conversion of γ- to α-tocopherol took place under both conditions. The high amounts of tocopherols detected in seedling tops, as compared to the bottoms, suggest that the tocopherols are located mainly in the leafy parts of the seedlings. Unlike the individual tocopherols, the individual sterols in seedlings did not show any distinct changes during the entire trial period. Furthermore, an even distribution of sterols in the seedling tops and bottoms was evident.

Under illuminated conditions, the absolute amount of total tocopherols in seedlings increased from about 370 ppm on day-0 to about 540 ppm on day-20 and in the extracted oil there was almost a 7-fold increase. Under dark conditions, within 5 days, the ratio of α-isomer: γ-isomer increased from 1:2.5 to 4:1. This increase in the proportion of α-isomer may improve the biological activity and commercial value of canola oil.

Although absolute amounts of phytosterols did not change in the seedlings during the whole process, the depletion of lipid reserves caused sterols to concentrate (4-5-fold increase compared to oil from dry seeds) in the extracted oil. Germination is an alternative natural means of concentrating phytosterols.

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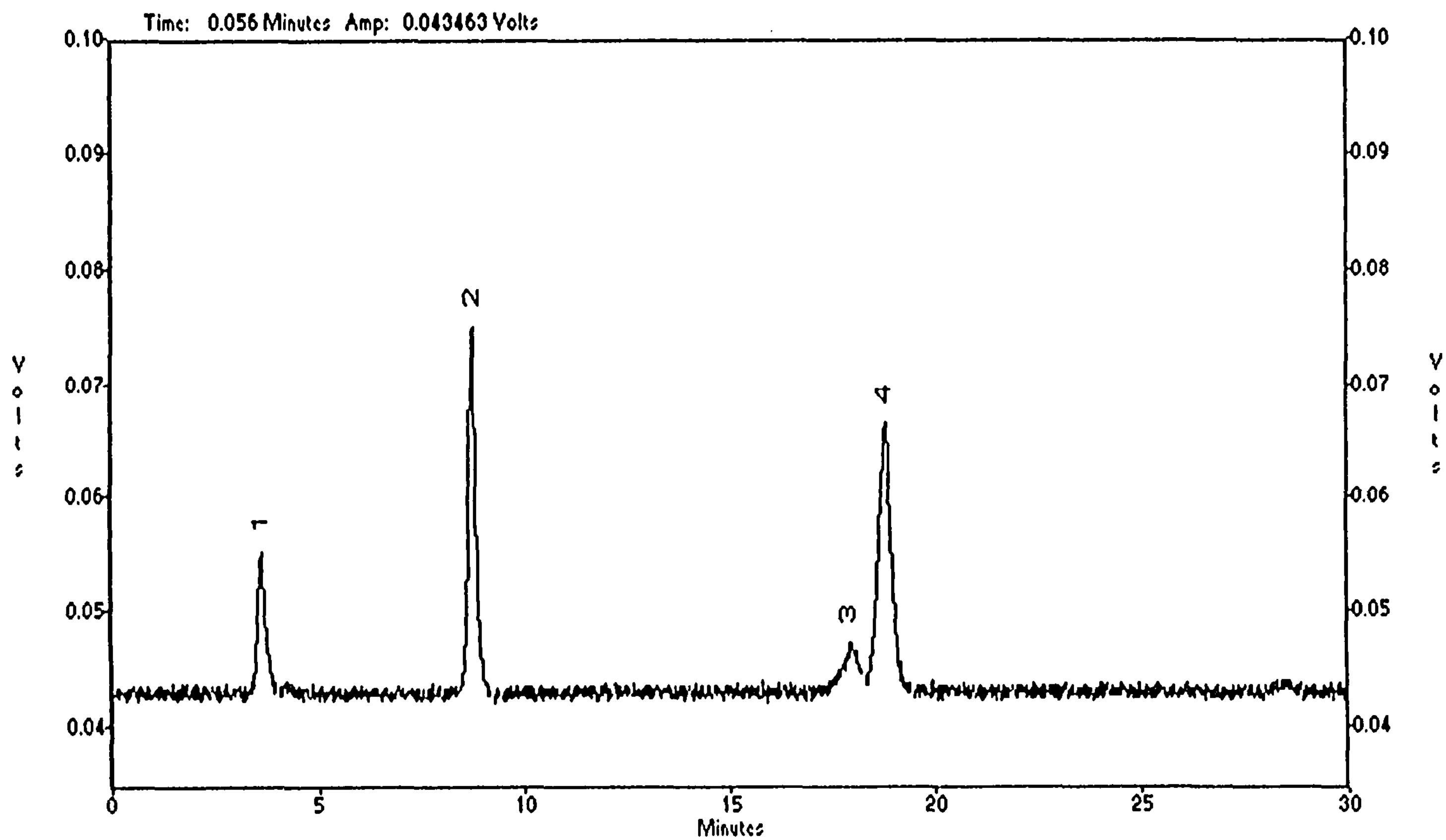

Figure 3.1 High performance of liquid chromatogram of canola seedling oil tocopherols. Peak assignment: (2) α - tocopherol, (3) β- tocopherol, (4) γ- tocopherol.

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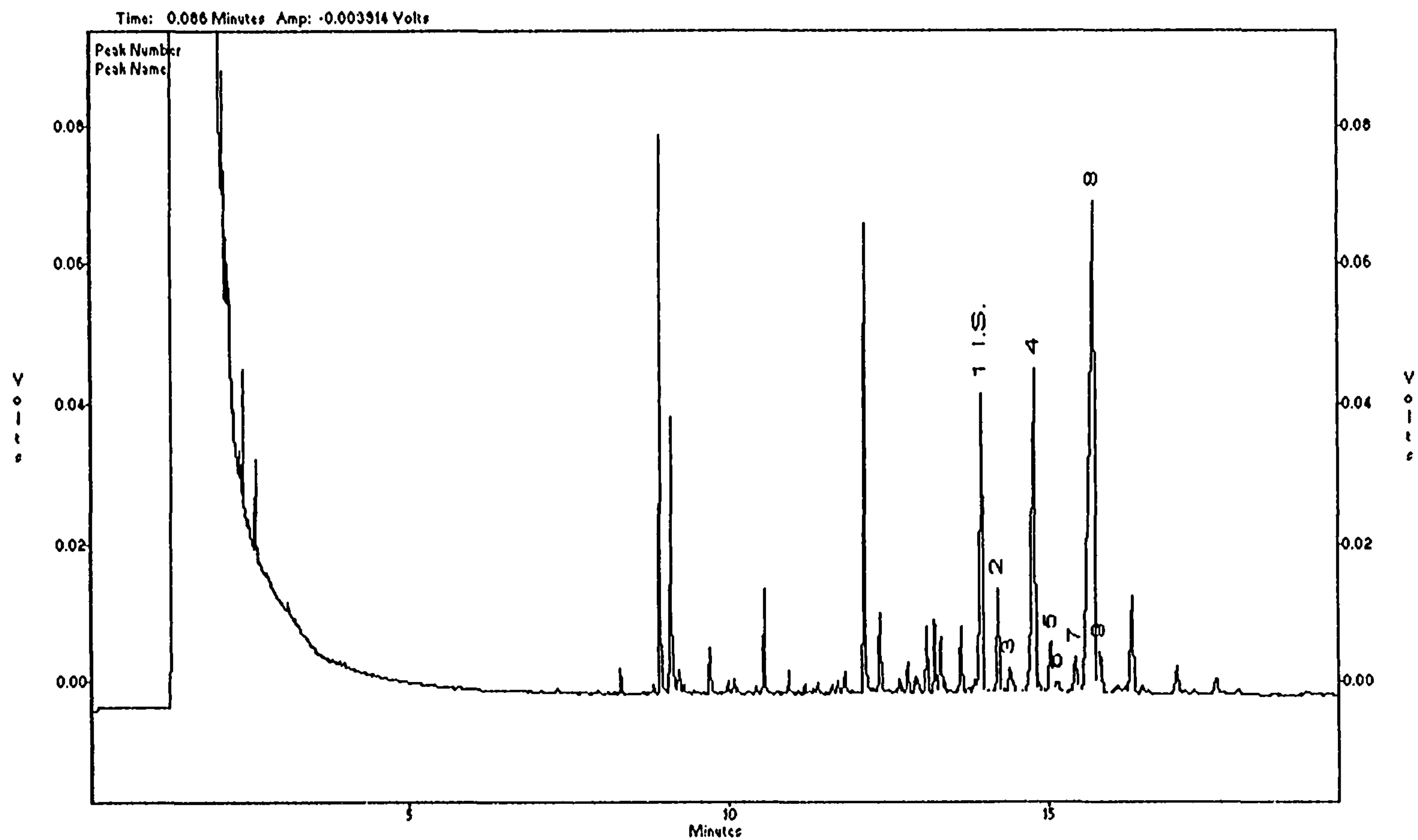

Figure 3.2 Gas chromatogram of canola seedling oil sterols. Peak assignment: (1) internal standard (I.S.): dihydrocholesterol, (2) brassicasterol, (4) campesterol, (5) stigmasterol, (8) sitosterol, (9) avenasterol.

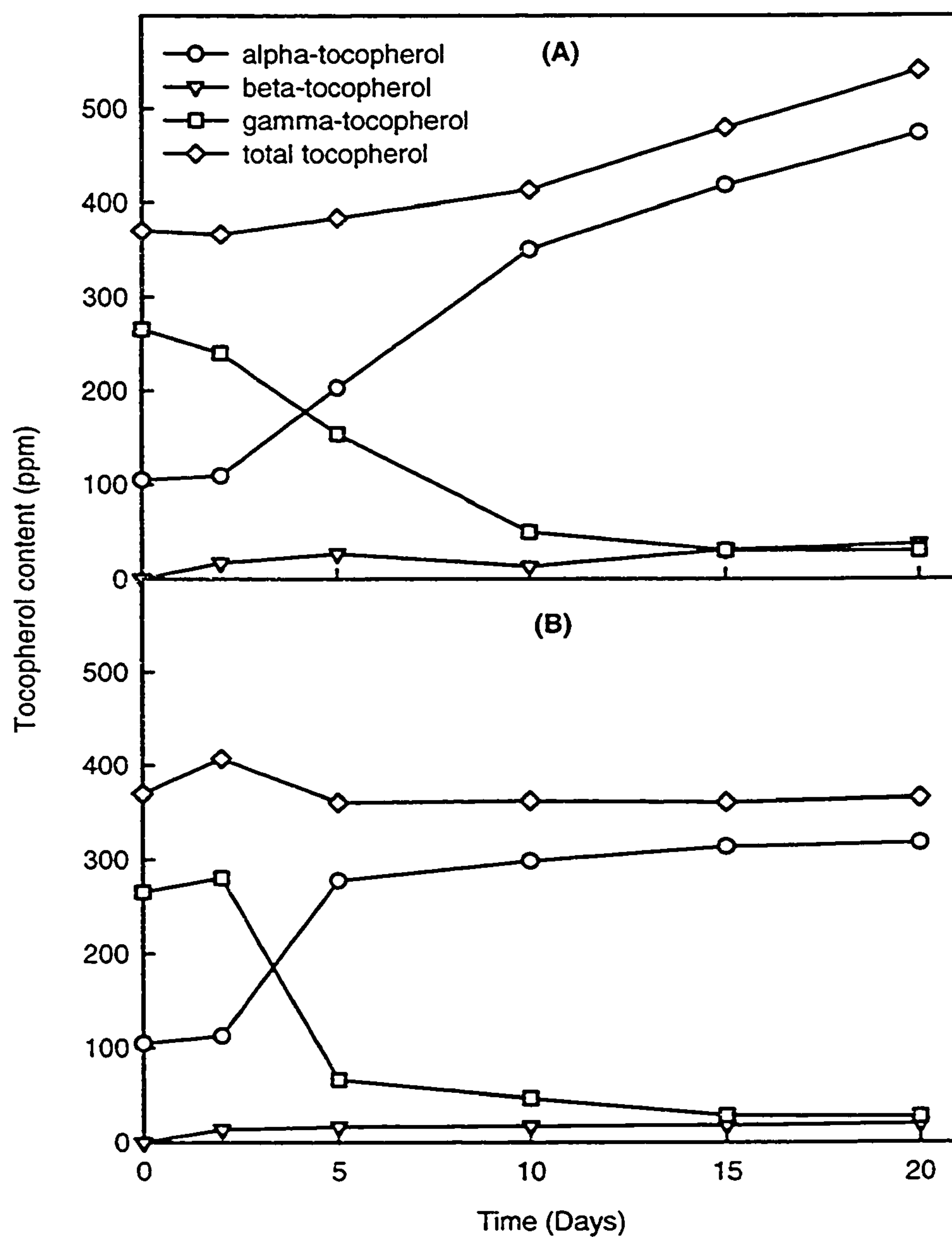

Figure 3.3 Changes in tocopherol content on seed dry wt. basis under illuminated (A) and dark (B) condition.

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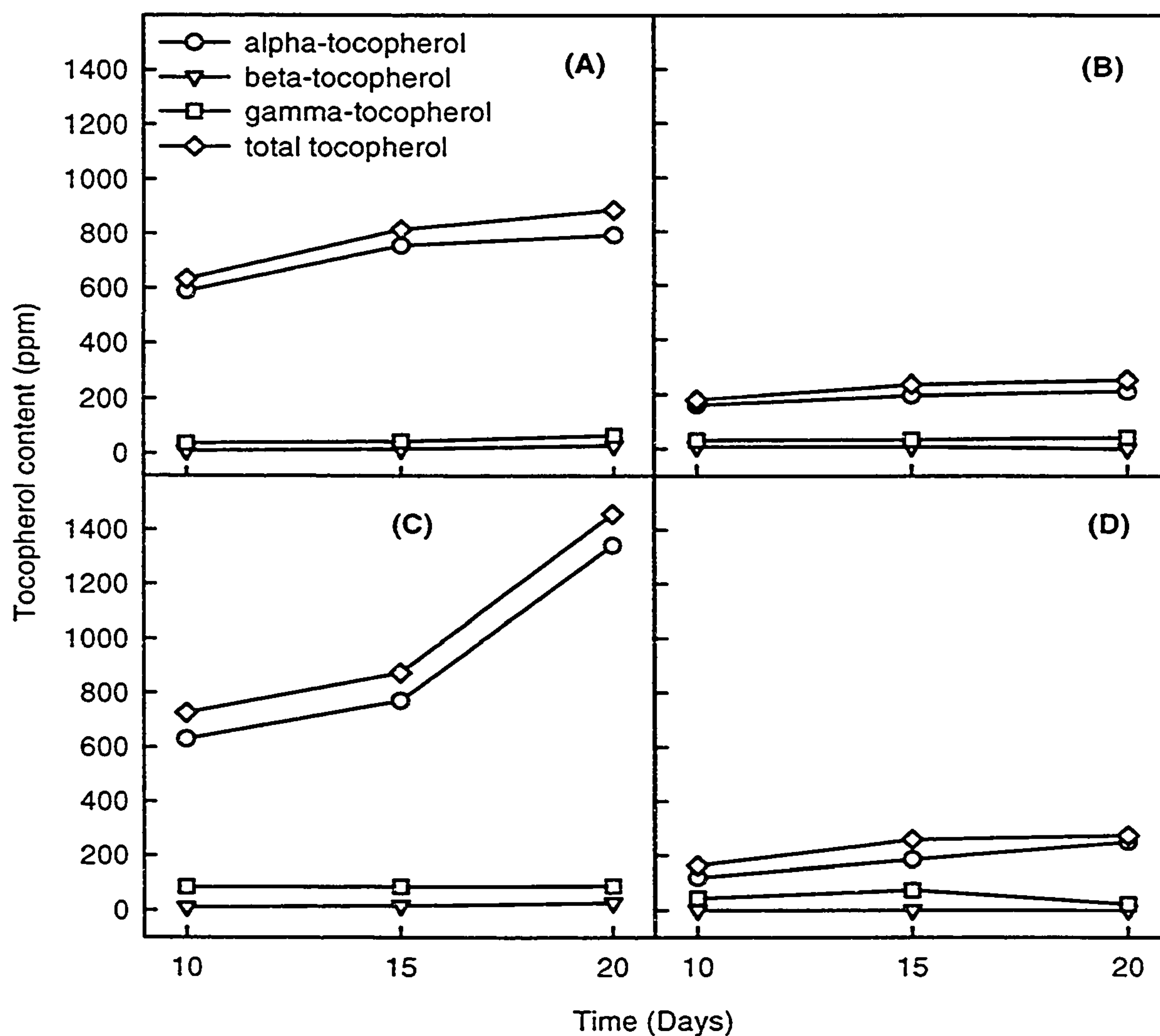

Figure 3.4 Changes in tocopherol in seedling tops and bottoms (dry wt. basis).
(A) and (C) depict seedling tops under illuminated and dark conditions, respectively.
(B) and (D) depict seedling bottoms under illuminated and dark conditions, respectively

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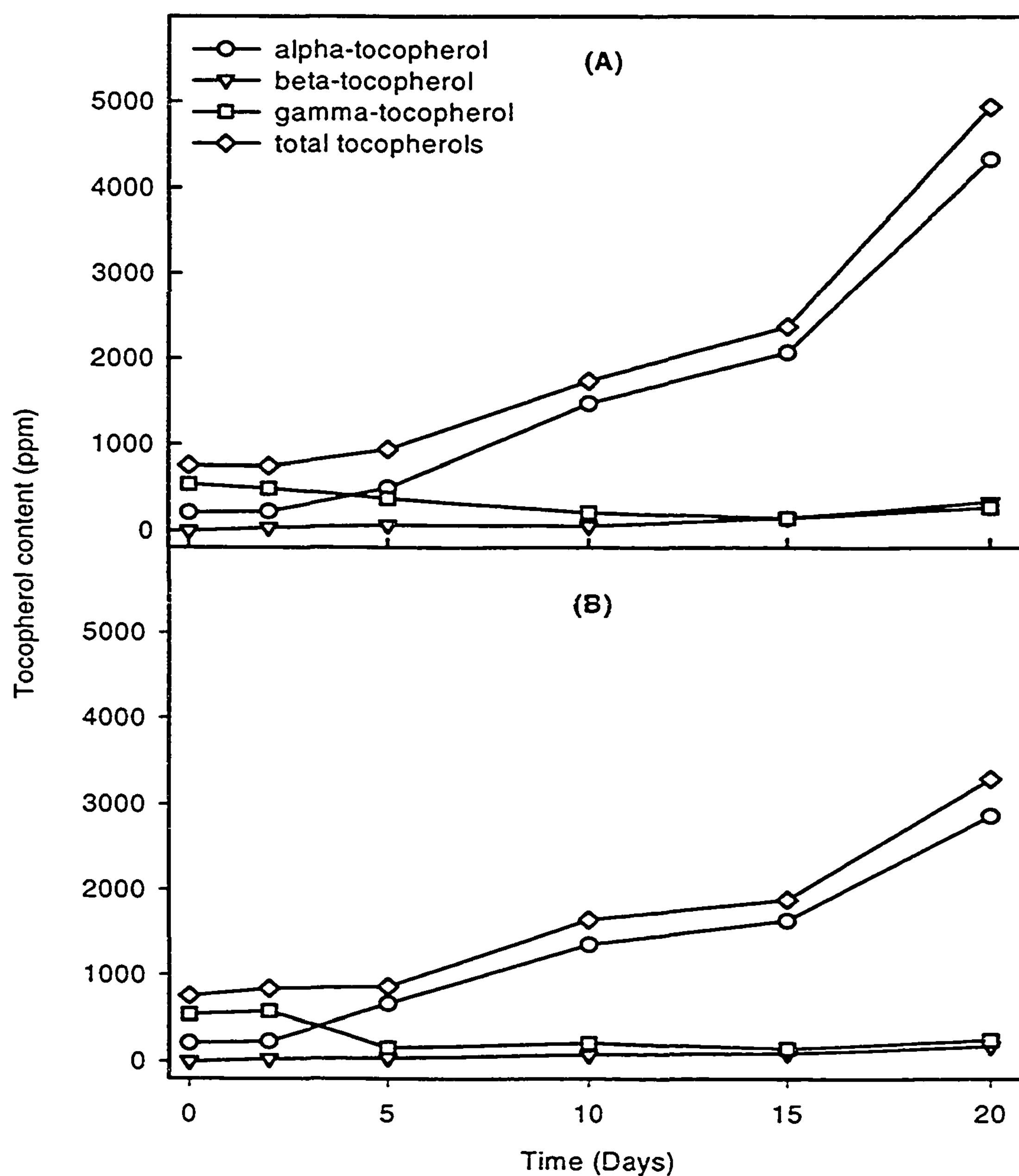

Figure 3.5 Changes in tocopherol content of canola seedling oil at different stages of germination under illuminated (A) and dark (B) condition.

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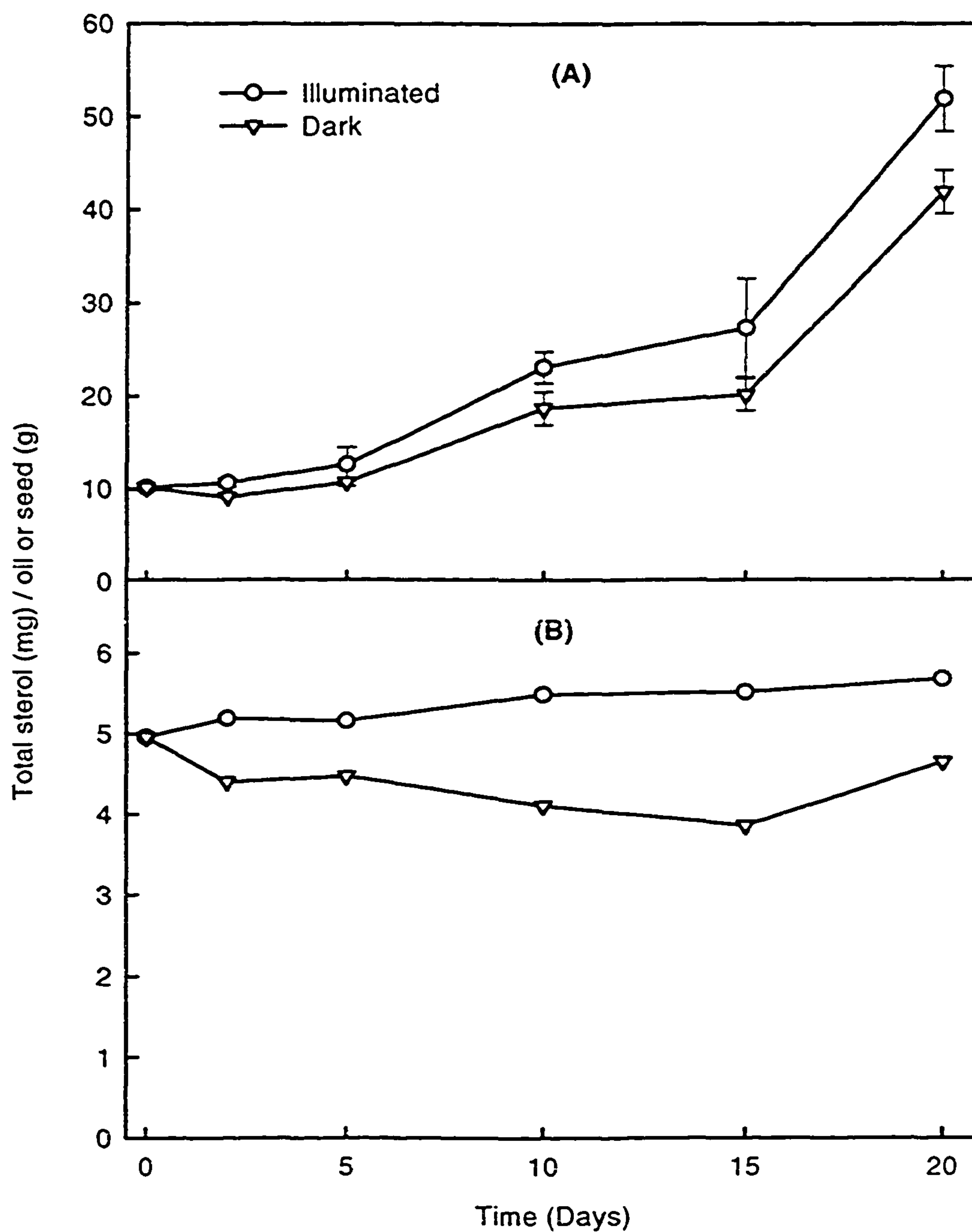

Figure 3.6 Changes in total sterol content during canola seed germination on oil basis (A) and on seed dry wt. basis (B).

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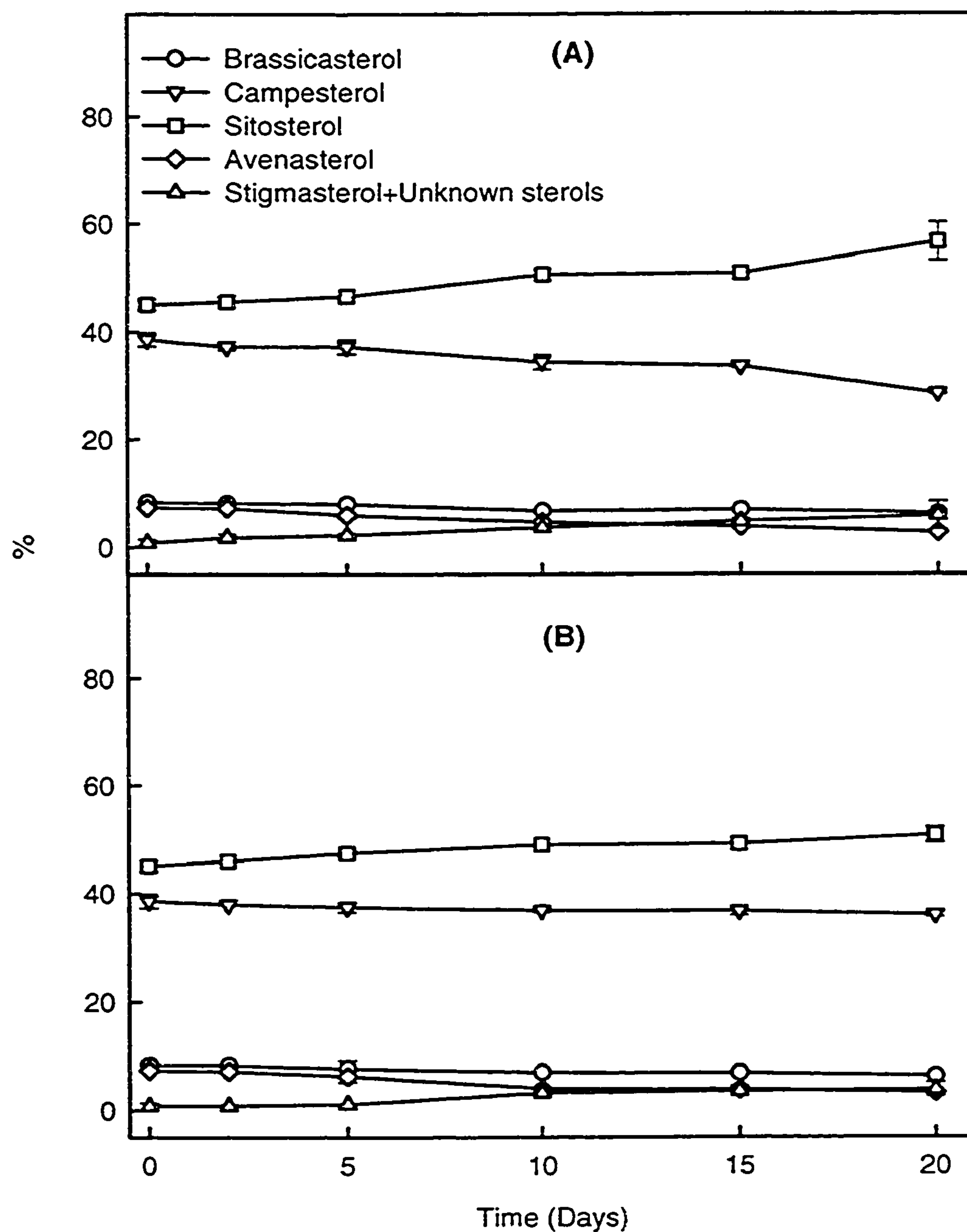

Figure 3.7 Changes in sterol composition during canola seed germination. (A) under illuminated conditions (B) under dark conditions.

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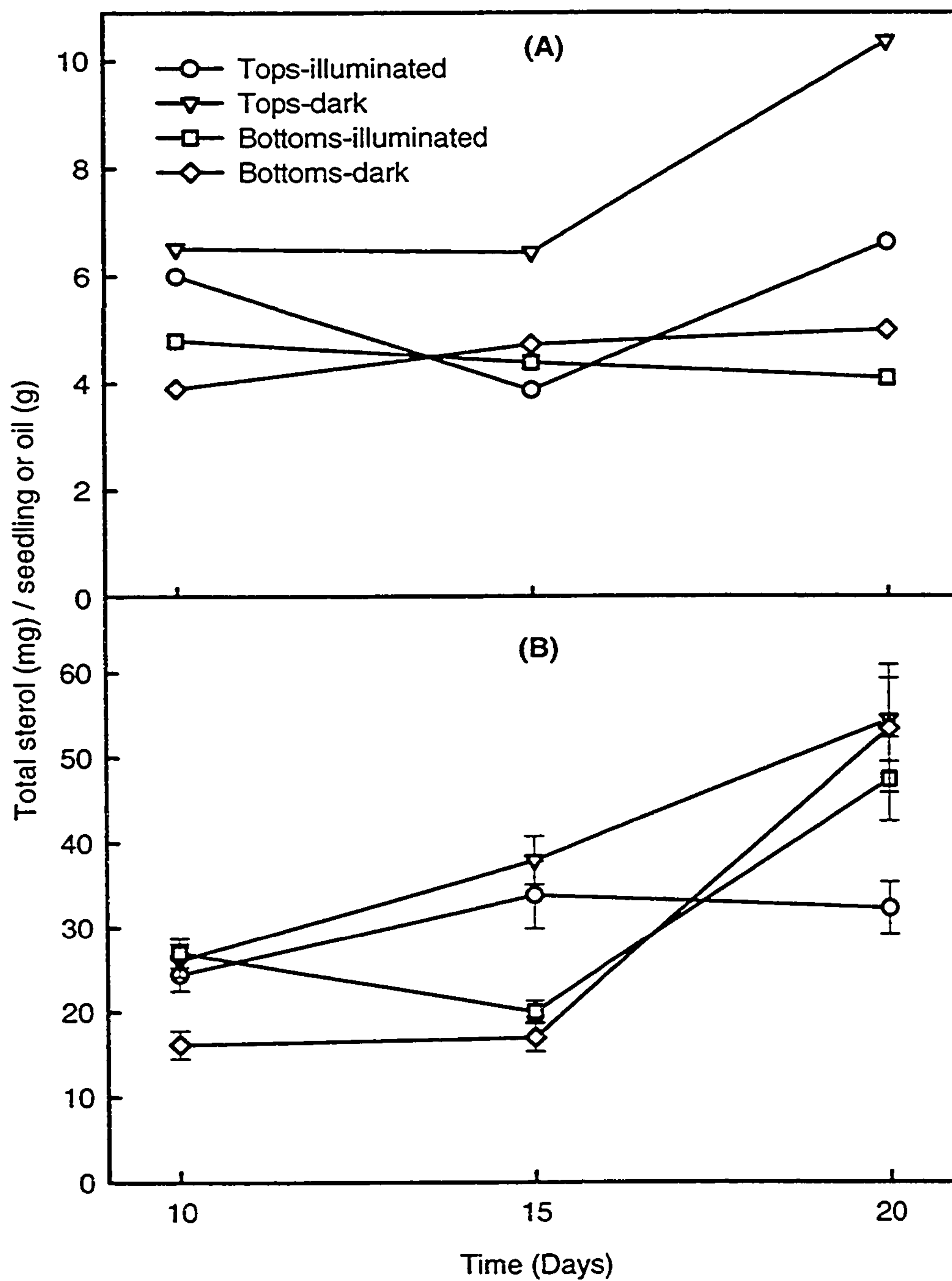

Figure 3.8 Changes in total sterol content in seedling tops and bottoms. Dry seedling wt. basis (A) and oil basis (B).

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4.0 CONCLUSIONS AND RECOMMENDATIONS

During the 20-day period of germination, oil and protein were depleted to supply the energy and building material for seed growth. The factor of light had no significant effect on this depletion. But light did cause different protein solubility and different photosynthesis rate, which caused a decrease of dry matter in the dark condition, and increase under light condition. The breakdown of triglycerides leads to a dramatic rise of free fatty acids, but not of mono- and diglycerides. The rate of breakdown in the dark is somewhat faster than that observed in the light. The change of fat profile in the "top" and "bottom" parts is consistent with the whole seedling. γ-Tocopherol and α-tocopherol are the only tocol isomers that exist in the original seed; however, in the seedling, β-tocopherol appeared. In the sample grown in the presence of light, new tocopherol was synthesized at later stage, which did not happen in the sample grown in the absence of light. Light seems to be a crucial factor for tocol synthesis, but not for isomer conversion, since the conversion of γ-tocopehrol to α-tocopherol also happened in the sample grown in the dark. The obvious higher amount of tocopherol in the "seedling top" than the "seedling bottom" implicated that tocopherols mainly exist in the leaves. Unlike tocopherol, the sterol content and composition did show obvious changes during the whole study period, and sterols are evenly distributed in "seedling top" and "seedling bottom".

As indicated in Chapter 1, the high crude fiber content of canola meal limits its use in feed formulations. Because most of the fiber is in the hull, many efforts are directed toward reducing the hull content of the meal but few prove to be efficient. The ease of dehulling depends mainly on adhesion of the hull to the endosperm. The seed

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coat of canola adheres tightly to the endosperm and embryo and is difficult to remove. In this study, it was observed that after 4 hours of soaking, the seed coat become very soft and loosely adhere to the endosperm so that it is easy to dehull. After 24 hours of germination, the seed coat was ruptured due to inbibition of water and swelling of seeds and growth of radicle. At this stage, no valuable nutrients are lost and therefore, this soaking process may be employed to remove seed coats, which, in turn, will result in a low fiber meal with increased palatability.

With the breakdown of triglyceride, large amount of free fatty acids accumulated in the seedling oil, from almost zero at the beginning to 50-60% at the end. The increase of free fatty acids leaves the oil with rancid odor and less palatable. However, on the other hand, germination can be an alternative method of FFA production for non-food industry.

Total tocopherol content of canola is medium to low compare to other oils, and therefore, higher levels of tocopherol are desirable in this species. Meanwhile α-tocopherol has the best vitamin effectiveness among all tocopherol derivatives, however, the main dietary source (i.e. soybean and corn oil) of tocopherols, typically contain α-tocopherol as a minor component and high levels of γ–tocopherol. The other oil crops such as corn, cottonseed and palm oil have similarly low α- to γ-tocopherol ratios (Eitenmiller, 1997). Substantial increase in the α-tocopherol content of major food crops are needed to provide the humanity with dietary sources of vitamin E that can approach the desired therapeutic levels.

It was observed that under illuminated conditions, the absolute amount of total tocopherols increased from ~ 370 ppm on day-0 to ~ 540 ppm on day-20 seedlings and in

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oil there was almost a 7-fold increase. Moreover, under dark condition, only within 5 days, the ratio of α-: γ- tocopherol increased from 1:2.5 to 4:1. Such increased proportion of α- tocopherol isomer will definitely improve the biological activity and commercial value of canola grain and oil.

The absolute content of phytosterol did not change in the seeds during the 20 days germination process. However, with the depletion of oil, sterols got concentrated in the final oil almost 4-5 fold higher than original oil. The oil with so high content of sterols as well as tocols (i.e. α- tocopherol) is a potential source of sterols and tocols production in Canada.

In general, germination of canola influences the grain composition and contents. Transformation of gamma-tocopherol to alpha tocopherol is beneficial as the latter has been shown to have higher physiological value. Significant increase of tocopherol and sterol contents in oils extracted from seedling provides a value-added opportunity to the functional food industry. In addition, the meal will be low in crude fiber content, which will be better preferred by the animal feeding industry. Appendix A depicts a procedure to produce natural vitamin E supplement which are also rich in phytosterols.

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5.0 FURTHER STUDIES

- Similar studies must be extended to other varieties of canola and other grains
- Separation of protein from seedlings and their functional characterization
- The present study investigated only the total sterols. Separation and characterization of free sterols and sterol esters warrant investigation
- Assessment of the antioxidant potential of the tocopherol enriched oil
- Commercial scale up and the economics of the value-added process suggested in Appendix A warrant investigation
- Potential food use of the low fiber canola seedling meal

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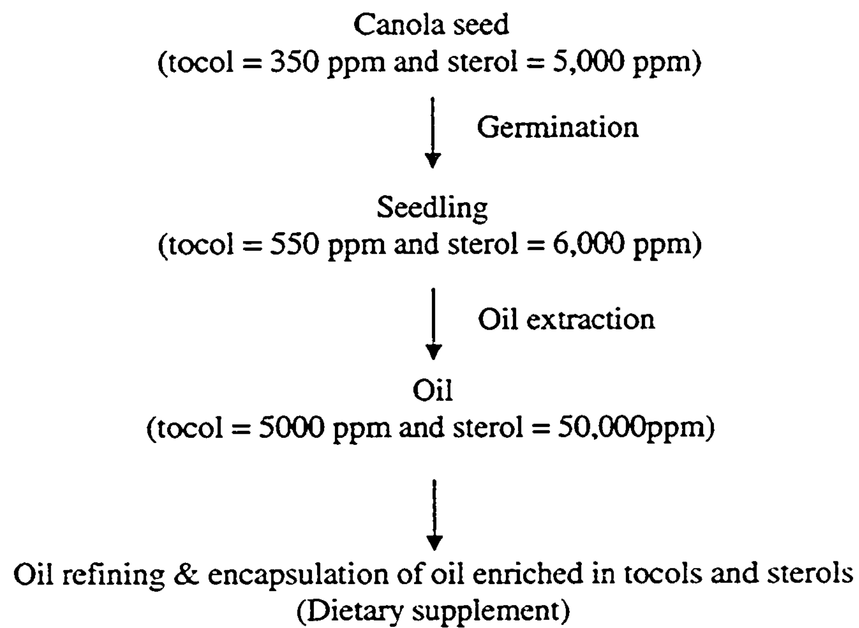
APPENDIX A

New value-added opportunity
(Enrichment of tocols and sterols)



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