• *Technical*

# Lipid Changes in Maturing Oil-Bearing Plants. I. Gross Changes in Safflower and Flax [1,2]

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Flax and safflower blossoms were tagged at the onset of fertilization. At intervals of approximately 10 days, samples of leaves, seeds, bolls, and bracts were collected, frozen in liquid nitrogen, and kept in deep-freeze until analyzed.

A comparison of the behavior during maturation of the seeds of Raja and Rocket flax and Indian safflower showed the following points of similarity and difference. Flax and safflower seed had similar patterns for changes in total extractable matter and true free and true bound lipid, dry matter, iodine absorbed by the seed oil, and isotopic carbon incorporation. The phosphorus and nitrogen contents of the free and bound lipid of flax and safflower seed had different patterns of variation. At a given stage of development, fresh flax seed tissue incorporated more acetate-1-$C^{14}$ than the same weight of fresh safflower tissue. The effect of adverse growing conditions was reflected more clearly by the behavior of safflower than by that of flax. Raja and Rocket flax developed similarly and differed only in regard to response to climatic conditions.

ONE OF THE PROJECTS of the Genetics and Plant Breeding Research Institute of the Canada Department of Agriculture is a study of the lipid changes that take place as an oil-bearing plant progresses from fertilization to maturity. The behavior of safflower and flax was therefore investigated to provide information on two very different types of oil-producing plants. In addition, two varieties of flax, an early- and a late-ripening variety were studied.

Since Ivanov's pioneer work in 1912 (2) many investigations have been made of the effect of environment and variety on oil quality and quantity. However detailed reports on lipid changes that occur during maturation of flax are few. The first intensive study of the physical and chemical changes in flax seed was not made until 1939 when Lehberg, McGregor, and Geddes followed the physical and chemical changes in flax seed at progressive stages of maturity. Oil content and iodine value of the oil were the lipid characteristics examined (4). In 1944 Painter (6) followed changes in the fatty acid composition of the oil from developing flax seed. Recently Vidal (9) analyzed the seed of flax, sunflower, sesame, and soybean at three stages of maturity, reporting on oil content, free fatty acid, iodine value, and refractive index. No reference to similar work with safflower could be found.

In the present work, free and bound lipid contents were estimated by extracting freeze-dried seed with nonpolar and polar solvents. The polar and nonpolar lipid content, the amount of nonsaponifiable material, the lipid phosphorus and nitrogen content, and the iodine value of the extracted oil were also determined. Information on the fatty acid composition of the oils will be published separately.

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[2] Contribution No. 43 from the Genetics and Plant Breeding Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.

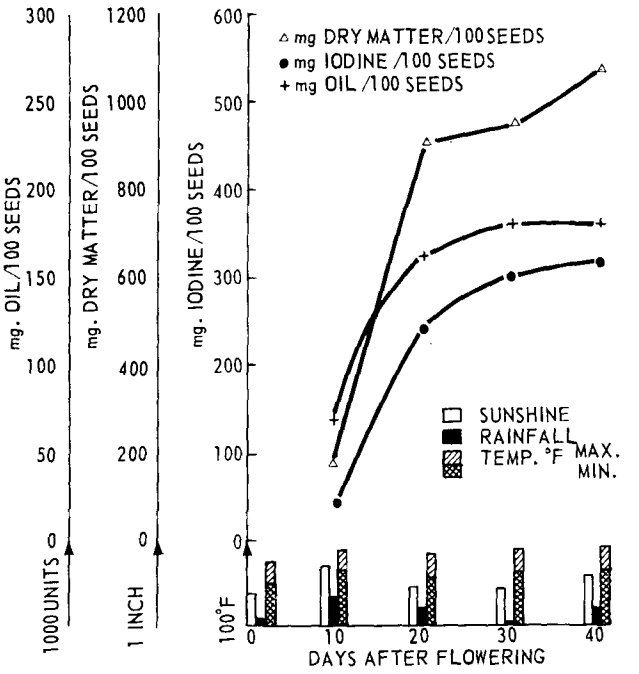


FIG. 1. Oil unsaturation, oil and dry matter in the seeds of Raja flax grown in 1955.

## Materials and Methods

*Materials.* Flax blossoms were tagged on the morning of their appearance, when fertilization occurs. Safflower blossoms were tagged when one to three florets were in bloom. Safflower belongs to the *Compositae* family, and each flower head contains from 20 to 80 florets, each capable of producing one seed. Flowering proceeds centripetally and requires from four to five days for completion; fertilization is extended over the same period of time.

The flax bolls and safflower heads were collected at intervals of approximately 10 days. The field-collected material was brought indoors immediately where the seeds were separated and immersed in liquid nitrogen. When a sufficiently large sample had been collected, the frozen material was transferred to double polythene bags and stored in a deep-freeze until analyzed. Unfrozen material was used in the isotope incorporation studies.

Indian safflower and Raja and Rocket flax were the varieties investigated. In general, Raja and Rocket flax are planted within a day of each other. Rocket flax takes about six days longer to flower and matures eight to 10 days later than Raja. Differences in harvesting dates between these two varieties there-

fore reflect a longer period between blossoming and maturation.

*Methods.* a) Moisture content by freeze-drying—The frozen material plus any ice crystals that sometimes sublimed onto the inside of the inner polythene bags were quickly transferred to tared freeze-dry tubes, and the sample weight was determined. The tubes and contents were chilled to −70°C., connected to the manifold, and kept over-night at a pressure of 5 to 1 microns Hg. The seed was then weighed and counted, and loss of weight was determined. Dry matter and all other like values were expressed as quantity per 100 seeds. Estimates of dry matter obtained by freeze-drying were identical to those found by vacuum drying at 100°C.

b) Total extractable matter, free lipid—Freeze-dried material was ground in a Micro-Wiley mill until it passed a 20-mesh screen. Samples of the ground material were then extracted with chloroform in specially-built Soxhlet extractors, fitted with a nitrogen sweep through the boiling liquid and a check valve on the exhaust manifold to prevent entry of air while the solvent was cooling down. Solvent was removed at 35–40°C. in a rotating evaporator exhausted by a rotary oil pump. Details of this method have been published previously (8).

Bound lipid—Plant material extracted with chloroform was re-extracted in the same apparatus with the ethanol-benzene azeotrope. The solvent was removed as before.

c) Extracted nonlipid material—To recover true lipid from the total extractable matter, petroleum ether, B.P. 30–60°C., was added to the residue after the extraction solvent had been removed and the mixture filtered through a Whatman No. 42 filter paper. Loss in weight, on evaporation of the petroleum ether, was reported as nonlipid extractable material.

d) Fat synthesis power—This was estimated by incubating in water, containing acetate-1-$C^{14}$, a fixed weight of tissue, cut into 2-mm. squares. The tissue was incubated in 30-ml. beakers containing 500 mg. of fresh tissue, 3 ml. of water, and $1.6 \times 10^{-2}$ microcuries of labelled acetate. The beakers were shaken in a Dubnoff Metabolic Incubator, operating at 37°C. and 100 strokes per minute. An atmosphere of oxygen was maintained within the incubator. To show changes in tissue metabolic activity, a fixed weight of fresh tissue, 500 mg., was used in all experiments.

Samples were removed from time to time, and the reaction was quenched by the addition of 5 ml. of 3 N alcoholic potassium hydroxide. This mixture was then transferred to a flask for saponification under reflux for 4 hrs. After transfer of the saponified fat to a separatory funnel, 75 mg. of carrier sodium palmitate were added and the mixture was acidified with hydrochloric acid. The fatty acids were collected in petroleum ether (2 extractions of 50 ml. each). The combined hydrocarbon extract was then washed free of residual acetate-1-$C^{14}$ (two washes of 20 ml. each of 0.1 M acetic acid, followed by one wash with 50 ml. of distilled water) and evaporated on a steam bath to dryness. All transfers were quantitative.

To determine specific activity, an aliquot sample of approximately 1.5 mg. was plated according to the method of Entenman (1), and counts per minute were measured by using a gas flow counter and binary scaler. Whenever the oil content of the original tissue was sufficiently high that endogenous oil would affect the sample weight, corrections were made before total activity in a sample was calculated.

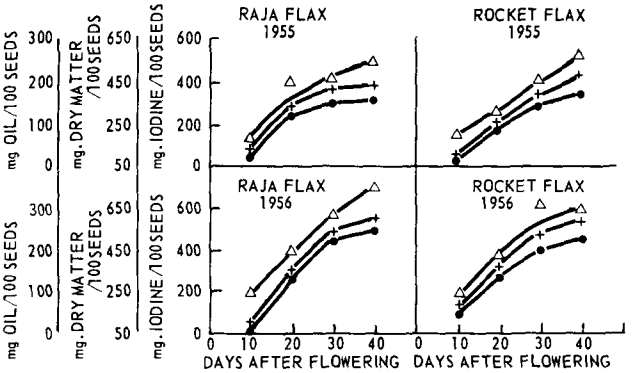


FIG. 2. Comparison of seed characteristics of Raja and Rocket flax grown in 1955 and 1956. Symbols: △ = dry matter; – = oil content; ● = unsaturation.

The slope of the rate plot made by plotting micromoles of acetate-1-$C^{14}$ incorporated as a function of time of contact with the labelled solution was used to denote fat synthesis activity. These slopes were then plotted as functions of extent of development of the plant.

e) Miscellaneous determinations—Iodine value was determined by the Wijs method as modified by Sims and Stone (8); phosphorus content by a local modification of the King method (3,7); and nonsaponifiable matter by the A.O.C.S. Official Method Ca6a-40 (5). Digestions for semi-micro Kjeldahl nitrogen determinations were catalyzed by selenium and copper. Released ammonia was determined by potentiometric titration.

## Results and Discussion

*Free Lipids in Flax and Safflower Seed.* The characteristics of Raja flax seed grown during the hot summer of 1955 are shown in Figure 1.

The amount of dry matter, free lipid, and double bonds in the lipid all increased as the seed matured. The rate of desiccation of the seed appeared to show little sign of slackening off whereas oil content and the amount of unsaturation in the oil seemed to tend toward stabilization. The large increase in fat content was accompanied by a large increase in total unsaturation.

A comparison of early-ripening and late-ripening flax is shown in Figure 2 and Table I, where data for the hot, dry summer of 1955 are shown with data for the cooler summer of 1956. A comparison of each variety between years shows the following differences. Raja seed in 1956 contained 1.39 times more oil than it did in 1955. The oil in the 1956 Raja seed contained 1.58 times more double bonds than the 1955 oil. Rocket seed in 1956 contained 1.25 times more oil

TABLE I

Changes in Raja Flax

| Days after flowering | Wt.(g.) /100 seeds | % dry matter | % Oil | Iodine value | % Phos. in oil |
|---|---|---|---|---|---|
| 1955 | | | | | |
| 10 | 0.592 | 31.28 | 13.51 | 141.2 | 0.141 |
| 20 | 0.789 | 58.00 | 30.29 | 174.1 | 0.116 |
| 30 | 0.506 | 93.76 | 36.86 | 170.0 | 0.053 |
| 40 | 0.566 | 98.17 | 33.48 | 171.5 | 0.046 |
| 1956 | | | | | |
| 10 | 0.938 | 26.03 | 4.66 | 122.9 | 0.095 |
| 20 | 0.991 | 45.00 | 33.28 | 172.8 | 0.040 |
| 30 | 0.997 | 61.53 | 39.82 | 180.8 | 0.026 |
| 40 | 0.788 | 89.81 | 38.47 | 181.0 | 0.025 |

than it did in 1955. The oil in the 1956 Rocket seed contained more unsaturation, 1.32 times more, than the 1955 oil.

Raja flax, with a normally-shorter growing season, was more affected by the different climates of the two years.

A comparison of the behavior of the two varieties within a given year reveals the following points of difference. In the hot summer of 1955 the amount of oil in the Raja seed was only 88% of that in the Rocket seed. Moreover this oil contained only 94% of the double bonds in the Rocket-produced oil. However, in 1956, Raja seed produced as much oil as the Rocket seed, and this oil contained 8% more unsaturation.

TABLE II

Changes in Indian Safflower

| Days after flowering | Wt.(g.) /100 seeds | % Dry matter | % Oil | Iodine value | % Phos. in oil |
|---|---|---|---|---|---|
| 1957 | | | | | |
| 20 | 4.800 | 67.55 | 15.00 | 115.3 | 0.021 |
| 30 | 4.770 | 76.00 | 20.18 | 136.3 | 0.019 |
| 40 | 4.491 | 85.74 | 21.86 | 144.1 | 0.021 |
| 50 | 4.423 | 89.31 | 22.08 | 146.2 | 0.020 |
| 1958 | | | | | |
| 10 | 4.035 | 49.86 | 0.85 | 104.5 | 0.008 |
| 20 | 5.110 | 55.57 | 2.09 | 139.4 | 0.016 |
| 20 | 4.885 | 68.42 | 21.84 | 136.4 | 0.032 |
| 40 | 4.665 | 71.72 | 18.65 | 149.4 | 0.026 |

The characteristics of safflower seed were compared for 1956, 1957, and 1958. These years were, respectively, cool, wet, and cloudy; cool but with a warm, moist September; and cool, wet, and cloudy. Safflower was more sensitive to adverse weather than flax. The seed produced during the favorable summer of 1957 contained twice as much oil as the 1956 crop. The curves for dry matter, oil content, and absorbed iodine as functions of the extent of maturity of the safflower seed resembled those obtained with flax. Again, no real equilibrium was observed for oil content or degree of unsaturation.

Polar lipid content is reported only as lipid phosphorus and nitrogen contents because, for the time being, only gross changes are under investigation. In Figure 3 trends in the polar lipid content of the true free lipid of Raja flax are shown for the years 1954, 1955, and 1956. The left-hand graph shows the lipid phosphorus content of the oil, and the right-hand graph shows the lipid phosphorus content of the seed. It would appear that the lipid phosphorus content of the oil decreases as maturity is approached. If phospholipids are assumed to contain an average of 4% of phosphorus, then the oil in the 10-day seeds could contain 3% of phospholipid and that in the 40-day seeds, 1% of phospholipid. During this period however the lipid phosphorus content of the individual seed increased.

In contrast, the lipid phosphorus content of the true free lipid of safflower was uniformly low during the development of the seed. The lipid nitrogen was low also during this period. As with the true free lipid of flax, the atomic ratios of nitrogen to phosphorus varied irregularly from 2.0 to 0.5.

A second extraction of the ground seed tissue, this time with the ethanol-benzene azeotrope (EBA), yielded further material which was then separated on the basis of solubility in petroleum ether into true bound lipid and nonlipid extractable material. The total EBA extract, the nonlipid extractable material, and the true bound lipid content of the seed all decreased as the seed matured. The 10-day value for the true bound lipid ranged from 1 to 2% of the weight of the dry seed, and this dropped to about 0.15% by the twentieth day. Both flax and safflower seed showed the same behavior.

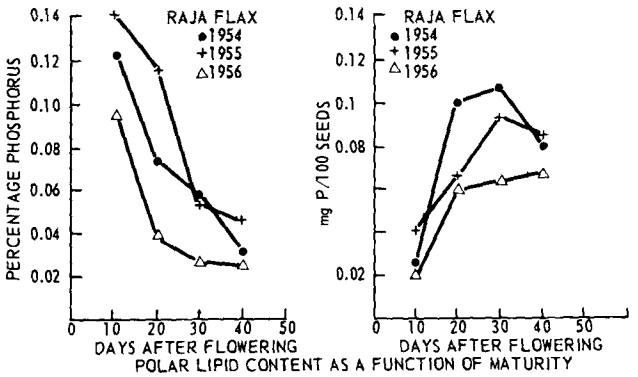


FIG. 3. Phosphorus content of the true free lipid in Raja flax as a function of extent of maturity.

The atomic nitrogen:phosphorus ratio of the bound lipids of both safflower and flax varied from 0.8 to 1.0. With flax, the lipid phosphorus content remained essentially constant during the maturation period at a value that ranged from 0.5 to 0.6%. This is roughly equivalent to 13% phospholipid in the bound lipid. The data for safflower showed a gradual increase in lipid phosphorus content, from 0.5% in 10-day oil to 0.8% in 50-day oil.

The true free and the true bound lipids were separated into saponifiable and nonsaponifiable material. The results obtained with both flax and safflower were uninteresting in that the nonsaponifiable matter appeared to stay at a low, rather constant value. No large amount of nonsaponifiable matter was found in the very immature seeds as reported by Ivanov (2).

Acetate incorporation ability was measured with boll and leaf tissue as well as with seed. The characteristics of the boll and leaf tissue, which will be described elsewhere, are mentioned at this point because their behavior is the inverse of that of the seed. The rate of $C^{14}$ incorporation of seed tissue (Figure 4) started from a low initial value and increased with increasing maturity. The 40-day flax seeds and the 50-day safflower seeds showed vigorous incorporation activity. This might suggest a dynamic state for the lipids in the mature seed. In contrast, leaf and boll tissue at the 0 and 10-day periods after fertilization had high acetate-incorporating power, which decreased almost linearly with time.

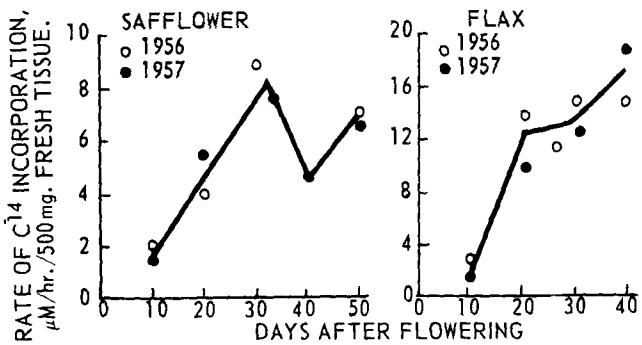


FIG. 4. Rate of incorporation of acetate-1-$C^{14}$ by safflower and flax tissue as functions of extent of maturity.

It is perhaps significant that a given weight of fresh flax seed tissue can incorporate in a given time almost twice as much acetate as the same weight of safflower seed tissue.

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# Lipid Changes in Maturing Oil-Bearing Plants. II. Changes in Fatty Acid Composition of Flax and Safflower Seed Oils[1,2]

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Changes in the fatty acid composition of the oil in flax and safflower seed that occur during the seed-ripening period have been measured. Concentrations of lipid or of specific fatty acid, expressed on a weight-per-seed basis, have been plotted as functions of days after fertilization and of percentage of oil development. Relations between these two independent variables have been established, and limitations to the usefulness of the latter variable have been pointed out. Days after fertilization proved to be the more useful abscissa.

Nonpolar solvents were used to remove free lipid from the tissue, and the total extractable matter was separated into true lipid and nonlipid components. With both flax and safflower, weight of true free lipid per seed and total unsaturation increased during the same period of growth. Nonlipid extractable matter was an inverse function of the extent of development.

In developing flax seed, oleic, linoleic, and linolenic acids all increased continuously; oil in immature seed however was more saturated than oil in more mature seed. Nevertheless the ratio of linolenic acid to linoleic acid that characterizes linseed oil was established by the 20th day after fertilization during a normal growing season.

In developing safflower seed, oleic acid concentration increased slowly during the first 30 days after fertilization and then appeared to level off in some cases as maturity was approached. Initially linoleic acid was present in almost the same amount as oleic acid, but by the 20th day after fertilization its concentration was three times that of oleic acid. This ratio of linoleic to oleic acid tended to increase steadily during the latter part of seed development.

THE FIRST PAPER of this series (1) described overall changes in the lipid synthesis patterns of flax and safflower seed. The true free lipid in the seed was shown to become more unsaturated during the period of greatest increase in oil content, and the acetate-incorporating ability of the tissues was shown to increas during this same interval. A detailed study of the changes in fatty acid composition of the seed oils was therefore undertaken to provide further information on the fat-synthesis program of these two very different genera.

Studies of this type have been made before. Simmons and Quackenbush followed fatty acid deposition in soybeans of increasing extents of maturity (2). They found that, although the amounts of unsaturated fatty acid per bean increased, the iodine value of the oil decreased. Crombie (3) has described fatty acid formation in the maturing kernel of the West African palm. The kernel oil is however of a limited degree of unsaturation. More recently Vidal (4) and Kartha (5) have both studied characteristics of oils formed at various stages of development, but their data are few. The investigations of Painter in 1942 (6) and by McGregor and co-workers in 1939 (7) still remain the most complete studies of changes in linseed oil as the seeds increase in maturity. Since then however new methods of analysis and new ideas on fat biosynthesis have made this present study desirable. Moreover no information was available on the fatty acid synthesis pattern in maturing safflower seed.

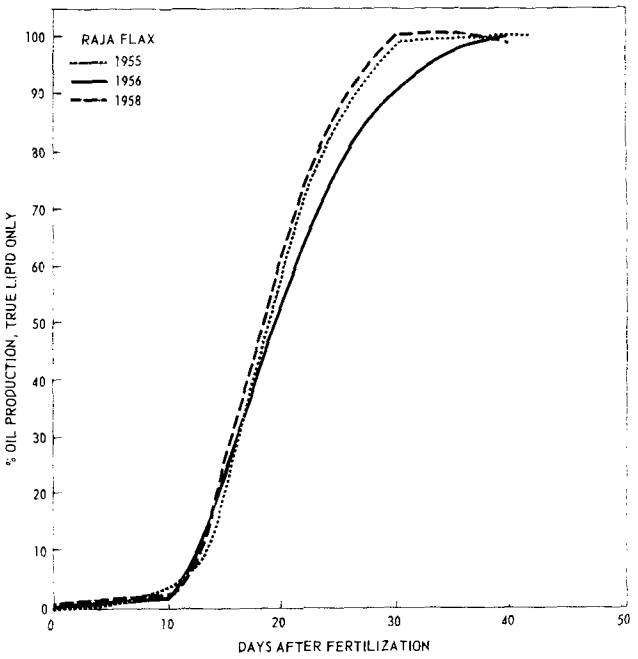


FIG. 1. Relation between % oil production and days after fertilization, Raja flax.

## Materials and Methods

Oils used in this investigation were from the same Raja and Rocket flax seed and Indian safflower seed

[1] Contribution No. 45 from the Genetics and Plant Breeding Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.
[2] Presented at the Fall Meeting, American Oil Chemists' Society, New York, October 17–19, 1960.